Title
Viral and host determinants of HIV-1-mediated CD4+ T-cell infection and depletion in human lymphoid tissue

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Publication Date
2000

Peer reviewed|Thesis/dissertation

Viral and Host Determinants of HIV-1-mediated CD4+ T-cell Infection and Depletion in Human Lymphoid Tissue: the Role of Viral Coreceptor Preference and CD4+ T-cell Phenotype

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

Date University Librarian

Degree Conferred: .....

Dedication

I thank the God for blessing me with the will and perseverance necessary for the completion of this body of work. This dissertation is dedicated to my parents Michael and Margaret Penn, Sr. who have showered me with love so complete and developed in me a mind so great that my reaching this educational pinnacle was merely a formality—I had been Dr. Penn in their eyes long before I entered graduate school.

Preface

The completion of my graduate work represents an amazing synergy between my own efforts and the support and guidance of many individuals. The most important contribution was from my mentor and friend Mark Goldsmith. Mark's passion for science has been the ultimate inspiration for me. He has provided me with the priceless "reagents and protocols" that are required for a rewarding and productive career in science. He has developed the keen ability to ascertain my needs: to promote my strengths and strengthen my weaknesses. He has also given me the freedom to explore my ideas and develop my own scientific identity. I will forever be indebted to him for creating the ideal environment for me to do my graduate work. I would like to thank the many folks in the lab over the years who have shared with me equally valuable tools for success. Roberto Speck was an impeccable example of discipline and a beacon of creativity. Sarah Gaffen was a great friend and exceptional mentor. Stephen Lai and Kathleen Liu served as my unofficial MSTP "big brother" and "big sister." Yun You gave me the inspiration to be technically perfect. Ninan Abraham, who could always answer my questions, served as my scientific encyclopedia. I thank Oliver Keppler for his interest in my work and fine example of truly thoughful and methodical science. Stephen Chan and Johnathan Snow were great friends, provided me with tremendous encouragement, and made the lab a fun place to be. Birgit Schramm has been the other half of my scientific mind and my histoculture right hand—her critical mind and scientific precision were invaluable. Dan Eckstein has quickly become my most trusted labmate, friend, and scientific sounding board. I thank Peggy

Chin for her tireless efforts and seemingly unlimited enthusiasm. I am indebted to the members of my qualifying exam committee (Tris Parslow, Warner Greene, Eric Verdin, and Rich Locksley) for challenging me to think both "in" and "out of the box."

I am very grateful for the financial support of the National Institutes of Health Medical Scientist Training Program, the Eugene Cota Robles Fellowship, the UCSF Chancellor's Fellowship, and the Universitywide AIDS Research Program Dissertation Fellowship. Additionally, I would like to thank all of the members of the J. David Gladstone Institute of Virology and Immunology for their generosity and assistance during my graduate training.

All of the work in this dissertation has been submitted and/or published in various scientific journals. Chapter 2 of this thesis is an article reproduced with permission from the Proceedings of the National Academy of Sciences. Chapters 3 and 4 were published in the Journal of Virology and reproduced with permission from the American Society for Microbiology. Chapter 5 is work that is in press at the Journal of Virology and is reproduced with permission from the American Society for Microbiology and B. Schramm. Chapter 6 has been submitted to AIDS Research and Human Retroviruses. Chapter 7 is a manuscript that was submitted to Nature Medicine.

Viral and Host Determinants of HIV-1-mediated CD4$^+$ T-cell Infection and Depletion in Human Lymphoid Tissue: the Role of Viral Coreceptor Preference and CD4$^+$ T-cell Phenotype

Michael L. Penn, Jr.

The central feature of human immunodeficiency virus type 1 (HIV-1) disease is the depletion of CD4$^+$ T-cells. The course of disease in HIV-1-infected individuals is determined by incompletely understood interactions between the virus and the host. The primary goal of this study was to examine how cellular entry, as determined by viral coreceptor preference, sets the stage for HIV-1-mediated depletion of CD4$^+$ T-cells. To accomplish this, we utilized a lymphoid histoculture system that recapitulated key aspects of HIV-1 infection *in vivo*. We inoculated human tonsil or spleen histocultures with a panel of paired HIV-1 strains that were isogenic except for envelope determinants that controlled coreceptor preference and measured the depletion of CD4$^+$ T-lymphocytes. We found that CXCR4-dependent HIV-1 strains depleted CD4$^+$ T-cells aggressively while CCR5-dependent strains depleted these cells only mildly. Furthermore, we determined that the ability to use additional coreceptors other than CCR5 and CXCR4 did not contribute significantly to increased HIV-1 pathogenicity in lymphoid tissue cultured *ex vivo*. Therefore, CCR5 and CXCR4 appear to be the dominant coreceptors influencing HIV-1-specific killing of T-cells in these tissues. A secondary goal of this study was to determine how host factors such as cellular activation, maturation, and proliferation impact HIV-1 replication and

depletion of $CD4^+$ lymphocytes within human lymphoid tissues. Despite considerable *in vitro* evidence that these cellular attributes regulate HIV-1 replication, the endogenous microenvironment within lymphoid histocultures permitted *de novo* HIV-1 replication and depletion in essentially all $CD4^+$ T-cells regardless of phenotypic classification or proliferative status. Therefore, lymphoid histocultures support the infection and depletion of virtually all recognized T-cell subsets, including resting, naïve $CD4^+$ T-cells. Collectively, these studies exploited a human lymphoid histoculture model to identify and characterize major HIV-1- and host-specific factors that likely contribute significantly to the widespread collapse of the $CD4^+$ T-cell repertoire that characterizes the acquired immunodeficiency syndrome.

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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-cells over time ultimately resulting in immunodeficiency. The kinetics of disease progression, or loss of $CD4^+$ T-cells, among infected individuals is variable and involves incompletely understood factors that are HIV-1- and host-specific. For example, differences in viral preference for chemokine receptors, necessary cofactors that permit cellular entry, have been shown to regulate viral replication and cell target specificity. In addition, specific cellular attributes are also believed to regulate HIV-1 target cell specificity and to determine permissivity for viral infection. The primary focus of this study was the examination of how HIV-1 coreceptor preference and cellular entry sets the stage for HIV-1 replication within and depletion of $CD4^+$ T-cells. A secondary focus of this study was the determination of how cellular attributes impact these parameters. Because, the majority of HIV-1 replication *in vivo* takes place within lymphoid tissues, we utilized an *ex vivo* lymphoid histoculture system that simulated specific elements of natural HIV-1 infection. The endogenous heterogeneity found within these tissues provided a relevant environment in which to study the impact of both viral and host factors on HIV-1 pathogenesis.

II. HALLMARKS OF HIV-1 DISEASE: $CD4^+$ T CELL COUNTS AND VIRAL QUASISPECIES

The human immunodeficiency virus type 1 (HIV-1) is the cause of the acquired immunodeficiency syndrome (AIDS). Clinical disease consists of a number of different phases: (1) primary infection, (2) asymptomatic infection,

and (3) AIDS. Primary infection is the period during which HIV-1 establishes itself within the host. A clinical syndrome resembling a flu-like illness often manifests indicating a concomitant surge in viral replication along with a transient decrease in $CD4^+$ lymphocyte count (82). Also during this period, the immune system builds its response to the infection by producing HIV-1 antibodies and generating virus-specific $CD8^+$ cytotoxic T-cells (45, 63, 68). Following this response, levels of detectable virus in plasma drop dramatically and remain stable at low to undetectable levels for months to years which characterizes the asymptomatic phase (2, 55, 56, 62). However, despite low levels of virus detectable in peripheral blood, viral replication continues relentlessly within lymphoid tissues and ultimately leads to their destruction (67). The asymptomatic phase ends with the appearance of signs of severe immunocompromise, high viral burden, and a fall in $CD4^+$ T-cell counts below 200 cells/mm^3, thus heralding the advanced stage of infection, or AIDS (86). This phase is characterized by a marked and rapid loss of $CD4^+$ T-cells and dysregulation of the immune system that ultimately leads to the acquisition of numerous opportunistic infections and eventual death (82). The progression of this disease process between individuals is highly variable. Some individuals progress to AIDS rapidly upon infection while others progress slowly, taking well over 10 years to reach advanced disease (2, 55, 56, 62). A number of factors, both host and virus specific, presumably contribute to this variability in disease progression.

III. HIV-1 POPULATION DYNAMICS DURING DISEASE

In 1995, two published studies showed that HIV-1 replication reached exceedingly high levels—producing approximately 1-10 billion new virions daily—during the asymptomatic and late-stages of disease (47, 102). Unfortunately, coupled with this rapid replication rate is a similarly high rate of spontaneous mutation due to an error-prone reverse transcriptase enzyme (104). Therefore, following primary infection, HIV-1 rapidly evolves thus creating a pool of genetic variants, or quasispecies, related to the transmitted strain but with random variations. These variations may dramatically alter the fitness, or replication potential, of the newly created viruses. This on-going process of replication and evolution creates a diverse pool from which more fit viruses can emerge in response to selection pressures exerted by the immune system and/or treatment regimens such as highly active anti-retroviral therapy (HAART). The consequences of this process often result in the evolution of more virulent strains with altered host cell specificity, resistance to immune attack or suppression by antiretroviral drugs.

By characterizing the different populations of virus present within HIV-1 infected patients, the viral basis for variability in disease progression can be studied. Upon expanding and cloning individual virus strains, it was observed that all strains replicated in stimulated peripheral blood mononuclear cell (PBMC) cultures. Some strains replicated very rapidly in these cultures while others replicated quite slowly (85). Further characterization of the replication potential of these viruses on other primary cells and transformed cell lines revealed that they were distinguishable in two additional ways. The rapidly replicating strains grew primarily in PBMC and T-cell lines and induced

syncytia, large multinucleated cellular structures in these cultures (85). The more slowly replicating strains did not induce syncytia, thus allowing a simple classification scheme according to the ability to form syncytia in PBMC cultures or T-cell lines. Furthermore, syncytium-inducing (SI) viruses targeted PBMC and T-cell lines but not monocyte-derived macrophages, while non-syncytium inducing viruses (NSI) replicated in macrophages and also PBMCs but not T-cell lines (39, 78). These replication properties and differences in tropism were mapped to the env gene, or gp120, of HIV-1 (11, 12, 22, 32, 33, 49, 95).

When looking at the representation of these two classes of viruses in HIV-1-infected patients, it was observed that NSI viruses often were virtually the only type that was transmitted and predominated throughout disease whereas SI viruses often emerged only in later stages of disease (39, 78). The basis for this switch in viral populations with different tropisms remains an open and actively debated topic in AIDS research.

IV. HIV-1 CELLULAR ENTRY: THE CORECEPTOR PARADIGM

The entry of HIV-1 into cells involves an interaction of gp120 with the human CD4 molecule. Conformational changes that ensue following this interaction ultimately results in exposure of gp41, the viral fusion peptide and subsequent fusion of viral and cellular membranes allowing viral entry into cells. Interestingly, not all $CD4^+$ cells were found to be susceptible to viral entry. While the interaction with CD4 is indispensable for HIV-1 entry into cells, it is not sufficient, demonstrating that additional cellular factors were necessary. These factors remained elusive for many years until 1996, when several groups discovered that the human chemokine receptors, CXCR4 and CCR5, were

necessary cofactors, or coreceptors, allowing HIV-1 entry into CD4-expressing cells (1, 14, 27, 29, 31). Further experiments demonstrated that all published HIV-1 strains utilized either CCR5 (designated R5 viruses) and/or CXCR4 (designated R5/X4 or X4 viruses). NSI viruses isolated from primary and asymptomatic stages of disease were specific for CCR5 while SI viruses isolated from late stage infection were specific for CXCR4 (6, 21, 81, 89). Expression studies of CXCR4 on PBMC, T-cell lines, and macrophages revealed that CXCR4 was ubiquitously expressed on PBMC and T-cell lines but only transiently during monocyte development into macrophages (7, 27, 30, 77, 107). CCR5 was more restricted in its expression being absent from nearly all T-cell lines, and expressed on only a small subset of PBMC but ubiquitously on human macrophages (7, 27, 77, 107). Thus, the molecular basis underlying the differences in cellular tropism and the SI/NSI phenotype among different HIV-1 strains corresponded to differences viral coreceptor preference for either CCR5 or CXCR4.

Numerous *in vitro* studies have revealed that HIV-1 coreceptor specificity is not limited to CXCR4 and CCR5. Several other human chemokine receptors and related orphan receptors exhibit coreceptor activity for select HIV-1 strains in cellular infection assays (reviewed in reference (4)). In addition to CCR5 and CXCR4, the potential coreceptor repertoire of HIV-1 includes the human chemokine receptors CCR2b, CCR3, CCR8, CCR9, and CX3CR1 (V28) and the orphan receptors GPR1, BOB (GPR15), Bonzo (STRL33), APJ, and ChemR23. In addition, the leukotriene B4 receptor and the human cytomegalovirus encoded, chemokine-like receptor US28 permit cellular entry by some HIV-1 isolates *in vitro*. To date, all HIV-1 isolates found to use any of these receptors also use

CCR5 and/or CXCR4. Although there is evidence that many of these receptors are expressed in relevant $CD4^+$ HIV-1 target cells (13, 28, 34, 43, 66, 69, 79, 88), the importance of these additional coreceptors for HIV-1 pathogenesis *in vivo* remains undefined.

V. CORECEPTOR PREFERENCE AS A VIRAL DETERMINANT OF HIV-1 DEPLETION OF $CD4^+$ T-CELLS

The biological significance of this new HIV-1 coreceptor classification scheme is highlighted by numerous observations. For example, individuals harboring certain mutations in coreceptors or their ligands can be protected from infection and/or have a delayed disease progression. For example, studies show that a homozygous deletion in the gene coding for CCR5 significantly lowers the risk of HIV infection by preventing receptor transport to the plasma membrane (24, 48, 60, 80). Furthermore, variants in the gene encoding for the CCR2 receptor and SDF-1, the ligand for CXCR4, independently are associated with slower progression to AIDS (54, 90, 105). The exact mechanisms by which these mutations delay disease is not known but likely influence the availability of coreceptors. HIV-1 transmission appears to be mediated primarily by R5 viruses that predominate throughout disease, while later in the course of disease viruses that use CXCR4 often evolve and become dominant (21, 39, 53, 81, 108). The evolution of X4 viruses is associated with disease acceleration and rapidly declining $CD4^+$ T-cell counts typically observed in rapid progressors (21, 53, 81, 84, 98, 99), whereas R5 viruses remain dominant in long term non-progressors (39, 52, 99). These observations suggest that X4 viruses may have a higher cytopathic potential for $CD4^+$ T-cells than R5 viruses. The clinical course of

patients harboring these viruses has also been studied. In general, patients with R5 viruses have a more delayed disease course compared to patients harboring X4 viruses (39, 53). In fact, X4 viruses often are only detected during or just before periods of rapid $CD4^+$ T-cell decline (87). An important question is whether the emergence of these viruses is a cause or an effect of accelerated disease progression. That X4 viruses were highly cytopathic for T-cells *in vitro* while R5 viruses were mildly cytopathic for these cells suggests that the former hypothesis is correct. However, a molecular explanation for these observations remained elusive, and discovering this explanation was one of the goals of this dissertation research. Chapters 2, 3, and 4 in this thesis describe different experimental approaches that provide compelling evidence that X4 viruses augment HIV-1 disease progression.

VI. A GENERAL STRATEGY FOR ASSESSING THE IMPACT OF CORECEPTOR PREFERENCE ON THE DEPLETION OF $CD4^+$ T-CELLS.

Previous studies have mapped the critical determinants for coreceptor preference to variable regions within the *env* gene (5, 18, 46, 92, 108). Specifically, the V3 loop region is the dominant envelope determinant for coreceptor preference with additional contribution from other regions. The importance of the V3 loop in determining HIV-1 coreceptor specificity is demonstrated by experiments testing the coreceptor specificity of recombinant viruses with chimeric *env* genes. Replacing the V3 loop from a CXCR4-dependent envelope with that from a CCR5-dependent envelope is sufficient to change coreceptor specificity from CXCR4 to CCR5 (92). In addition, very subtle changes in V3

loop sequence (e.g. a single amino acid) can similarly switch coreceptor preference (92).

To determine precisely the contribution of HIV-1 coreceptor preference to $CD4^+$ T-cell depletion, we used multiple pairs of isogenic viruses differing exclusively in envelope determinants (e.g. V3 loop) that control preferences for individual coreceptors. By comparing pairs of isogenic recombinant viral strains with well characterized coreceptor utilization profiles, contributions from other potentially confounding HIV-1 gene products were excluded, thus allowing precise determination of the specific contribution of coreceptor preference to $CD4^+$ T-cell depletion. These experiments are described in detail in Chapters 2, 3, and 4 of this dissertation.

VII. CORECEPTOR PREFERENCE AS A VIRAL DETERMINANT OF HIV-2 DEPLETION OF $CD4^+$ T-CELLS

Human immunodeficiency virus type 2 (HIV-2) infection is primarily found in West Africa but is also found in Asia, Western Europe, and the United States. Interestingly, following the discovery of human chemokine receptors as necessary cofactors for HIV-1, several studies established that this paradigm was also applicable to HIV-2. Nearly all strains of HIV-2, like HIV-1, use CD4 together with CCR5 and/or CXCR4 to gain entry into cellular targets (41, 59, 61, 65, 91). These observations provided an important rationale for the experiments presented in Chapter 5 where we tested how coreceptor preference impacted the ability of HIV-2 to deplete $CD4^+$ T-cells in lymphoid histocultures. Another compelling rationale for these experiments is provided by observations that HIV-2 infection frequently displays a clinical course in infected individuals that is

distinct from HIV-1 infection. Disease progression, as measured by decreasing $CD4^+$ T-cell counts and development of AIDS, is markedly delayed in HIV-2-infected cohorts compared with HIV-1-infected cohorts (50, 58, 71, 103), suggesting that HIV-2 has an attenuated pathogenic potential. Therefore, in Chapter 5 we used the lymphoid histoculture system to test this hypothesis by investigating the relationship between coreceptor preferences and cytopathicity of HIV-2.

VIII. IMPACT OF HIV-1 RESISTANCE TO PROTEASE INHIBITORS ON THE DEPLETION OF $CD4^+$ T-CELLS

As described in Section III of this introduction, mutations in the HIV-1 genome caused by an error-prone reverse transcriptase may dramatically alter the virulence of virons released during successive rounds of viral replication. Inhibitors of the viral protease gene (PI) contained within typical HAART regimens exert a strong selection pressure that, in some patients, results in the evolution of viral strains that exhibit resistance to suppression by these drugs (19, 20, 25, 26, 70, 74). Interestingly, a subset of patients harboring these resistant viruses continue to accumulate $CD4^+$ T-cells despite rebounding viral loads (25, 70), suggesting that these strains might have diminished pathogenicity. In Chapter 6 of this thesis, we tested this hypothesis by determining what impact PI resistance-conferring mutations in the HIV-1 protease gene have on the depletion of $CD4^+$ T-cells in human lymphoid histocultures. While this chapter is not directly related to the major focus of this thesis, it indirectly tests the strength of the coreceptor paradigm by determining whether mutations in the HIV-1 protease gene can significantly alter the pathogenicity dictated by viral

coreceptor preference. These experiments are described in detail in Chapter 6 of this dissertation.

IX. CELLULAR CHARACTERISTICS AS DETERMINANTS OF HIV-1 DEPLETION OF CD4$^+$ T-CELLS

Not all CD4$^+$ T-cells are believed to be equally susceptible to infection and depletion by HIV-1. Several clinical studies have suggested that particular CD4$^+$ T-cell compartments are lost selectively during the course of HIV disease (3, 23, 40, 51, 75, 100, 111), implying that certain cellular characteristics regulate susceptibility to productive infection and depletion by HIV-1. This hypothesis is controversial because other studies have failed to detect preferential loss of individual subsets as total numbers of CD4$^+$ T-cells decline *in vivo* (8, 15, 35, 42, 57, 73).

PBMC studies have also suggested that specific cellular characteristics govern the susceptibility of cells to infection by HIV. First, cellular activation has been shown to be required for the completion of reverse transcription and/or proviral integration in cultured cells (10, 93, 96, 109, 110). In particular, activated cells, defined by cell surface molecules like CD25 and HLA-DR, are uniquely susceptible to lentivirus infection in this system (16, 72, 101) while non-activated cells are not. Second, cell maturation has also been shown to be another factor that determines the host cell specificity of HIV-1 infection of CD4$^+$ T-cells *in vitro*. Specifically, cultured memory cells are preferentially infected (44, 83, 94, 106) while resting and/or naïve cells are highly resistant to infection (16, 76, 109, 110). Third, cellular proliferation can regulate HIV-1 infection in PBMC, with reverse transcription and viral integration being incomplete or inefficient in the absence

of cellular proliferation in PBMC (9, 10, 93, 97, 110). However, despite this extensive evidence of multiple cellular restrictions in the *in vitro* context, recent work has revealed that non-activated, naïve, and/or non-proliferating lymphocytes in HIV-1 infected patients exhibit active HIV-1 gene expression (64, 112). The basis for these paradoxical observations is not well established, but one possibility is that 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 made it difficult to conclude with certainty whether these seemingly resistant cell populations were infected by HIV-1 *de novo* or became infected during an earlier phase of the cellular lifetime. Therefore, the secondary aim of this study, presented in Chapter 7 of this dissertation, was to determine how cellular activation, maturation, and proliferation impact HIV-1 replication and depletion of $CD4^+$ T-cells.

X. HUMAN LYMPHOID HISTOCULTURES AS A MODEL SYSTEM FOR HIV-1 PATHOGENESIS

Traditionally, studies investigating mechanisms of HIV-1-mediated T-cell depletion were performed in PBMC cultures. However, these cells represent only 2% of all the lymphocytes in the human immune system (17). In contrast, the primary areas of HIV-1 replication *in vivo* occur within lymphoid organs, representing the remaining 98% of lymphocytes within the human immune system. Developed by Glushakova and colleagues, the lymphoid histoculture system (36, 37) was therefore an attractive and relevant model system in which to study coreceptor determinants of HIV-1 pathogenesis. Importantly, these tissues are immunologically active such that they produce antibodies specific for recall

antigens like tetanus (38). Tissues are cultured *ex vivo* as 2-3 millimeter blocks cut from surgical specimens of human tonsil or spleen and do not require exogenous stimulation for permissivity to HIV-1-infection that is typically required of PBMC cultures. Typical stimulation protocols necessary for HIV-1 replication in PBMC cultures alter chemokine receptor expression and cellular phenotypes, two important factors potentially affecting the replication of HIV-1. Thus the lack of exogenous stimulation in these cultures is a critically important aspect of the design of this study. Tissue blocks are three-dimensional and heterogeneous containing many different cell types thus closely mimicking the native *in vivo* environment. Importantly, histocultures are viable throughout typical culture periods of 12-15 days and they recapitulate key aspects of HIV-1 infection *in vivo*, namely the replication of virus and depletion of $CD4^+$ lymphocytes.

Thus, the present studies were designed to achieve two major goals: (1) to determine the role of coreceptor preference in determining the extent of $CD4^+$ T-cell depletion within lymphoid histocultures and (2) to determine how cellular activation, maturation, and proliferation impact viral replication and depletion within human lymphoid tissues. The work in this dissertation focused upon comparing the replication and depletion characteristics of various HIV-1 strains with differing coreceptor specificities in human lymphoid histocultures and determining the replication and depletion potential of HIV-1 within specific $CD4^+$ T-cell subsets described above.

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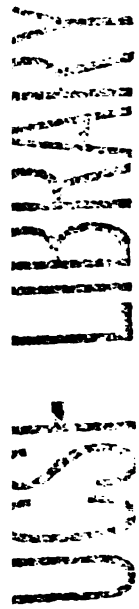

Chapter 2

CXCR4 Utilization Is Sufficient to Trigger CD4+ T-cell Depletion in HIV-1-infected Human Lymphoid Tissue

Prologue

Both *in vitro* and *in vivo* studies suggest that CXCR4 utilization is linked to a stronger cytopathic phenotype and that evolution toward CXCR4 use by HIV-1 *in vivo* directly contributes to disease acceleration. However, these studies have not fully excluded potential contributions of other viral characteristics because neither the basis for the switch in coreceptor usage nor a causal relationship between the switch and disease progression was established. To rigorously test the hypothesis that CXCR4 utilization is sufficient to cause T-cell depletion, we infected human PBMC and lymphoid tissue *ex vivo* with pairs of isogenic viruses differing only in their coreceptor utilization profiles. A direct comparison of the extent of $CD4^+$ T-cell depletion caused by infection of these systems with X4 viruses and R5 viruses demonstrated a causal role for CXCR4-dependent envelopes in augmenting virus pathogenicity. This study provided the first formal proof that HIV-1 specificity for CXCR4 is a cause of aggressive $CD4^+$ T-cell depletion in a relevant lymphoid tissue model system.

This study was an important collaboration with the Margolis Lab from whom I learned the histoculture system. I subsequently established and adapted this system, along with fellow graduate student Birgit Schramm, for use in our own laboratory. Jean-Charles Grivel and I contributed equally to these studies. My contributions included experimental data (Figures 1, 2, 4, and 5), intellectual input, and significant writing and editing of the manuscript. L. Margolis and M. Goldsmith jointly supervised this work.

Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 663–668, January 1999
Medical Sciences

CXCR4 utilization is sufficient to trigger CD4+ T cell depletion in HIV-1-infected human lymphoid tissue

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Communicated by Malcolm A. Martin, National Institutes of Health, Bethesda, MD, November 13, 1998 (received for review August 12, 1998)

ABSTRACT **The human chemokine receptors CCR5 and CXCR4 have emerged as the predominant cofactors, along with CD4, for cellular entry of HIV-1 *in vivo* whereas the contribution of other chemokine receptors to HIV disease has not been yet determined. CCR5-specific (R5) viruses predominate during primary HIV-1 infection whereas viruses with specificity for CXCR4 (R5/X4 or X4 viruses) often emerge in late stages of HIV disease. The evolution of X4 viruses is associated with a rapid decline in CD4+ T cells, although a causative relationship between viral tropism and CD4+ T cell depletion has not yet been proven. To rigorously test this relationship, we assessed CD4+ T cell depletion in suspensions of human peripheral blood mononuclear cells and in explants of human lymphoid tissue on exposure to paired viruses that are genetically identical (isogenic) except for select envelope determinants specifying reciprocal tropism for CXCR4 or CCR5. In both systems, X4 HIV-1 massively depleted CD4+ lymphocytes whereas matched R5 viruses depleted such cells only mildly despite comparable viral replication kinetics. These findings demonstrate that the coreceptor specificities of HIV-1 are a causal factor in CD4+ T cell depletion *ex vivo* and strongly support the hypothesis that the evolution of viral envelope leading to usage of CXCR4 *in vivo* accelerates loss of CD4+ T cells, causing immunodeficiency.**

HIV-1, the causative agent of AIDS, represents a large number of diverse variants that differ genetically and phenotypically from one another. Until recently, HIV-1 isolates were classified according to their ability to infect and induce syncytia in various cells lines or monocyte-derived macrophages in culture. Although all isolates are capable of infecting cultures of human peripheral blood mononuclear cells (PBMCs), some isolates also can infect cultured macrophages but fail to infect and induce syncytia in established T-cell lines [referred to as non-syncytium-inducing (non-SI) or M-tropic strains]. In contrast, other variants do not infect macrophages but are capable of infecting T-cell lines and inducing syncytia in these cultures (referred to as SI or T-tropic). These properties are determined largely by the envelope gene of HIV-1 (reviewed in ref. 1).

The molecular mechanism(s) underlying differences in both cellular tropism and the SI/non-SI phenotype among different strains of HIV-1 became apparent with the discovery of the human chemokine receptors CCR5 and CXCR4 (2–6) as coreceptors along with human CD4 for the cellular entry of most strains of HIV-1. Expression studies of these receptors revealed that both CXCR4 and CCR5 are expressed by PBMC (5, 7–10), but most T-cell lines express only CXCR4 (7). Monocyte-derived macrophages express high levels of CCR5 but much lower levels of CXCR4 (11, 12). In fact, all reported SI and non-SI viruses segregate according to CXCR4 (designated X4 viruses) or CCR5 (designated R5 viruses) utilization, respectively (13–16). Although many other chemokine receptors demonstrate some degree of HIV-1 coreceptor activity *in vitro*, their importance to viral spread and pathogenesis *in vivo* remains to be elucidated.

The biological importance of the HIV-1 coreceptor classification scheme (17) is highlighted by changes in the phenotypes of HIV-1 strains that predominate *in vivo* during progression of HIV-1 disease to AIDS. HIV-1 transmission appears to be mediated by non-SI/R5 viruses (18, 19), which predominate throughout early stages of HIV-1 disease (13, 14, 20, 21). Later in the course of HIV disease, viruses that use CXCR4—either exclusively or in addition to CCR5—often evolve and become dominant (13, 14). The evolution of SI (X4 or R5X4) variants is associated with disease acceleration, in particular with a rapid drop in T cell counts (22, 23). Such a switch of viral phenotype and coreceptor tropism occurs typically in rapid progressors and in some slow progressors (13, 14, 20–23) whereas non-SI (R5) viruses remain dominant in long-term nonprogressors (22, 24, 25).

Thus, both *in vitro* and *in vivo* studies might suggest that CXCR4 utilization *per se* is linked to the stronger cytopathic phenotype and that the evolution toward CXCR4 use by HIV-1 *in vivo* directly contributes to disease acceleration. However, earlier studies have not fully excluded potential contributions of other viral characteristics because neither the basis for the switch in coreceptor usage nor a causal relationship between the switch and disease progression were established (26, 27). To rigorously test the hypothesis that CXCR4 utilization is sufficient to cause T cell depletion, we infected human PBMC and lymphoid tissue *ex vivo* with pairs of isogenic viruses differing only in their coreceptor utilization profiles. A direct comparison of the extent of CD4+ T cell depletion caused by infection of these systems with X4 viruses and R5 viruses demonstrated a causal role for CXCR4-dependent envelopes in augmenting virus pathogenicity.

MATERIALS AND METHODS

Cells and Transfections. PBMC were isolated from whole blood buffy coats (Stanford Blood Bank, Palo Alto, CA) by Ficoll-Hypaque density gradient centrifugation (Histopaque 1077, Sigma), were cultured for 48 h in medium (RPMI 1640) (Mediatech, Washington, DC) supplemented with 10% fetal calf serum (FCS, Gemini Biological Products, Calabasas, CA) and 2 μg/ml phytohemagglutinin (Sigma), and were stored at −80°C. Before their use in experiments, thawed cells were cultured for 2 days in RPMI medium 1640 with 10% FCS and

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Abbreviations: PBMC, peripheral blood mononuclear cells; SI, syncytium-inducing; FCS, fetal calf serum.

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5 units/ml recombinant human interleukin-2 (courtesy of Chiron). COS-7 cells (American Type Culture Collection) were maintained and transfected as described (28) with combinations of expression vectors encoding human CD4 (pCD4Neo) (29), CCR5 (pCMVFCCR5) (30), or CXCR4 (pCMVFCXCR4, provided by K. Neote, Pfeizer, Inc.).

Preparation of Viral Stocks. Except where noted otherwise, all proviral plasmids, envelope expression constructs, and viruses were from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The primary isolate BaL was prepared by culturing on monocyte-derived macrophages derived from human PBMC by plastic adherence. The molecular clones 49–5, 61–21, and JR-CSF were generous gifts from B. Chesebro (Rocky Mountains Laboratories, National Institute of Allergy and Infectious Diseases). To prepare viruses for infections, proviral plasmids were transfected into 293T cells by using the $CaPO_4$ method (Promega) as described (30), and viral stocks were harvested after both 36- and 60-h incubations. The p24 Gag concentration (ng/ml) was assessed by ELISA (New England Nuclear). To prepare pseudotype virus carrying the luciferase gene, 2 μg of pNL-Luc-E-R- (5) and 2 μg of the NL4–3 envelope expression vector pDOLenv or 2 μg of 49–5 proviral plasmid were cotransfected into 293T cells growing as described (30), and supernatants were harvested.

Coreceptor Utilization Assay for Single-Round Infection. COS-7 cells transfected with pCD4neo alone or pCD4neo in combination with pCMVFCCR5 or pCMVFCXCR4 were infected with 200 μl of pseudotype viruses. After 48 h, luciferase expression was assessed by enzymatic activity (relative light units) as described (5) according to the manufacturer's instructions (Analytical Luminescence Laboratory, San Diego).

PBMC Infections. To titrate viruses to achieve similar viral replication kinetics among different viruses for depletion studies, varying amounts of each were added to 500,000 PBMCs and were incubated overnight at 37°C. Cells then were washed extensively with PBS and were cultured in medium (RPMI 1640), 10% FCS and interleukin-2 as above. Supernatant (20 μl) was harvested every 2 days for p24 determinations and was replenished with an equal amount of RPMI medium 1640, 10% FCS, and interleukin-2 medium. For depletion studies, cultures infected with virus doses selected to achieve similar viral production during the assay were harvested 10 days after infection and were analyzed.

mAbs, Immunofluorescence Staining, and Flow Cytometry. PBMC were washed in 4 ml of PBS and 3% FCS before immunostaining. Cells were incubated on ice for 30 min in 100 μl of 1:20 dilutions of various fluorochrome-conjugated anti-CD4 (PE-conjugated), anti-CD8 (PerCP-conjugated), and anti-CD3 (FITC-conjugated) mAbs (clones SK3, SK1, and SK7, respectively) or the Tritest kit combining all three antibodies (Becton Dickinson). Cells were washed again with 4 ml of PBS and 3% FCS serum and were analyzed with a FACScan (Becton Dickinson). Lymphocytes (20,000) were counted by forward/side scatter characteristics, and the data were analyzed with CELLQUEST software (Becton Dickinson). CD4+ lymphocytes were sorted by positively selecting CD4+/CD8− lymphocytes using a FACS Vantage (Becton Dickinson).

Infection of Human Lymphoid Tissue *ex Vivo*. Human tonsil tissue removed during routine tonsillectomy and not required for clinical purposes was received within 5 h of excision and was sectioned into 2- to 3-mm blocks. These tissue blocks were placed onto collagen sponge gels in the culture medium at the air-liquid interface and were infected as described (31–33). In a typical experiment, equal amounts of various isolates (0.5 ng of p24 per block) were applied as 3–5 μl of clarified virus-containing media to the top of tissue blocks from the same donor. Productive HIV-1 infection was assessed by measuring p24 in the culture medium by using an HIV-1 p24 antigen ELISA (Cellular Products and AIDS Vaccine Program, National Cancer Institute, Frederick, MD): specifically, the concentration of p24 accumulated in 3 ml of culture medium bathing six tissue blocks during the 3 days between the successive medium changes was used as a measure of virus replication. Similar methods were used with human spleen samples derived at autopsy (provided by National Diabetes Research Interchange, Philadelphia).

Flow cytometry was performed as described above on cells mechanically isolated from control and infected tissue, and CD4+ T cell depletion is expressed as a ratio of CD4+ to CD8+ T cells blocks as described earlier (31). For characterization of productively HIV-infected cells, the following cell surface markers were used in combination with anti-p24 RD1 (Coulter); anti-CD3 PerCP (Becton Dickinson), anti-HLA-DR APC, anti-CD68 FITC, and anti CD25 APC (Caltag, Burlingame, CA); and anti-CD64 Cy5 and anti-CD69 FITC (PharMingen). Cells first were stained for the cell surface antigens, were fixed and permeabilized by using Cytofix-Cytoperm (PharMingen), and were stained for intracellular p24.

RESULTS AND DISCUSSION

To test the hypothesis that CXCR4 tropism alone is sufficient to produce HIV-1-induced pathogenesis, we used pairs of isogenic viruses differing exclusively in envelope determinants that control preferences for the coreceptors CXCR4 and CCR5 leading to distinct cellular tropisms. NL4–3 (34) is a well known, molecularly cloned strain of HIV-1 that exhibits strict CXCR4-tropism (35, 36). 49–5 (37) is a recombinant virus based on NL4–3 that contains the envelope V3 loop sequence derived from that of BaL (38), a molecularly cloned primary isolate that exhibits selectivity for CCR5 (5, 6). Because the V3 loop has been shown to be the predominant (4, 35, 39–43) though not exclusive (44, 45) determinant of HIV coreceptor specificity, 49–5 was expected to be CCR5-restricted. This phenotype was verified by transiently transfecting COS-7 cells with plasmids encoding human CD4 along with human CXCR4 or CCR5 and infecting them with pseudotype HIV-1 preparations, 49–5 and NL4–3, that encoded the firefly luciferase gene. As expected, NL4–3 utilized CXCR4 and not CCR5 as a coreceptor whereas 49–5 used CCR5 and not CXCR4 (Fig. 1). This distinction provides a basis for examining the relationship between coreceptor utilization by HIV-1 and CD4+ T cell depletion while holding constant the remainder of the viral genome.

We therefore compared CD4+ T cell depletion in phytohemagglutinin-blasted PBMCs cultured with interleukin-2 and infected with NL4–3 or 49–5. Viral replication was monitored by measuring the HIV-1 capsid protein (p24) in the culture supernatants over time, which revealed very similar replication kinetics for NL4–3 and 49–5 (Fig. 2 *Inset*). Cells were harvested 10 days after infection, were immunostained with anti-CD4 and anti-CD8 antibodies, and were analyzed by fluorescence-based flow cytometry. In three independent experiments, NL4–3 severely depressed the CD4/CD8 ratios in these cultures whereas 49–5 exerted significantly less effect (Fig. 2). The differences in CD4+/CD8+ ratios indicate that infection with a CXCR4-dependent virus induces significantly greater CD4+ T lymphocyte depletion in PBMC suspension cultures than does the isogenic CCR5-tropic strain, despite comparable viral replication kinetics.

In vivo viral replication occurs in whole tissues rather than in isolated cell populations. Furthermore, general cell activation itself can modulate coreceptor expression (8) and also can contribute to cell death (46). We therefore extended our study of CD4+ T cell depletion to integral human tonsillar tissue infected *ex vivo* by using a system that has been shown to

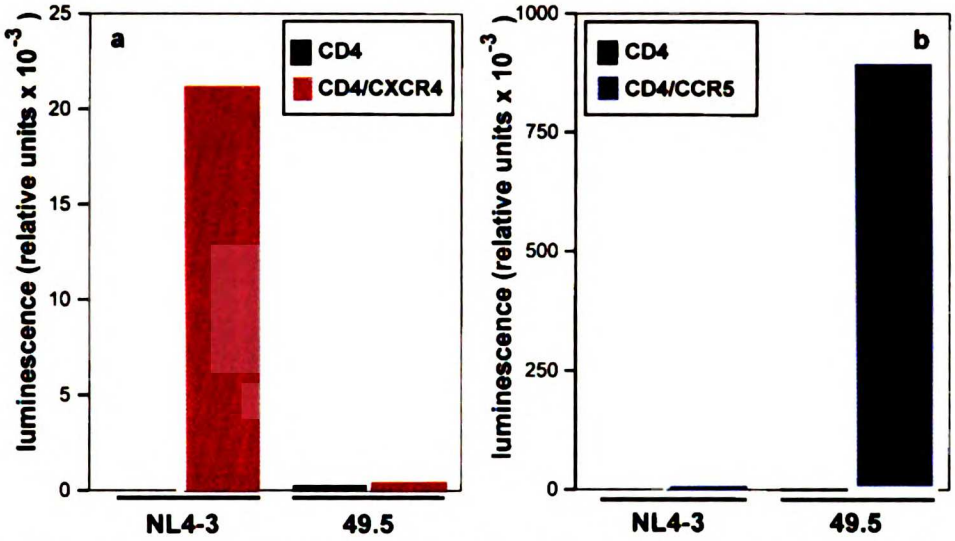

FIG. 1. Reciprocal utilization of CXCR4 and CCR5 by paired, isogenic HIV-1 strains differing in envelope determinants. COS-7 cells were transfected with plasmids encoding CD4 alone or CD4 in combination with CCR5 or CXCR4. Cells subsequently were infected with pseudotype NL4-3 or 49-5. After 48 h, luciferase expression was assessed by enzymatic activity (relative light units). (*a*) Transfection with CD4 (black bars) alone or with CD4/CXCR4 (red bars). (*b*) Transfection with CD4 (black bars) alone or with CD4/CCR5 (blue bars).

preserve tissue cytoarchitecture and to support productive infection by all HIV-1 isolates without exogenous stimulation (31, 32). We tested NL4-3, BaL, and the chimeric virus 49-5 for immunodepletion in these three-dimensional cultures. All three viruses replicated well in the tonsillar histocultures as evaluated by the increase of p24 in the culture medium (Fig. 3). Replication of each became evident, starting approximately from day 6 after infection, and continued to increase during the course of experiment. The absolute level of replication varied 2- to 3-fold from tissue donor to donor. In experiments with tissue from five donors, there were no consistent differences among different viral variants in the rate of replication or in the amount of total virus produced during the 12-day experiments (Fig. 3).

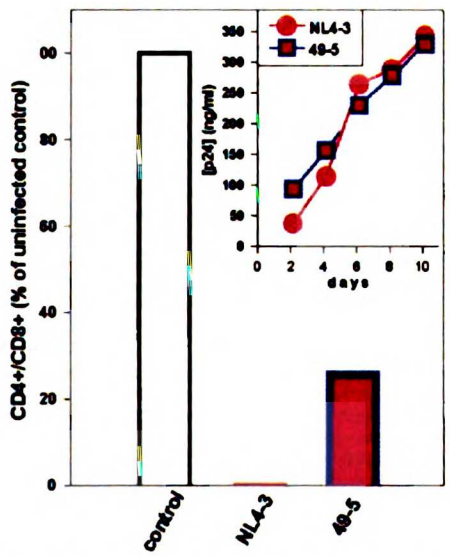

FIG. 2. CD4+ T lymphocyte depletion in PBMC cultures by paired HIV-1 strains. Shown are CD4+/CD8+ ratios in PBMC infected with NL4-3 (red bars) or 49-5 (red/blue bars) after a 10-day infection. The ratios are expressed as a percentage of that in a control (uninfected) PBMC culture. (*Inset*) The concentration of p24 in culture medium of PBMC infected with NL4-3 (red circles) or 49-5 (red/blue squares). These experiments are representative of three independent experiments using PBMC isolated from different donors. Color code: An individual color was assigned to each viral isolate as shown under the bar graph. The bar colors for isogenic chimeras correspond to the colors of parental viruses whereas the color of the edges correspond to the sources of Env sequences.

Despite comparable replication properties, marked differences in the abilities of different viruses to deplete CD4+ T cells were observed. NL4-3 severely depleted the tonsil tissues of CD4+ T cells (Fig. 3*a*) as assessed by the CD4+/CD8+ ratio (31). In contrast, BaL and the chimeric virus 49-5 containing the V3 loop derived from BaL exhibited only minimal depletion effects on CD4+ T lymphocytes (Fig. 3*b*).

To verify that the genomic environment of the envelope sequences does not affect CD4+ T cell depletion in this system, we performed experiments with a second, reciprocal set of matched viruses. JR-CSF (47) represents a molecularly cloned, CCR5-dependent primary isolate, and chimeric virus 61-21 is its isogenic partner containing the backbone of JR-CSF and the V1-V3 loop segments derived from NL4-3 that have been shown previously to exhibit strict CXCR4-dependence (35). In these experiments, NL4-3, JR-CSF, and 61-21 replicated with kinetics similar to those of other tested viruses (Fig. 3). Despite comparability in replication properties, only the CXCR4-dependent viruses (NL4-3 and 61-21) caused prominent depletion of CD4+ T cells (Fig. 3). In these and in the earlier experiments, the decline of CD3+/CD4+ cells by depleting viruses was not accompanied by a relative increase of CD3+/CD8−/CD4− cells. Thus, viral infections in these cultures trigger CD4+ T cell loss rather than down-regulation of cell surface CD4 expression accounting for these changes.

We also tested these viruses for effects on CD4+ T cells in noninflammatory human spleen tissue. Because spleen tissue

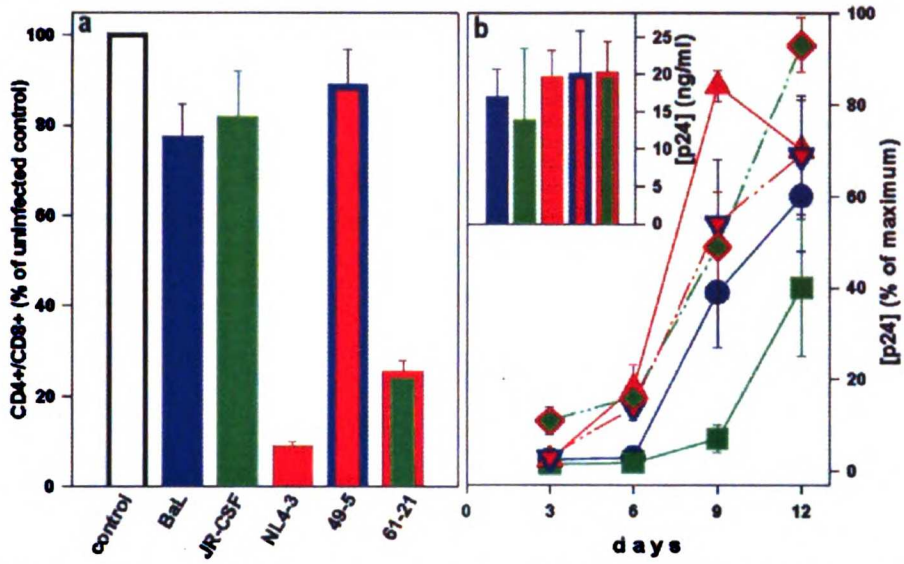

FIG. 3. CD4+ T cell depletion and viral replication in human tonsillar tissue infected *ex vivo* by matched HIV-1 strains. (*a*) CD4+ T cell depletion, as assessed by CD4+/CD8+ ratio on day 12 after infection. For each measurement, cells were isolated from 6–10 tissue blocks and were analyzed by flow cytometry. Mean values (± SEM) are shown from experiments with tissues from three to six different donors infected in parallel with a panel of tested viruses. (*b*) Viral replication, as assessed by p24 values in the histoculture supernatants. Tissues from three to five different donors were infected as indicated, and viral replication was monitored. To compare replication kinetics in tissues from different donors, the absolute value for viral replication for a given donor tissue was normalized by the maximum value of viral replication for the entire tested panel. Mean values ± SEM are shown for experiments with tissues from three to six different donors. (*Inset*) Absolute value of viral replication of the same histocultures, as assessed by measurements of p24 concentration in the culture medium measured on day 12 after infection. For each measurement, medium bathing six blocks of tissue was pooled. Presented are mean values ± SEM for experiments with tissues from three to six different donors. The same tissue blocks were used for measurements presented in *a* and *b*. The color code is the same as in Fig. 2.

persists longer as viable histocultures than does tonsillar tissue, use of this system further allowed us to determine whether R5 viruses cause CD4+ T cell depletion over greater lengths of time. After prolonged infection of the spleen samples, the CCR5-tropic virus 49–5 had only modest effects on CD4+ T cell survival whereas parental NL4–3 in the same time frame massively depleted the samples of CD4+ T cells (Fig. 4). As in the earlier experiments, the difference in CD4+ T cell depletion could not be attributed to differences in viral replication rates (Fig. 4 *Inset*). Indeed, the total amount of virus produced in tissue infected with the depleting NL4–3 was even lower than that produced in tissue infected with its nondepleting 49–5 derivative.

Therefore, these reciprocal experiments collectively demonstrate that the preference for CXCR4 is a major causative factor in accelerating depletion of CD4+ T cells. One hypothesis to account for these results is that differences in cellular tropism among X4 and R5 viruses cause different pathologic outcomes. However, both X4 and R5 viruses were found to infect T cells in these tissues. This fact was established by flow cytometry of cells mechanically isolated from infected tissues and immunostained for CD3 and p24. The frequency of CD3+/p24+ cells varied between 0.4 and 2.8% of all T cells regardless of coreceptor properties, which is in accordance with the data reported for tissues from asymptomatic HIV-infected patients (48, 49). These findings indicate that the less pathogenic behavior of R5 variants is not attributable to their inability to infect T cells.

To confirm that other cells are not required for differential CD4+ T cell depletion, we compared the effects of infection and cell depletion by NL4–3 and its CCR5-dependent derivative 49–5 in a sorted population (98% purity) of peripheral blood CD4+ T cells. In agreement with the results presented above, CXCR4-dependent NL4–3 nearly obliterated the sorted CD4+ T cell cultures whereas the CCR5-dependent derivative 49–5 depleted only mildly. Counts of viable (trypan blue-excluding) cells demonstrated that NL4–3 depleted to 0.6% of the uninfected control whereas 49–5 depleted to 61% of the control (Fig. 5). The replication of both viruses in the cultures was comparable (Fig. 5 *Inset*), again excluding replication rates as the basis for these differences in CD4+ T cell depletion.

Other attempts have been made to establish a correlation between cellular tropism and CD4+ T cell depletion. Studies using human PBMC transplanted into immunodeficient mice have found complex relationships among viral replication rates, cellular tropism, and CD4+ T cell depletion (27, 50, 51). Recent work comparing X4/R5 and R5 strains was highly suggestive regarding the possible contribution of CXCR4, although it did not fully distinguish between the pathogenic effects of CXCR4 itself versus the combined effects of CCR5 with CXCR4 (27). Other experiments with human lymphoid tissue *ex vivo* also have demonstrated a consistent relationship between T-cell tropism and CD4+ T cell depletion (31, 32), but, as in natural human infections, diverse genomic differences among the tested viruses obscured the ability to assign a singular causative role to the coreceptor preferences. Thus, the present recombinant strategy utilizing pairs of viruses that differ only in these select envelope sequences and replicate to similar levels provides definitive evidence that viral use of CXCR4 is sufficient to produce severe CD4+ T lymphocyte depletion in multiple human lymphoid culture systems.

R5 viruses also cause some degree of CD4+ T cell depletion in these experimental systems and *in vivo*, but the more aggressive behavior of X4 strains in the present studies appears to recapitulate their clinical properties in human infection. An

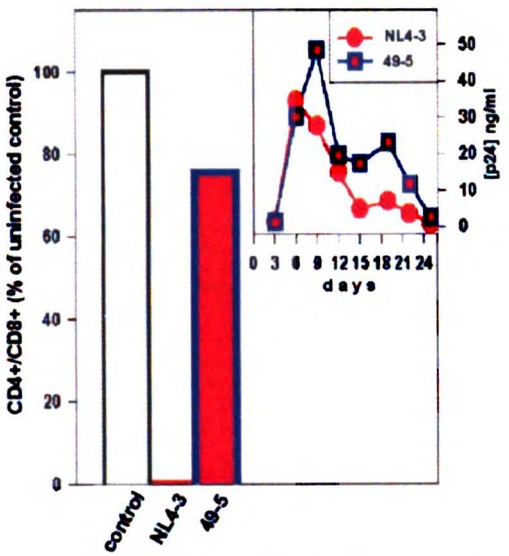

FIG. 4. CD4+ T lymphocyte depletion in human spleen histocultures by paired HIV-1 strains. Mean CD4+/CD8+ ratios from duplicate samples are shown from an experiment performed with human spleen histocultures infected with NL4–3 or 49–5. The p24 concentrations of these viruses in the culture medium over time are indicated as in Fig. 3 (*Inset*). The color code is the same as in Fig. 2.

interesting question regards the mechanism of virus-specific lymphocyte depletion. One possibility is that the ranges of

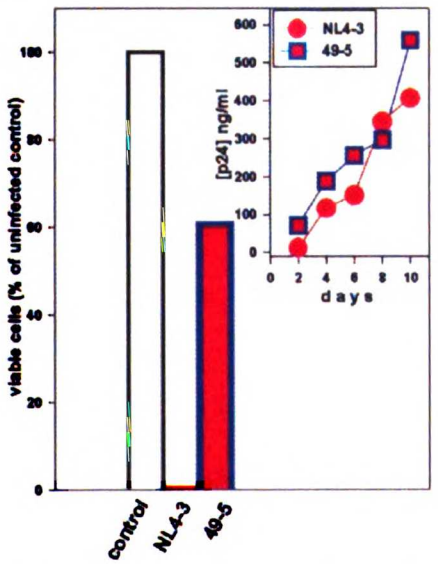

FIG. 5. Viability of purified CD4+ T lymphocyte cultures after infection by paired HIV-1 strains. CD4+ T lymphocyte cultures were infected with NL4–3 or 49–5. Viability of the cultures also was assessed 10 days after infection by counting cells that excluded trypan blue, and results are expressed as a percentage of the viable cell counts in the uninfected control cultures; this experiment was performed in duplicate, and mean values are shown. (*Inset*) The p24 concentrations of these viruses in the culture medium over time. The color code is the same as in Fig. 2.

specific targets among the T cells differ for X4 and R5 strains, which may lead to distinct consequences for the lymphocyte population overall. An intriguing alternative hypothesis is that viral usage of a particular coreceptor is a direct factor in the fate of an infected cell. Further investigation will be needed to define the relevant mechanisms. In any event, as patients harboring X4 viruses progress to AIDS more rapidly than do similar cohorts harboring exclusively R5 viruses (13, 14, 20–22, 24), these findings strongly support the postulate that the emergence of HIV-1 strains that utilize CXCR4 is a causative factor in disease acceleration.

The authors thank Dr. Joshua Zimmerberg for many helpful discussions, encouragement, and support, and Dr. Bruce Chesebro for kindly providing recombinant viruses. The authors acknowledge the technical assistance of Yang He, Dr. Eric Wieder, and Lisa Gibson in the conduct of these experiments and the administrative assistance of Heather Livesay in the preparation of this manuscript. M.L.P. is supported by the Biomedical Sciences Graduate Program and the National Institutes of Health Medical Scientist Training Program at the University of California, San Francisco, and B.S. is supported by the Boehringer Ingelheim Fund. This work was supported in part by the National Aeronautics and Space Administration/National Institutes of Health Center for Three-Dimensional Tissue Culture (L.M. and J.-C.G.), the J. David Gladstone Institutes (M.A.G.), National Institutes of Health Grant R21-AI42654 (to M.A.G.), a grant from Pfizer, Inc. (to M.A.G.), and the University of California, San Francisco AIDS Clinical Research Center (M.L.P.).

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Chapter 3

Viral Entry through CXCR4 Is a Pathogenic Factor and Therapeutic Target in Human Immunodeficiency Virus Type 1 Disease

Prologue

The aim of this study was to elucidate the contributions of distinct coreceptor specificities to HIV-1 disease by evaluating these properties in human lymphoid histocultures. We reported previously that the specificity of recombinant HIV-1 strains for CXCR4 promotes aggressive depletion of $CD4^+$ T-cells in this model system, which illustrates the potential *in vivo* impact of coreceptor specificity. The present study demonstrates that the X4 phenotype of recombinant HIV-1 strains and primary HIV-1 isolates is linked to a highly pathogenic phenotype in *ex vivo* lymphoid tissues, which is substantially inhibited by interfering with CXCR4-mediated entry. Furthermore, we show that promiscuous use of the additional coreceptors CCR3, CCR8, BOB, or Bonzo *in vitro* does not promote accelerated $CD4^+$ T-cell depletion in *ex vivo* lymphoid tissues. Finally, we provide evidence that *in vitro* coreceptor specificity does not necessarily reflect relevant coreceptor affinities in biological systems. This study stresses the importance of the gp120-CXCR4 interaction and suggests that antagonism of this interaction may have therapeutic value for suppressing the pathogenic effects of HIV-1 contributing to HIV-1 disease progression.

This chapter represents a collaborative effort with Birgit Schramm, a fellow graduate student in the lab with whom I shared first-authorship. E. De Clercq and D. Schols provided AMD3100, R. Speck contributed intellectually, S. Chan developed and assisted with coreceptor specificity assays, and R. Connor provided the sequential primary isolates. My contributions to this chapter involved preparing and titering many of the primary isolate viral stocks, developing a flow cytometric approach for looking at coreceptor-specific $CD4^+$ T-

cell depletion, testing the CXCR4-inhibitors, performing the experiments presented in Figures 1 and 2, providing intellectual input, and some editing of the manuscript. M. Goldsmith supervised this work.

JOURNAL OF VIROLOGY, Jan. 2000, p. 184–192
0022-538X/00/$04.00+0

Vol. 74, No. 1

Viral Entry through CXCR4 Is a Pathogenic Factor and Therapeutic Target in Human Immunodeficiency Virus Type 1 Disease

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Received 7 September 1999/Accepted 17 September 1999

The chemokine receptors CCR5 and CXCR4 function as the principal coreceptors for human immunodeficiency virus type 1 (HIV-1). Coreceptor function has also been demonstrated for a variety of related receptors in vitro. The relative contributions of CCR5, CXCR4, and other putative coreceptors to HIV-1 disease in vivo have yet to be defined. In this study, we used sequential primary isolates and recombinant strains of HIV-1 to demonstrate that CXCR4-using (X4) viruses emerging in association with disease progression are highly pathogenic in ex vivo lymphoid tissues compared to CXCR4-independent viruses. Furthermore, synthetic receptor antagonists that specifically block CXCR4-mediated entry dramatically suppressed the depletion of CD4⁺ T cells by recombinant and clinically derived X4 HIV-1 isolates. Moreover, in vitro specificity for the additional coreceptors CCR3, CCR8, BOB, and Bonzo did not augment cytopathicity or diminish sensitivity toward CXCR4 antagonists in lymphoid tissues. These data provide strong evidence to support the concept that adaptation to CXCR4 specificity in vivo accelerates HIV-1 disease progression. Thus, therapeutic intervention targeting the interaction of HIV-1 gp120 with CXCR4 may be highly valuable for suppressing the pathogenic effects of late-stage viruses.

Human immunodeficiency virus type 1 (HIV-1) typically uses CD4 and a coreceptor to gain entry into target cells (reviewed in reference 3). The chemokine receptors CCR5 and CXCR4 are considered the major coreceptors in HIV-1 infection, since most or all isolates exhibit specificity for one or both of these receptors. The discovery of these entry cofactors unraveled the molecular basis underlying the differences in cellular tropism in vitro among HIV-1 strains. Whereas non-syncytium-inducing (NSI) macrophagetropic viruses use CCR5 to enter primary CD4⁺ T cells and macrophages, syncytium-inducing (SI) viruses infect primary CD4⁺ T cells and T-cell lines via CXCR4. NSI CCR5-using (R5) viruses represent the predominant virus population early after infection and persist throughout all stages of disease, whereas SI CXCR4-using (X4) viruses and/or viruses with dual tropism (R5/X4) typically emerge later in disease in temporal association with rapid CD4⁺ T-cell decline and progression to AIDS (11, 23, 29, 47, 51, 54, 56). The importance of CCR5 as an HIV-1 coreceptor in vivo became apparent with the discovery that individuals homozygous for a mutant CCR5 allele (CCR5 Δ32) are highly resistant to HIV-1 infection (12, 27, 34, 46). These findings collectively suggest an important contribution of X4 variants to HIV-1 pathogenesis in later stages of disease, whereas R5 variants appear to be crucial for transmission and establishment of infection.

Numerous in vitro studies have revealed that HIV-1 coreceptor specificity is not limited to CXCR4 and CCR5. Several other human chemokine receptors and related orphan receptors exhibit coreceptor activity for select HIV-1 strains in cellular infection assays (reviewed in reference 3). In addition to CCR5 and CXCR4, the potential coreceptor repertoire of HIV-1 includes the human chemokine receptors CCR2b, CCR3, CCR8, CCR9, and CX_3CR1 (V28) and the orphan receptors GPR1, BOB (GPR15), Bonzo (STRL33), APJ, and ChemR23. In addition, the leukotriene B4 receptor and the human cytomegalovirus-encoded, chemokine-like receptor US28 permit cellular entry by some HIV-1 isolates in vitro. To date, all HIV-1 isolates found to use any of these receptors also use CCR5 and/or CXCR4. Although there is evidence that many of these receptors are expressed in relevant CD4⁺ HIV-1 target cells (9, 13, 19, 26, 38, 39, 45, 52), the importance of these additional coreceptors for HIV-1 pathogenesis in vivo remains undefined.

The discovery of HIV-1 coreceptors provided fundamental insights into the mechanism of viral entry and viral evolution, rendering the virus-coreceptor interaction a novel and highly attractive target for therapeutic intervention in HIV-1 disease. In view of the large number of potential alternate coreceptors and the ability of the virus to change its coreceptor specificity readily through evolution within the *env* gene (16, 36, 53), defining the in vivo significance of the various coreceptors in HIV-1 transmission and disease progression is crucial for the design of such antiviral strategies.

The aim of this study was to elucidate the contributions of distinct coreceptor specificities to HIV-1 disease by evaluating these properties in human lymphoid histocultures. This ex vivo system recapitulates key aspects of viral pathogenesis within the tissue context of dominant HIV-1 replication sites in vivo (20, 21). We reported recently that the specificity of recombinant HIV-1 strains for CXCR4 promotes aggressive depletion of CD4⁺ T cells in this model system (40), which illustrates the

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potential in vivo impact of coreceptor specificity. The present study demonstrates that the X4 phenotype of recombinant HIV-1 strains and primary HIV-1 isolates is linked to a highly pathogenic phenotype in ex vivo lymphoid tissues, which is substantially inhibited by interfering with CXCR4-mediated entry. Furthermore, we show that promiscuous use of the additional coreceptor CCR3, CCR8, BOB, or Bonzo in vitro does not promote accelerated $CD4^+$ T-cell depletion in ex vivo lymphoid tissues. Finally we provide evidence that in vitro coreceptor specificity does not necessarily reflect relevant coreceptor affinities in biological systems. Together, our findings further highlight the critical role of gp120-CXCR4 interactions in the depletion of $CD4^+$ T cells by HIV-1 and provide direct evidence that antagonism of this interaction may have therapeutic value for suppressing the pathogenic effects of HIV-1 contributing to disease progression.

MATERIALS AND METHODS

Cell lines and receptor plasmids. 293T cells and COS-7 cells were cultured as described previously (5). Plasmids expressing CD4 (pCD4neo), CXCR4 (pCMVFCXCR4), CCR5 (pCMVFCCR5), CCR3 (pCDNA3-CCR3p), and CCR8 (pAW-CCR8F) were previously described (1, 5, 22, 40). Plasmids expressing BOB/GP15 (pcDNA-GP15) and Bonzo/STRL33 (pcDNA-STRL33) were a gift of Kuldeep Neote.

Construction of recombinant viruses. NL4-3 provirus expression plasmid p4-14 (7), containing unique restriction sites flanking the C2 and V3 regions of the viral *env* gene, was used as the isogenic viral backbone for constructing C2- and V3 replacements. C2-V3 regions were obtained by PCR with oligonucleotide primers containing the *Sta*I and *Nhe*I cloning sites from plasmids holding the envelope sequences of the primary isolates HIV-1 92UG037-8 and 92HT593-1 (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) and HIV-1 ADA (55). Cloned fragments were ligated into the *Sta*I/*Nhe*I-digested backbone of plasmid p4-14, a gift of Bruce Chesebro. Construction of V3 recombinants 4-9 and 6-4 has been described elsewhere (5).

Preparation of virus stocks. Molecular clones pNL4-3 and pNL-Luc-R E were obtained from Malcolm Martin and Ned Landau, respectively, via the AIDS Research and Reference Reagent Program. The molecular clones 49-5 (7), 242 (8), and 241 (8) were generous gifts from Bruce Chesebro. Infectious virus stocks were prepared as previously described (5, 22). The p24 Gag concentration was assessed by enzyme-linked immunosorbent assay (NEN Life Sciences, Boston, Mass.). Pseudotyped virus stocks carrying the luciferase gene with the various envelopes were prepared similarly by cotransfecting pNL-Luc-R E along with full-length proviral plasmids, and supernatant was harvested as described earlier (5). The primary isolates 1/85, 7/86, 12/86, and 11/89 (11) were expanded by infection of heterologous peripheral blood mononuclear cells (PBMC). PBMC from four different donors were isolated from whole blood buffy coats (Stanford Blood Bank, Palo Alto, Calif.) by Ficoll-Hypaque density gradient centrifugation (Histopaque 1077; Sigma, St. Louis, Mo.) and cultured for 48 h in medium (RPMI 1640; Mediatech, Washington, D.C.) supplemented with 10% fetal calf serum (Gemini Biological Products) and phytohemagglutinin (1 μg/ml; Sigma). The PBMC were then washed and cultured for at least 7 days in RPMI 1640 medium with 10% fetal calf serum and recombinant human interleukin-2 (5 U/ml; courtesy of Chiron Corp.).

Coreceptor utilization assay for single-round infection. 293T cells were seeded in six-well plates and transfected by the $CaPO_4$ method (Promega, Madison, Wis.) with pCD4neo alone or in combination with pCMVFCXCR4, pCMVFCCR5, pcDNA-CCR3p, pAW-CCR8F, pcDNA-GP15, or pcDNA-STRL33 according to the manufacturer's instructions. COS-7 cells were transfected by the Lipofectamine method as described earlier (1, 5). After 24 h, cells were infected with pseudotype viruses, and luciferase expression was assessed 48 h postinfection by measuring enzymatic activity (relative light units) as instructed by the manufacturer (Analytic Luminescence Laboratory, San Diego, Calif.).

Selectivity of CXCR4 antagonists assessed in single-round infections. COS-7 cells were transiently transfected with pCD4neo in combination with pCMVFCXCR4 or pCMVFCCR5 as described above. Transfected cells were inoculated with pseudotype reporter viruses in the presence and absence of the CXCR4 antagonist AMD3100 (250 nM) or T22 (1 μM) (custom preparation by American Peptide Company, Inc., Sunnyvale, Calif.) and subsequently infected with luciferase-expressing reporter viruses. Luciferase expression was assessed 48 h postinfection.

Infection of human lymphoid tissues ex vivo. Human noninflammatory spleen tissue derived postsurgery or postautopsy (provided by National Diabetes Research Interchange, Philadelphia, Pa.) and tonsil tissue removed during routine tonsillectomy (provided by San Francisco General Hospital and Kaiser-San Francisco, San Francisco, Calif., and Kaiser-San Rafael, San Rafael, Calif.) were prepared and inoculated with HIV-1 strains (approximately 0.1 ng for primary isolates and up to 5 ng/block for HIV-1 recombinants) as described previously (21, 40). Productive HIV-1 infection was assessed by measuring the amount of p24 antigen that accumulated in the culture medium between the successive changes of medium; 10 to 14 days postinfection, cells were mechanically isolated from infected and uninfected control tissue and analyzed by flow cytometry (fluorescence-activated cell sorting [FACS]) (see below).

Inhibition of CXCR4-mediated entry in ex vivo lymphoid histocultures by CXCR4-specific antagonists. At 12 to 24 h prior to infection, the culture medium was supplemented with the CXCR4 antagonist AMD3100 (250 nM) or T22 (1 μM). Virus stocks were diluted 1:1 with medium containing the relevant antagonist in the indicated concentrations and applied on top of the tissue blocks. Untreated tissue was infected with equal amount of virus diluted 1:1 with plain culture medium. Plain medium or medium containing the relevant antagonist was successively changed in the untreated or treated cultures. Viral replication was assessed by measuring the p24 concentration in the culture medium that accumulated between medium changes.

Assessment of $CD4^+$ T-cell depletion by flow cytometry. Dispersed cells from infected and uninfected lymphoid histocultures were stained for cell surface markers CD3, CD4, CD8, and CCR5 as described previously (40) by using anti-CD3 (clone SK7, allophycocyanin conjugated), anti-CD4 (clone SK3, fluorescein isothiocyanate conjugated), anti-CD8 (clone SK1, peridinin chlorophyll protein conjugated) (Becton Dickinson, San Jose, Calif.), and anti-CCR5 (clone 2D7, phycoerythrin conjugated; Pharmingen, San Diego, Calif.) monoclonal antibodies; 5,000 to 10,000 lymphocytes positive for CD3 surface marker were counted, and the data were analyzed with CellQuest software (Becton Dickinson). To facilitate comparison among experiments, $CD4^+$ T-cell depletion was assessed by measuring the ratio of $CD4^+$ to $CD8^+$ T cells. This value was normalized to the CD4/CD8 ratio of control (uninfected) samples to yield the mean relative CD4/CD8 ratio. Results are reported as mean relative CD4/CD8 ratio with standard error of the mean (SEM).

RESULTS

Minimal changes in the HIV-1 envelope are associated with increased depletion of $CD4^+$ T cells. In a prior study, we found that CXCR4 coreceptor specificity alone is sufficient for HIV-1 to cause severe $CD4^+$ T-cell depletion in human lymphoid histocultures (40). A cloned HIV-1 strain (NL4-3) that exhibits selectivity for CXCR4 (53) induced severe $CD4^+$ T-cell depletion in ex vivo tonsil cultures, as represented by a profound decrease of the CD4/CD8 ratio among $CD3^+$ lymphocytes, whereas an isogenic R5 strain (49-5) (7) induced only mild depletion (Fig. 1A). This finding was extended by assessing T-cell depletion in tonsil histocultures upon infection with a second isogenic virus pair, 242 and 241, which differ by a single amino acid substitution within the V3 loop (R5 [242] or R5/X4 [241]) (8, 53). The CCR5-restricted recombinant 242 caused only mild depletion in tonsil histocultures, whereas the dualtropic virus 241 severely depleted $CD4^+$ T cells (Fig. 1A). Comparable virus-specific effects were observed in spleen tissue (data not shown). Viruses 241 and 242 replicated with similar kinetics in the histoculture system, as demonstrated by progressive p24 secretion into the medium (Fig. 1B). These results indicate that minimal changes in the *env* gene that enable HIV-1 to engage CXCR4 in addition to CCR5 result in dramatic enhancement of $CD4^+$ T-cell depletion compared to the variant that is restricted to CCR5. In this setting of a dualtropic envelope, the aggressive T-cell depletion phenotype linked to CXCR4 usage dominates over the mild depletion phenotype linked to CCR5 usage.

The emergence of X4 variants in vivo is associated with heightened virulence. The results obtained with recombinants 242 and 241 may reflect the phenotypic evolution of HIV-1 in vivo. To test directly the hypothesis that the X4 variants emerging in patients accelerate $CD4^+$ T-cell decline, we assessed the depletion potential of longitudinal primary HIV-1 isolates derived from an infected individual. These previously described biological isolates reflect the evolution from CCR5-restricted viruses early in infection to viruses with expanded coreceptor specificities, including CXCR4, with disease progression (11). $CD4^+$ T-cell depletion and viral replication were assessed upon infection of tonsil histocultures with the sequential iso-

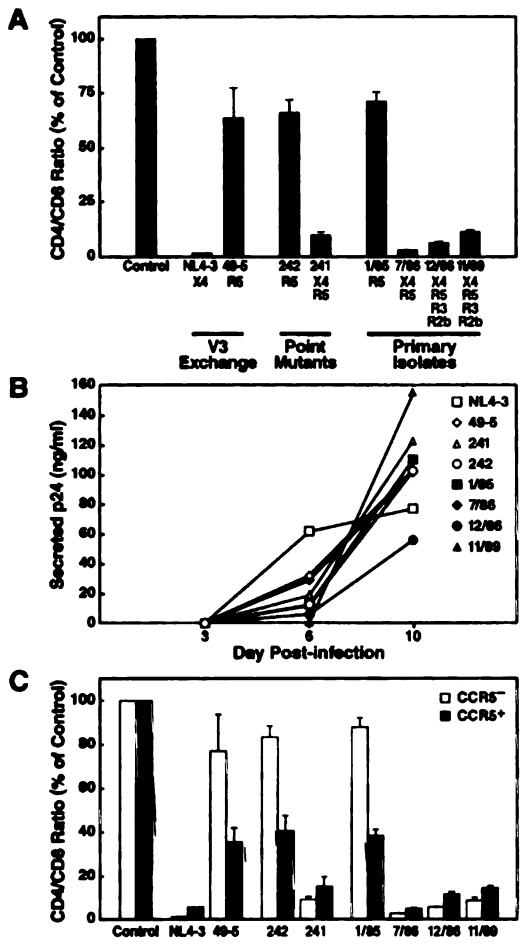

FIG. 1. Coreceptor specificity for CXCR4 by recombinant and primary isolates is linked with heightened virulence in ex vivo lymphoid tissues. (A) Previously determined coreceptor specificities (11, 40, 53) for each recombinant or primary virus are indicated. CD4⁺ T-cell depletion in ex vivo tonsil cultures was assessed by FACS analysis on day 10 postinfection; shown are mean relative CD4/CD8 ratios (n = 3) with SEM. (B) Viral replication was monitored by assessing the accumulation of p24 in the culture medium between successive medium changes on days 3, 6, and 10 postinfection. (C) In the same infection, T-cell depletion within the CCR5⁺ and CCR5⁻ subsets of CD4⁺ T cells was analyzed by multiparameter FACS on day 10 postinfection. Shown are mean relative CD4/CD8 ratios (n = 3) with SEM for CCR5⁺ and CCR5⁻ CD4⁺ T cells.

lates. The earliest isolate tested, 1/85 (R5), induced mild depletion of CD4⁺ T cells, whereas all subsequent isolates (7/86 [R5/X4], 12/86 [R5/X4/R3/R2b], and 11/89 [R5/X4/R3/R2b]) that had adapted to use CXCR4 aggressively depleted CD4⁺ T cells in these cultures (Fig. 1A). Despite differences in coreceptor specificity (11), all of these primary isolates replicated to comparable levels, as measured by the amount of viral p24 protein released into the culture medium (Fig. 1B). No significant difference in the CD4⁺ T-cell depletion potential was found among isolate 7/86 (R5/X4) and the later isolates (12/86 and 11/89 [R5/X4/R2b/R3]), indicating that specificity for CXCR4 is sufficient to cause profound CD4⁺ T-cell depletion. These results strongly suggest that based on increased T-cell depletion in ex vivo lymphoid tissue, the adaptation of viral strains in vivo to use CXCR4 is likely to promote disease progression and rapid CD4⁺ T-cell loss.

Target cell specificity is linked to coreceptor expression. Assessment of CXCR4 and CCR5 expression via flow cytometry in human spleen and tonsil specimens revealed differential expression of the viral coreceptors (24; J. C. Grivel, M. L. Penn, D. Eckstein, B. Schramm, R. F. Speck, N. W. Abbey, B. Herndier, L. Margolis, and M. A. Goldsmith, submitted for publication). Whereas CXCR4 typically is present on the vast majority of the CD4⁺ T lymphocytes, CCR5 is found on the surface of a minority of CD4⁺ T cells and varies with different donors. Therefore, most CD4⁺ T cells are potential targets for X4 HIV-1 strains, whereas fewer cells are predicted to be targets for R5 strains. To determine whether coreceptor specificity is linked to depletion of select subsets of CD4⁺ T cells by individual strains, multiparameter flow cytometry was used to detect the CD4, CD3, and CCR5 surface markers. This analysis revealed that R5 recombinant viruses (49-5 and 242) preferentially depleted the CCR5⁺ T-cell subset relative to the CCR5⁻ subset, while X4 recombinants (NL4-3 and 241) potently depleted both subsets (Fig. 1C). Comparable results were obtained for the longitudinal primary isolates. The early CCR5-dependent isolate (1/85) preferentially depleted CCR5⁺ cells, whereas isolates with expanded coreceptor tropism, including CXCR4, depleted aggressively in both CCR5⁺ and CCR5⁻ subsets (Fig. 1C). Therefore, the depletion patterns generated by the recombinant and primary strains correspond to the expression patterns of CCR5 and CXCR4 among CD4⁺ T cells.

Interference with CXCR4-mediated entry blocks CD4⁺ T-cell depletion. Based on these findings, we predicted that interfering with CXCR4-mediated entry by recombinant or primary HIV-1 isolates would attenuate the severe depletion of CD4⁺ T cells. To test this hypothesis, we infected spleen histocultures with the cloned X4 strain NL4-3 and the primary isolate 7/86 in the presence and absence of two independent CXCR4 antagonists, T22 (1 μM), a synthetic peptide that specifically and potently inhibits infection by X4 HIV-1 strains (37), and the bicyclam AMD3100, which is highly potent in blocking X4 HIV-1 replication in suspension cultures (14, 50). CD4⁺ T-cell depletion was assessed on day 14 postinfection. As expected, in the absence of CXCR4 antagonists, NL4-3 markedly depleted CD4⁺ T cells, as represented by a decrease of the CD4/CD8 ratio to 11% of the uninfected control sample (Fig. 2A). Both depletion (Fig. 2A) and viral replication (Fig. 2B) by NL4-3 were completely abrogated in the presence of either T22 or AMD3100. Furthermore, cellular depletion by the primary isolate 7/86 was substantially, although not completely, suppressed in the presence of T22 or AMD3100 (Fig. 2A). In the absence of the CXCR4 antagonists, isolate 7/86 decreased the CD4/CD8 ratio to 18% of uninfected control tissue, whereas CD4⁺ T-cell depletion in the presence of T22 or AMD3100 was markedly attenuated, as indicated by CD4/CD8 ratio of 66 or 47%, respectively, of uninfected control cultures (Fig. 2A), demonstrating the impact of CXCR4 usage on the cytopathic effect of this isolate.

Compared with the influence on cellular depletion, the effect of the CXCR4 antagonists on viral replication of the primary isolate was more modest, causing only a slight delay of p24 output compared to untreated infections (Fig. 2C). The molecular basis of this discordance is uncertain, although it may represent selective outgrowth of CXCR4-independent and/or inhibitor-insensitive strains within the uncloned isolate. In any event, this finding implies that virus replication per se does not

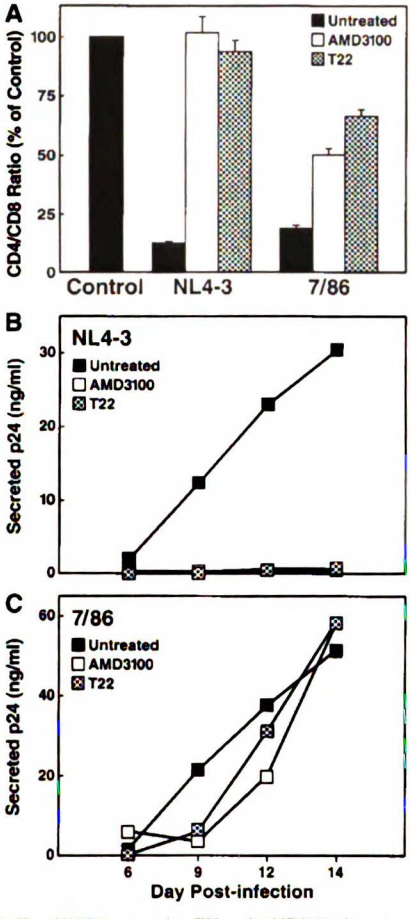

FIG. 2. The CXCR4 antagonists T22 and AMD3100 abrogate replication and depletion of the cloned HIV-1 strain NL4-3 and substantially diminish virulence of the primary isolate 7/86. (A) $CD4^+$ T-cell depletion in ex vivo cultures of human spleen tissue was assessed on day 14 postinfection in the absence or presence of the CXCR4 antagonist AMD3100 (250 nM) or T22 (1 μM) in the culture medium. Shown are mean relative CD4/CD8 ratios ($n = 3$) with SEM. Viral replication was monitored for NL4-3 (B) and 7/86 (C) by assessing the accumulation of p24 in the culture medium between medium changes on days 6, 9, 12, and 14 postinfection.

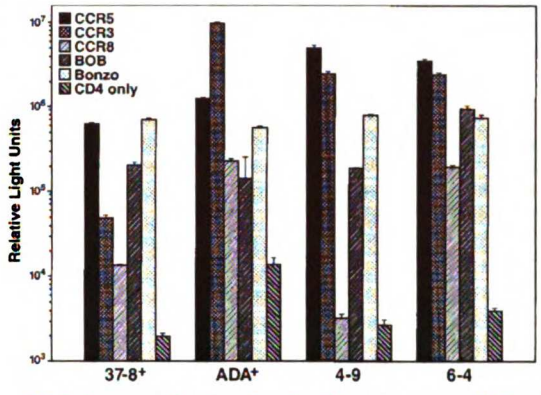

FIG. 3. Coreceptor utilization pattern of gp120 exchange mutants. 293T cells transiently expressing CD4 alone or in combination with the coreceptor CCR5, CCR3, CCR8, BOB, or Bonzo were infected with a pseudotype reporter virus carrying the envelope of the recombinant ADA^+, $37\text{-}8^+$, 4-9, or 6-4; 48 h postinfection, luciferase expression was assessed by measuring enzymatic activity (relative light units). The relative signals obtained for different coreceptors varied somewhat between multiple experiments, but the coreceptor pattern for a given virus was consistent.

necessarily equate to pathogenesis and emphasizes the importance of monitoring both virologic and cellular responses to a potential therapy.

Collectively these depletion studies with the CXCR4 antagonists confirm that the specificity for CXCR4 is a major determinant for the aggressive virulence of recombinant and primary isolates, and they provide additional evidence that the emergence of viruses with CXCR4 specificity contributes directly to acceleration of $CD4^+$ T-cell depletion in vivo.

Contribution of additional coreceptors to $CD4^+$ T-cell depletion. HIV-1 coreceptor usage is not strictly limited to CXCR4 and CCR5, since a number of other human chemokine receptors and related orphan receptors have been shown to serve as coreceptors for HIV-1 in vitro (reviewed in reference 3). To investigate the contribution of these additional coreceptors to pathogenicity of HIV-1, we assessed $CD4^+$ T-cell depletion in lymphoid histocultures upon infection with multitropic viruses. For these experiments we used recombinant HIV-1 strains differing only in coreceptor determinants in the *env* gene in order to assign a singular causative role to coreceptor preferences if enhanced T-cell depletion was observed. Isogenic viruses were constructed by subcloning select envelope determinants of coreceptor specificity into a constant viral background (NL4-3). NL4-3 recombinants encoding the C2-V3 regions of the NSI R5 primary isolate 92UG037.8 and NSI R5 HIV-1 ADA (ADA^+ and $37\text{-}8^+$) were characterized for their coreceptor utilization patterns in a standard single-round infection assay. 293T cells transiently expressing CD4 alone or in combination with various coreceptors were infected with luciferase reporter viruses pseudotyped with the corresponding envelopes of viruses ADA^+ and $37\text{-}8^+$. Both recombinants efficiently infected cells expressing CCR5 along with CD4, whereas only background level signals were obtained with CD4 alone (Fig. 3). In addition, $37\text{-}8^+$ infected cells via Bonzo and to a somewhat lesser extent via BOB, CCR3, and CCR8 (Fig. 3). The ADA^+ recombinant exhibited specificity for CCR5, CCR3, CCR8, BOB, and Bonzo (Fig. 3). The relative signals obtained for infections with different coreceptors varied somewhat among multiple assays, but the utilization patterns were consistent. We also used two R5 NL4-3 recombinants that we recently described, 4-9 and 6-4, encoding the V3 regions of colon-derived HIV-1 envelope sequences (5). Both 4-9 and 6-4 used CCR5 and CCR3 in the entry assay, whereas 6-4, but not 4-9, exhibited entry via CCR8 (Fig. 3). In addition, both viruses exhibited specificity for BOB and Bonzo (Fig. 3). To determine whether the ability to enter cells via additional coreceptors in vitro affects the depletion potential of such viruses in biological systems, we infected tonsil and spleen histocultures with these V3 exchange mutants and assessed $CD4^+$ T-cell depletion. The recombinant $37\text{-}8^+$ only mildly depleted $CD4^+$ T-cells in ex vivo spleen cultures, whereas the CXCR4-specific parent NL4-3 profoundly depleted $CD4^+$ cells in the same tissues (Fig. 4A). In analogous experiments performed with tonsil tissues, the multitropic recombinants ADA^+, 4-9, and 6-4 likewise displayed a modest T-cell deple-

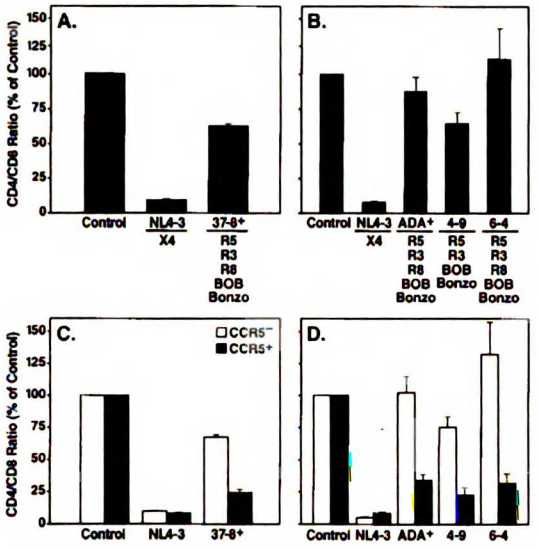

FIG. 4. CD4+ T-lymphocyte depletion by multitropic HIV-1 recombinants. (A) CD4+ T-cell depletion in human spleen tissue infected with HIV-1 recombinant NL4-3 or 37-8+. Shown are mean relative CD4/CD8 ratios with SEM determined on day 14 postinfection. (B) CD4+ T-cell depletion in human tonsil tissue infected by the recombinants NL4-3, ADA+, 6-4, and 4-9 as measured on day 12 postinfection. (C and D) Subset analysis of data from panels A and B, respectively, with CD4+ T-cell depletion stratified into CCR5+ and CCR5− subsets. The results shown are representative of two to three experiments performed with spleen and tonsil tissues from different donors for the viruses. All viruses replicated to similar levels in the corresponding spleen (total of accumulated p24 in panel A, 134 [NL4-3] and 127 [37-8+] ng/ml) and tonsil (total of accumulated p24 in panel B, 126 [NL4-3], 212 [ADA+], 196 [4-9], and 160 [6-4] ng/ml) tissue.

tion potential in contrast to the severe depletion of $CD4^+$ T cells by NL4-3 (Fig. 4B). All NL4-3 recombinants replicated to levels comparable to those for NL4-3, as assessed by p24 release in the culture supernatant (see the legend to Fig. 4). These results demonstrate that the ability of R5 strains of HIV-1 to enter cells via CCR3, CCR8, BOB, or Bonzo in vitro does not correspond to enhanced T-cell depletion potential as assessed in ex vivo spleen and tonsil histocultures.

As another strategy for determining the pathogenic significance of alternate coreceptor usage, we measured specific cellular subset depletion patterns as described earlier. To this end, $CD4^+$ T cells from experiments shown in Fig. 4A and B were analyzed for expression of CCR5. Viruses $37\text{-}8^+$, ADA^+, 4-9, and 6-4 exhibited preferential depletion of $CCR5^+$ $CD4^+$ T cells in spleen (Fig. 4C) and tonsil (Fig. 4D) tissues irrespective of their ability to use additional coreceptors in vitro, in contrast to the depletion of both $CCR5^+$ and $CCR5^-$ $CD4^+$ T-cell subsets by NL4-3 (Fig. 1C, 4C, and 4D).

Overall, these results demonstrate that the ability to use CCR3, CCR8, BOB, and Bonzo in in vitro entry assays does not alter the preference of R5 viruses to deplete the $CCR5^+$ subset of $CD4^+$ T cells, suggesting that the use of alternate coreceptors does not confer additional virulence in ex vivo lymphoid tissues.

Blockade of CXCR4 abrogates replication and depletion by a multitropic HIV-1. Our results demonstrated that aggressive depletion of $CD4^+$ T cells by HIV-1 infection in lymphoid histocultures requires the use of CXCR4 as a coreceptor, whereas specificity for CCR5 in combination with additional coreceptors results in mild depletion of $CD4^+$ T cells. We sought to confirm the linkage between CXCR4 specificity and high pathogenicity in the context of a multitropic HIV-1 recombinant. We therefore created the recombinant HT^+, encoding the C2-V3 envelope regions of the primary HIV-1 isolate 92HT593.1 in the NL4-3 backbone. The in vitro coreceptor utilization pattern of HT^+ was determined in the single-round luciferase reporter assay using COS-7 cells transiently expressing CD4 and the respective coreceptors. HT^+ exhibited specificity for CXCR4, CCR5, and Bonzo, with less pronounced usage of CCR3 (Fig. 5A); usage of CCR8 and BOB was in the range of background levels obtained with CD4 alone. When spleen cultures were infected with HT^+, strong depletion of $CD4^+$ T cells was observed, which was similar to the effect of the NL4-3 control virus (Fig. 5C). To determine the relative contribution of individual coreceptors to this effect, we performed infections in the presence and absence of two independent CXCR4 antagonists, T22 (37) (1 μM) and AMD3100 (14, 50) (250 nM). Somewhat surprisingly, cellular depletion and viral replication by the multitropic HT^+ was completely suppressed in the presence of T22 and AMD3100, similar to results for the CXCR4-restricted NL4-3 (Fig. 5C and D). In single-round infection assays using luciferase reporter viruses, we verified the selective effects of both antagonists in the concentrations used in the histoculture infections. Infection of COS-7 cells transiently expressing CD4 and CXCR4 by NL4-3 and HT^+ was completely blocked in the presence of either T22 (1 μM) or AMD3100 (250 nM), while infection of $CD4^+$ $CCR5^+$ cells by HT^+ was not affected (Fig. 5B). Together, these findings imply that the $CD4^+$ T-cell depletion and replication by HT^+ in ex vivo lymphoid tissue is solely a result of its specificity for CXCR4. Therefore, the qualitative ability to enter cells via certain receptors in vitro does not necessarily enable the virus to infect productively or to deplete lymphocytes via these receptors in biological systems in which quantitative or relative usage may be an important variable.

DISCUSSION

A compelling relationship between HIV-1 coreceptor specificity and disease pathogenesis has been suggested by prior epidemiologic evidence (11, 12, 27, 28, 34, 46, 47). A valuable system in which to elucidate this relationship directly is ex vivo human lymphoid histocultures (20, 21) since lymphoid tissues are crucial sites of viral replication and immune system destruction in vivo. We have shown that HIV-1 envelope specificity for CXCR4 is linked to aggressive depletion of the $CD4^+$ T-cell population in such cultures (40). The profound influence of coreceptor specificity on virulence appears to be largely attributable to differences in the availability of target cells expressing individual coreceptors (24; Grivel et al., submitted).

In this study, we used lymphoid histocultures to elucidate further the linkage between coreceptor specificity and the pathogenic potential of HIV-1. Our earlier work demonstrated that subtle changes in the HIV-1 envelope glycoprotein, gp120, are sufficient to drive drastic changes in coreceptor specificity (53). We found here that a single amino acid substitution causing a shift from R5 to R5/X4 phenotype dramatically enhanced HIV-1-induced pathogenicity as defined by $CD4^+$ T-cell depletion, which parallels the typical viral evolution pattern in vivo. Thus, the heightened virulence linked to the X4 phenotype is dominantly expressed, as has been observed in another model system (36). To verify the biological relevance of these experimental viruses, we also assessed the pathogenic potential of primary viral isolates that had been obtained lon-

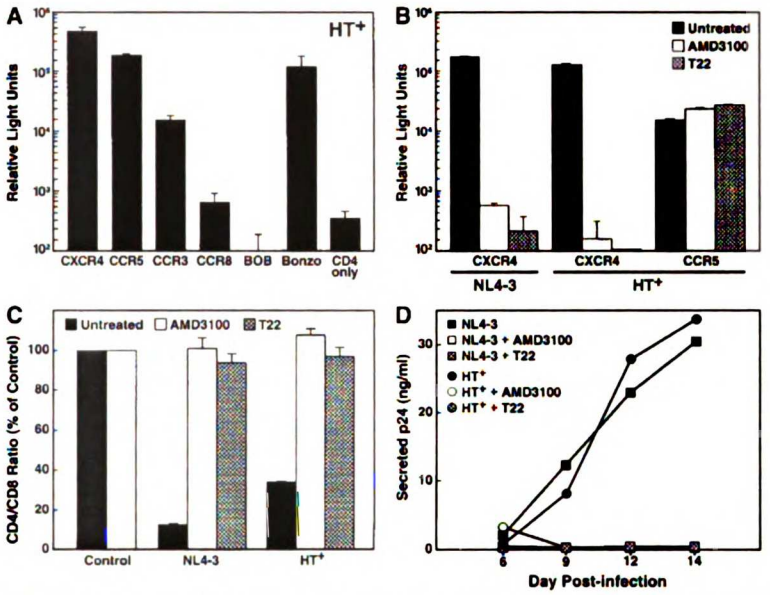

FIG. 5. Inhibitory effect of CXCR4 antagonists on CD4+ T-cell depletion and replication by the multitropic HIV-1 recombinant HT+. (A) In vitro coreceptor specificity of HT+ assessed by infection of COS-7 cells transiently expressing CD4 alone or in combination with the corresponding coreceptor. Pseudotype reporter viruses expressing luciferase and carrying the envelope of HT+ were used, and luciferase activity was measured. (B) Selective inhibition of CXCR4-mediated entry by CXCR4 antagonists AMD3100 and T22. Transfected COS-7 cells were infected with the HT+ reporter viruses in the absence or presence of the antagonist AMD3100 (250 nM) or T22 (1 μM). (C) CD4+ T-cell depletion in spleen histocultures upon infection with replication-competent NL4-3 or HT+ in the absence or presence of the antagonist AMD3100 (250 nM) or T22 (1 μM). Shown are mean relative CD4/CD8 ratios with SEM determined on day 14 postinfection. (D) Viral replication of NL4-3 and HT+ in the experiment shown in panel C was monitored by assessing the accumulation of p24 in the culture medium between successive changes of medium.

gitudinally from an HIV-1-infected individual who eventually progressed to AIDS (11). This panel of sequential clinical isolates reflects the typical broadening of coreceptor specificity in association with disease progression. The results of this analysis demonstrate that the expansion to R5/X4 phenotype in vivo is similarly linked to a highly pathogenic phenotype for lymphoid tissues ex vivo, confirming that the emergence of viral specificity for CXCR4 likely accelerates CD4+ T-cell depletion in vivo.

The interaction between HIV-1 and its coreceptor is an attractive target for antiviral therapy, since it may be possible to disable HIV-1 infection at the level of initial cellular entry. Both the remarkable resistance to HIV-1 infection found for individuals homozygous for a CCR5-null allele (12, 27, 32, 34, 46) and the inhibitory effects of the natural receptor ligands or modified chemokines in vitro (4, 6, 10, 33) emphasize the possible therapeutic value of targeting virus-coreceptor interactions. However, the potential of HIV-1 to evade antiviral strategies was suggested by the recent observation that anti-CCR5 agents can select for variants with CXCR4 specificity in a xenotransplant model of HIV disease (36). Our observation that CXCR4 utilization is a key factor in HIV-1 virulence further emphasizes the need to consider this as a possible target for antiviral therapy. Certain synthetic CXCR4 antagonists selectively and potently block CXCR4-mediated cell entry of HIV-1 in vitro (14, 37, 50). Here we investigated the effects of antagonists on recombinant and primary viral isolates in ex vivo cultures of lymphoid tissue. In the presence of the CXCR4 antagonists T22 (37) and AMD3100 (14, 50), replication and CD4+ T-cell depletion by the X4 clone NL4-3 were completely abrogated and the pathogenicity of the R5/X4 primary isolate 7/86 was markedly suppressed, demonstrating that interference with CXCR4-mediated entry dramatically reduces pathogenicity of X4 HIV-1 isolates in lymphoid tissues. Together these data provide additional evidence that specificity for CXCR4 is a major determinant of the greater virulence of viruses from late-stage patients, and they underscore the causative relationship between viral specificity for CXCR4 and rapid CD4+ T-cell decline in vivo. Furthermore, these findings emphasize the potential therapeutic value of blocking CXCR4-mediated entry in combating HIV-1 disease progression in vivo.

Interestingly, replication of the primary isolate 7/86 was only slightly delayed in the presence of the CXCR4 antagonist T22 or AMD3100 despite marked suppression of CD4+ T-cell depletion. Based on the reported dualtropic character of isolate 7/86 (11), which likely reflects a heterogeneous virus population, robust viral replication and rather mild depletion of CD4+ T cells in the presence of the inhibitor might be due to the outgrowth of variants with specificity for CCR5 that are less aggressive in depleting CD4+ T cells. This observation is in agreement with a recent study performed in vitro, demonstrating that the X4 phenotype of clinical isolates was completely suppressed in the presence of the CXCR4 antagonist AMD3100, resulting in the outgrowth of R5 variants (16). Incomplete inhibition of X4 strains as a result of differences in receptor affinity and/or receptor engagement also cannot be excluded, as it might contribute to some extent to the residual T-cell depletion and the overall viral replication potential of isolate 7/86 observed here. Precedence for differential engagement of CXCR4 as a cause of CXCR4 ligand or antagonist insensitivity was shown in an earlier study by Schols et al., demonstrating that a T-cell line-adapted X4 HIV-1 strain was

able to overcome the inhibitory effects of SDF-1 and AMD3100 through the accumulation of mutations in gp120 that preserved its dependence on CXCR4 (49). These observations are potentially of great interest with respect to the development of potent antiviral agents targeting virus-coreceptor interactions but require further investigation including a broader test range of primary isolates.

In addition to CCR5 and CXCR4, more than 10 chemokine receptors and related orphan receptors have been shown to confer HIV-1 entry in vitro (reviewed in reference 3). Although the in vivo expression pattern of these additional coreceptors is not well defined, preliminary reports based mainly on detection of mRNA expression levels have suggested that most of these candidate receptors are expressed on $CD4^+$ HIV-1 target cells (9, 13, 19, 26, 38, 39, 45, 52). In general, alternative coreceptors are irregularly used by HIV-1 and appear to be less efficient than CCR5 and CXCR4 (5, 15, 42, 45, 57). Contradictory results for HIV-1 coreceptor specificities of certain HIV-1 strains have been obtained depending on the assay system used (2, 15, 42, 44). Furthermore, differences in coreceptors expression levels can influence coreceptor activity (30, 41, 44). Therefore, most in vitro studies on coreceptor usage should be interpreted qualitatively rather than quantitatively. In view of the high mutation rate of HIV-1, high viral turnover in vivo, and the resulting potential of HIV-1 to escape coreceptor antagonism by simply changing its coreceptor specificities, it is critical to elucidate the in vivo relevance of alternative coreceptors to the natural history of disease and/or possible therapeutic intervention. We addressed this issue by assessing the lymphocyte depletion potential of isogenic viruses differing only in their envelope-specified in vitro coreceptor utilization patterns, which allowed us to focus on cytopathic effects driven by coreceptor specificity. The recombinants used were specific for the principal coreceptor CCR5 and displayed additional usage of CCR3, CCR8, BOB, or Bonzo based on conventional single-round luciferase reporter assays in vitro. Infection of lymphoid tissues with these multitropic viruses resulted in rather mild $CD4^+$ T-cell depletion in contrast to severe $CD4^+$ T-cell depletion caused by X4 strains. Another strategy for detecting changes in target cell specificity is the stratification of $CD4^+$ T cells based on coreceptor expression to seek differences in the subsets of cells depleted by such viruses. Subset analysis based on CCR5 expression following infection with these multitropic viruses revealed no evidence for an expansion of the target cell population as a result of additional specificity for alternate coreceptors. Therefore, CCR5 and CXCR4 appear to be the dominant coreceptors influencing killing of T cells in these lymphoid tissues.

We also extended the analysis to a multitropic gp120 recombinant HIV-1 (HT^+) with specificity for CXCR4, CCR5, CCR3, and Bonzo in vitro and high pathogenic potential in ex vivo lymphoid tissues. To uncover the coreceptor specificities that are responsible in this multitropic context for high virulence in lymphoid cultures, we sought to inhibit viral entry via CXCR4. Somewhat surprisingly, this multitropic strain displayed great sensitivity toward two CXCR4 antagonists, T22 (37) and the bicyclam AMD3100 (14, 50). Both $CD4^+$ T-cell depletion and viral replication were completely abrogated in the presence of the inhibitors, similar to the effects obtained with the CXCR4-restricted strain NL4-3. A dominant interaction between the CXCR4 antagonists and the multitropic envelope that would result in interference with viral entry via coreceptors other than CXCR4 can be excluded as explanation for this effect since $CCR5^-$, $CCR3^-$, or Bonzo-mediated cellular entry was not abrogated by either T22 or AMD3100 in vitro (Fig. 5B, data not shown, and reference 31). These results imply that replication and pathogenicity of the recombinant HT^+ in lymphoid tissues derive largely from CXCR4-dependent processes, despite additional coreceptor specificity in vitro. Since CCR5 is clearly expressed in these tissues at levels that confer productive infection by CCR5-restricted viruses, the coreceptor utilization of this particular virus in vitro apparently does not reflect its coreceptor preferences in the relevant biological context. This discordance leads us to conclude that coreceptor activity as assessed in highly sensitive cell-cell fusion and/or reporter entry assays does not necessarily reflect relevant coreceptor affinities of HIV-1 in vivo, since these conventional assays do not allow assessment of the relative preference of one coreceptor versus another. Therefore, although virus phenotyping is of some clinical and scientific utility, the results must be viewed as qualitative and thus be interpreted with caution.

Overall, no evidence was found in our studies for a contribution by the additional coreceptors to viral pathogenicity and/or replication in lymphoid tissues cultured ex vivo. Similarly, a recent study found that in vitro usage of additional coreceptors other than CCR5 or CXCR4 was irrelevant for replication of HIV-1 in activated human peripheral blood cultures (58). In addition, viruses isolated longitudinally from an HIV-1-infected individual who is homozygous for the CCR5 Δ32 null allele exhibited exclusive specificity for CXCR4 (35). These findings collectively argue that additional coreceptors should not preclude the development of therapeutic strategies that aim to interfere with HIV-1 cellular entry. However, it remains possible that alternative coreceptors play important roles for viral replication and pathogenicity in select tissue compartments where HIV-1 infection is manifest, such as thymus, brain, and mucosal surfaces (17, 18, 25, 43, 48).

In conclusion, we have shown with recombinant and biological strains that the in vivo adaptation toward CXCR4 use is linked to heightened virulence in lymphoid tissues as detected in ex vivo histocultures. Furthermore, interference with CXCR4-mediated entry significantly reduces the aggressive $CD4^+$ T-cell depletion potential of recombinant and primary HIV-1 in lymphoid tissues, demonstrating the impact of CXCR4 specificity on pathogenicity. Together, these findings provide strong evidence that the acquisition of CXCR4 specificity in vivo directly contributes to acceleration of HIV-1 disease progression. No evidence was found that coreceptors other than CXCR4 and CCR5 contribute to HIV-1 pathogenicity in ex vivo lymphoid tissue. Although potential side effects must be examined carefully, these findings indicate that specific inhibition of CXCR4-mediated cellular entry may be highly valuable for suppressing HIV-1 pathogenicity in key sites of viral replication in vivo. A combined therapeutic strategy targeting the principal HIV-1 coreceptors CCR5 and CXCR4 in conjunction with established antiviral agents affecting other steps in the viral life cycle should potently combat HIV-1 infection and disease.

ACKNOWLEDGMENTS

B.S. and M.L.P. contributed equally to this work.

We thank Bruce Chesebro, Malcolm Martin, Ned Landau, and Kuldeep Neote for kindly providing plasmids and Dee J. Holthe, Cecilia Stewart, Claudette Delphis, Ursula Perotti, Nancy W. Abbey, Mark D. Weinstein, and the surgical staffs at Kaiser hospitals (San Rafael and San Francisco, Calif.) and San Francisco General Hospital for generous assistance in obtaining posttonsillectomy samples.

We acknowledge the technical assistance of Eric Wieder and Lisa Gibson in the conduct of these experiments and the assistance of Heather Gravois, John Carroll, and Neile Shea in preparation of the manuscript.

M.L.P. and S.Y.C. were supported by the Biomedical Sciences

Graduate Program and the National Institutes of Health Medical Scientist Training Program at UCSF. R.F.S. was supported by the UCSF-GIVI Center for AIDS Research, B.S. was supported by the Boehringer Ingelheim Fund, and R.I.C. was supported by the Irene Diamond Fund. This work was supported in part by NIH grant R01-AI43695 (M.A.G.) and the J. David Gladstone Institutes (M.A.G.).

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Chapter 4

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

Prologue

We previously found that molecular determinants of coreceptor preference for CXCR4 or CCR5 within the HIV-1 envelope glycoprotein (gp120) regulate the ability of HIV-1 to deplete $CD4^+$ T-cells in PBMC cultures and human lymphoid histocultures. In these studies, X4 viruses severely depleted $CD4^+$ T-cells while matched R5 viruses depleted such cells only mildly, suggesting that either X4 viruses are intrinsically more cytopathic than R5 viruses or that different viruses specifically target $CD4^+$ T-cells expressing CCR5 or CXCR4 thereby causing their death. Indeed, earlier studies have established that CXCR4 is expressed widely on lymphocytes while CCR5 is expressed on a small minority of these cells. Based on these results, one likely hypothesis is that R5 viruses are less efficient at depleting $CD4^+$ T-cells overall due to a small target lymphocyte pool, while X4 viruses cause rapid progression because of access to a broad target cell pool. This study sought to determine how usage of coreceptors by HIV-1 dictates cell tropism and depletion of $CD4^+$ T-cells in human lymphoid tissues cultured *ex vivo*. Our findings demonstrate that $CD4^+$ lymphocyte depletion in lymphoid histocultures is largely controlled by HIV-1 gp120-determined selective killing of lymphocytes expressing either CXCR4 or CCR5. In addition, we found a strong, but not absolute, preference of X4 viruses for lymphocytes and of R5 viruses for macrophages.

The experiments presented in this chapter are the product of a continued collaboration with the Margolis Lab. Jean-Charles Grivel and I again shared first authorship in this manuscript. D. Eckstein contributed substantially to the development of the flow cytometry assay to macrophage infection, B. Schramm

and R. Speck made intellectual contributions, and N. Abbey and B. Herndier provided the immunohistochemical analysis of infected histoculture specimens. My contributions consisted of performing the experiments presented in Figures 1, 2, 3, and 4C and drafting the manuscript. L. Margolis and M. Goldsmith jointly supervised this work.

JOURNAL OF VIROLOGY, June 2000, p. 5347–5351 Vol. 74, No. 11
0022-538X/00/$04.00+0

NOTES

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

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Received 22 July 1999/Accepted 25 February 2000

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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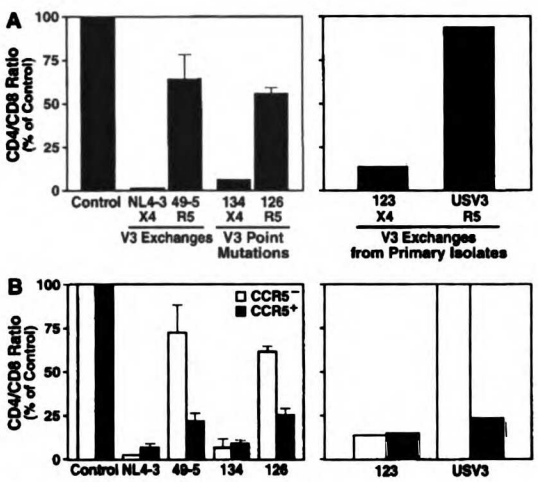

FIG. 1. CD4⁺ T-cell depletion in human tonsil histocultures infected ex vivo by matched HIV-1 strains. (A) Left: CD4⁺ T-cell depletion as indicated by mean relative CD4/CD8 ratio on day 15 after infection by recombinant viruses NL4-3, 49-5, 134, and 126. For each data point, cells were pooled from 6 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

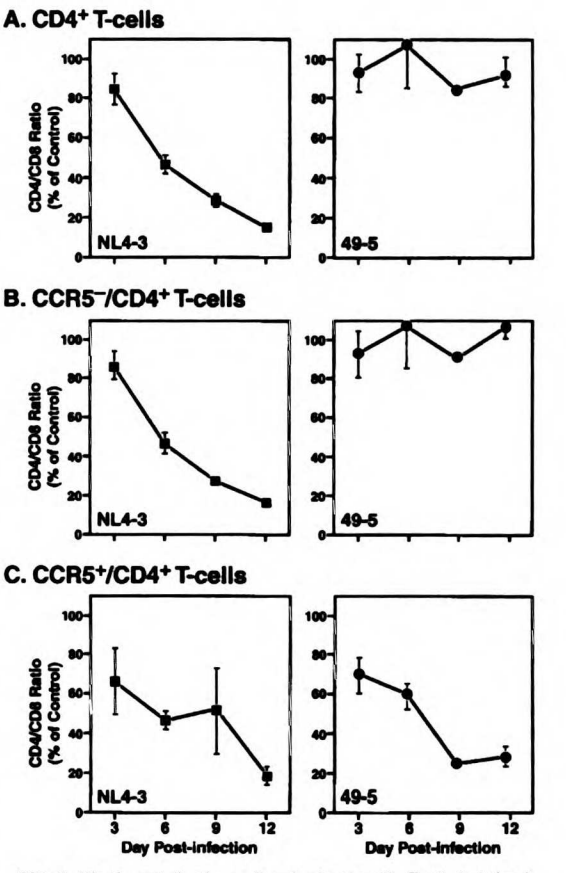

FIG. 2. Kinetic analysis of overall and subset-specific T-cell depletion in human tonsil histocultures in NL4-3- and 49-5-infected tonsillar tissue (left and right panels, respectively). Mean values (± standard errors of the means) from a representative experiment ($n = 3$) are shown.

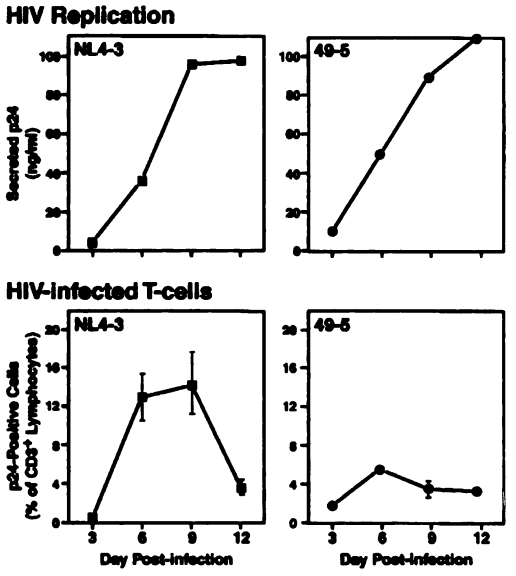

FIG. 3. Kinetic analysis of HIV-1 replication and lymphocyte infection in human tonsil histocultures. (A) HIV-1 p24 concentration in the culture medium of NL4-3- and 49-5-infected histocultures (Fig. 2) and sampled at various time points. (B) Frequencies of productively infected lymphocytes in these 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-

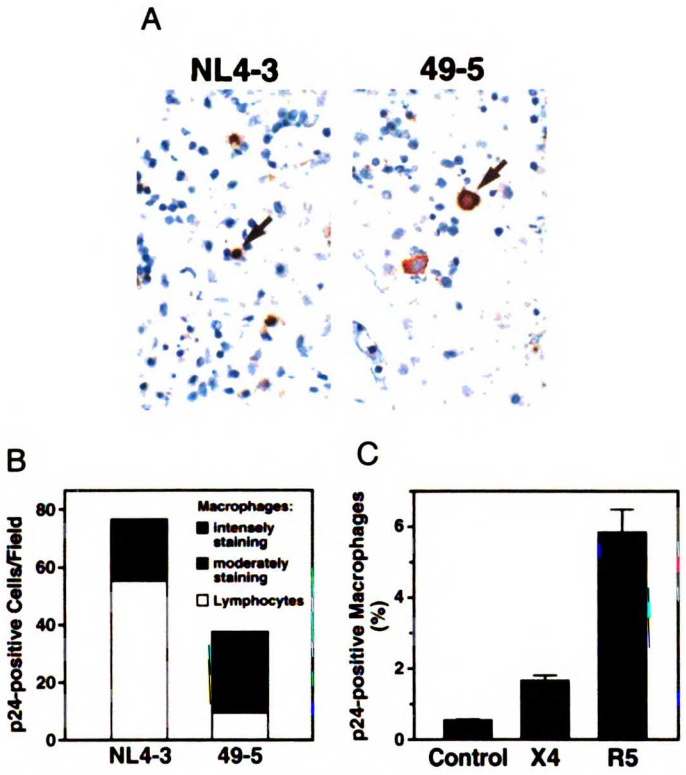

FIG. 4. Immunohistochemical and flow cytometric detection of intracellular HIV-1 p24 in infected tonsil histocultures. (A) Representative examples of p24-positive lymphocytes and macrophages in tonsil tissue infected ex vivo with NL4-3 and 49-5. Arrows indicate a typical lymphocyte (left) or macrophage (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.

We thank Bruce Chesebro and Malcolm Martin for kindly providing plasmids and Dee J. Holthe, Cecilia Stewart, Sharon Hall, Claudette Delphis, Ursula Perotti, Mark Weinstein, and the surgical staffs at Kaiser Hospitals (San Rafael and San Francisco) and San Francisco General Hospital for generous assistance in obtaining posttonsillectomy samples. We acknowledge the technical assistance of Eric Wieder and Lisa Gibson in these experiments and the assistance of Heather Gravois, John Carroll, and Neile Shea in the preparation of the manuscript.

M.L.P. was supported by the California Universitywide AIDS Research Program, the Biomedical Sciences Graduate Program, and the National Institutes of Health Medical Scientist Training Program at UCSF. D.A.E. was supported by the National Institutes of Health Medical Scientist Training Program at UCSF. B.S. was supported by the Boehringer Ingelheim Fund, and R.F.S. was supported in part by the UCSF-GIVI CFAR. This work was supported in part by NIH grant R01-AI43695 (M.A.G.), the J. David Gladstone Institutes (M.A.G.), and the NASA/NIH Center for Three-Dimensional Tissue Culture.

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Chapter 5

Cytopathicity of Human Immunodeficiency Virus Type 2 (HIV-2) in Human Lymphoid Tissue is Coreceptor-Dependent and Comparable to that of HIV-1

Prologue

Human immunodeficiency virus type 2 (HIV-2) is prevalent mainly in West Africa and is also found less frequently in some Asian countries, Western Europe, and the United States. Human infection with HIV-2 is associated with eventual immunologic failure and AIDS. However, disease progression is strikingly delayed when cohorts of HIV-2 infected individuals are compared to similar cohorts of HIV-1 infected individuals. This clinical difference has been attributed to an attenuated pathogenic potential of HIV-2, a hypothesis we sought to test in the histoculture model. With the discovery of the role of chemokine receptors in cellular entry by HIV-1, several studies have revealed that nearly all strains of HIV-2 use CD4 together with CCR5 and/or CXCR4 to gain entry into cellular targets. The contribution of distinct coreceptor specificities to HIV-2 infection and pathogenesis *in vivo* nevertheless remains to be established. Given the similarities in CCR5 and CXCR4 utilization and the striking difference regarding disease progression for HIV-1 and HIV-2, an important question is how coreceptor preferences relate to the cytopathic potential of HIV-2. In the present study, we therefore used an *ex vivo* human lymphoid histoculture system to investigate the relationship between coreceptor preferences and cytopathicity of HIV-2. Our results demonstrate that coreceptor specificity markedly influences the cytopathic potential of HIV-2, and that HIV-2 can be as cytopathic as HIV-1 in mature lymphoid tissue despite its distinct clinical characteristics.

Chapter 5 is a manuscript in which I am second author. B. Schramm performed all of the experiments and drafted the manuscript, and E. Palacios, F.

Kirchoff, and B. Grant provided reagents and intellectual input. My participation in this study involved developing two key experimental methods used in these studies: (1) measurement coreceptor-specific depletion of T-cells (presented in Figures 1, 3, 4, and 5) and (2) comparison of pathogenicity of viral strains with the same coreceptor preference (i.e., the kinetic analysis of T-cell depletion presented in Figure 2). In addition, I performed some data analysis, assisted in the editing of the manuscript, and made intellectual contributions. This project was performed under the direction of M. Goldsmith.

Cytopathicity of Human Immunodeficiency Virus Type 2 (HIV-2) in Human Lymphoid Tissue is Coreceptor-Dependent and Comparable to that of HIV-1

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ABSTRACT

Epidemiological studies have shown that human immunodeficiency virus type 2 (HIV-2) is markedly less pathogenic than human immunodeficiency virus type 1 (HIV-1) *in vivo*. Individuals infected with HIV-2 exhibit a remarkably slow rate of disease development, and these clinical properties have been attributed presumptively to an "attenuated" phenotype of HIV-2 itself. Here we investigated the impact of coreceptor usage on the cytopathicity of HIV-2 and compared its pathogenic potential with that of HIV-1 in a unique human lymphoid histoculture model. We found that HIV-2 strains, as well as closely related simian immunodeficiency viruses (SIV), displayed mildly or highly aggressive cytopathic phenotypes depending on their abilities to use the coreceptors CCR5 or CXCR4, respectively. A side-by-side comparison of primary X4 HIV-1 and HIV-2 strains revealed similar, high degrees of cytopathicity induced by both HIV types. Furthermore, we found that HIV-2 coreceptor specificity for CCR5 and CXCR4 determined the target cell population for T-cell depletion in lymphoid tissue. Finally, utilization of the alternate coreceptors BOB and Bonzo did not significantly increase the cytopathic properties of HIV-2. These findings demonstrate that coreceptor preference is a key regulator of target cell specificity and the cytopathic potential of HIV-2, with indistinguishable rules compared with HIV-1. Moreover, HIV-2 strains are not characterized by an intrinsically lower cytopathicity compared to HIV-1 strains. Therefore, direct cytopathic potential *per se* does not explain the unique behavior of HIV-2 in

INTRODUCTION

Human immunodeficiency virus type 2 (HIV-2) is prevalent mainly in West Africa and is also found less frequently in some Asian countries, Western Europe and the United States. Human infection with HIV-2 is associated with eventual immunologic failure and the acquired immunodeficiency syndrome (AIDS). However, disease progression has been reported to be much slower in HIV-2-infected individuals compared with HIV-1-infected individuals as evidenced by significantly slower rates of development of abnormal $CD4^+$ T-cell counts and progression to AIDS (20, 25, 32, 40). This attenuated virulence of HIV-2 infection *in vivo* is a compelling area of investigation in which to elucidate the mechanisms of HIV-induced pathogenesis.

With the discovery of the role of viral coreceptors in cellular entry by HIV, several studies have revealed that nearly all strains of HIV-2 use CD4 together with the chemokine receptors CCR5 and/or CXCR4 (18, 26-28, 37), the molecules that have been defined as the major coreceptors for HIV-1 (reviewed in (2)). In comparison with HIV-1, HIV-2 typically is more promiscuous in its coreceptor utilization profile (18, 26-28). Specifically, the majority of HIV-2 isolates can use the orphan receptors BOB and/or BONZO with efficiencies comparable to their usage of CCR5 in *in vitro* assays, a behavior that is similar to that of the closely related simian immunodeficiency viruses (SIV) (7, 28, 39). The contribution of distinct coreceptor specificities to HIV-2 infection and pathogenesis *in vivo*

nevertheless remains to be established. In HIV-1 infection, viral coreceptor phenotypes typically evolve during the course of infection in that CCR5-specific (R5) viruses predominate in early stages and persist throughout the course of disease, while CXCR4-using (X4) viruses emerge frequently in temporal association with rapid $CD4^+$ T-cell decline and onset of AIDS (10, 33, 35). Several studies have indicated a similar shift of viral coreceptor specificity for CCR5 and CXCR4 during the course of HIV-2 infection (27, 28, 37).

We have shown in previous studies that coreceptor specificity substantially determines the cytopathic potential of HIV-1, and that the X4 phenotype dramatically enhances HIV-1 virulence in mature lymphoid tissue *ex vivo*. These findings indicated that the emergence of X4 viruses *in vivo* likely accelerate disease progression in HIV-1-infected individuals due to enhanced cytopathic effects in peripheral lymphoid tissue (29). We also found that additional coreceptors appear to have little impact on the T-cell depletion potential of HIV-1 strains in lymphoid tissues *ex vivo* (34). Given the similarities in CCR5 and CXCR4 utilization patterns and the striking difference regarding the pathogenic potential compared to HIV-1, an important question is how coreceptor preferences relate to the cytopathic potential of HIV-2.

In the present study we therefore used an *ex vivo* human lymphoid histoculture system to investigate the relationship between coreceptor preferences and cytopathicity of HIV-2. Histocultures of spleen or tonsil specimens recapitulate important aspects of HIV pathogenesis in the tissue

microenvironment of major HIV production sites *in vivo*, and have served in previous studies as a valuable model in which to study HIV-1 infection (15, 16, 29, 34). A key feature of this system for studying the impact of HIV coreceptor utilization is that lymphoid histocultures are readily susceptible to HIV infection without requiring exogenous stimulation that can alter chemokine receptor expression patterns (4). Moreover, in this system the expression levels of CCR5 and CXCR4 on T-lymphocytes remain stable throughout the culture period. In the present study we exploited the lymphoid histoculture system to investigate the impact of coreceptor preferences on the cytopathicity of HIV-2. Our results demonstrate that coreceptor specificity markedly influences the cytopathic potential of HIV-2, and that HIV-2 can be as cytopathic as HIV-1 in mature lymphoid tissue despite its distinct clinical characteristics.

MATERIALS AND METHODS

Viruses and Preparation of virus stocks

The primary HIV-2 isolates A1958 and SLRHC (28) were provided by Beatrice Hahn, and the HIV-1 primary isolate 12/86 (10) by Ruth I. Connor. The HIV-2 strain CBL20 (36) was provided by Robin Weiss and the primary isolate HIV-2 7924A (14) by Feng Gao and Beatrice Hahn via the NIH AIDS Research and Reference Reagent Program. Virus stocks of HIV-1 and HIV-2 isolates were established by infection of heterologous phytohemagglutinin (PHA) -activated peripheral blood mononuclear cells (PBMC) that were propagated with interleukin-2 (IL-2) as described previously (34). Viral stocks of primary SIVsmm FKl and FBo were established by cocultivation of PBMC derived from two naturally infected sooty mangabey monkeys from the Yerkes Regional Primate Research Center with activated mixed heterologous PBMC from uninfected sooty mangabeys. Donor cells for FBo cultivation were activated with Concanavalin A (5 μg/ml, Sigma) for 48 h. Donor cells for FKl cultivation were depleted of CD8 cells (Dynal) and activated with anti-monkey-CD3 antibody (1 μg/ml, Biosource) for 48 h. Both isolates were cocultured in RPMI containing 20% fetal calf serum and recombinant human IL-2 (100 U/ml, Chiron). The molecular clone of HIV-2 ST/SXB1 (λJSP4-27) (24) was provided by Beatrice Hahn, the molecular clone pNL4-3 by Malcom Martin via the AIDS Research and Reference Reagent Program, and the molecular clone 49-5 (9) by Bruce Chesebro. The molecular clones SIVmac239,

SIVmac316 and SIVmac239PS were described previously (22). Infectious virus stocks were prepared as previously described (6). The p24 and p27 GAG concentration, respectively, of HIV-1, HIV-2 and SIV virus stocks were assessed by enzyme-linked immunosorbent assay (NEN Life Sciences, Beckman Coulter, Inc.).

Infection of human lymphoid tissue *ex vivo*

Human tonsil and adenoid tissue removed during routine tonsillectomy (provided by San Francisco General Hospital, Kaiser-San Francisco, Calif. and Kaiser-San Rafael, Calif.) was received within 5h of excision and was sectioned into 2- to 3-mm blocks. The tissue blocks were placed onto collagene sponges in the culture medium as described (29) and 5µl of clarified virus-containing media were applied on top of these tissue blocks (0.1-0.5 ng p24 or p27, respectively, for HIV-1, HIV-2 and SIVsmm primary isolates and up to 5 ng p24 or p27 per tissue block for HIV-1, HIV-2 and SIVmac recombinants) as described previously. Productive HIV infection was assessed by measuring the amount of p24 and p27 antigen, accumulated in the culture medium during the 3 days between successive changes of medium. Infections in the depletion-kinetic experiment (Fig. 2) were performed with 5µl virus-containing media per tissue block, which represented 7-40 50% tissue culture infectious doses ($TCID_{50}$) as determined by terminal dilution of

the virus stocks in quadruplicate on heterologous PHA-activated PBMC propagated with human IL-2 (5U/ml).

Assessment of $CD4^+$ T-cell depletion by FACS analysis

On day 12 following infection, cells were mechanically isolated from infected and uninfected control tissue and analyzed by flow cytometry (fluorescence activated cell sorting [FACS]). Dispersed cells from infected and uninfected lymphoid histocultures were stained for cell surface markers CD3, CD4, CD8 and CCR5 as described previously (29, 34), by using the mAbs: anti-CD3 (clone SK7, PE-conjugated), anti-CD4 (clone SK3, FITC-conjugated), anti-CD8 (clone SK1, PerCP-conjugated) (Becton Dickinson) and anti-CCR5 (clone 2D7, APC-conjugated; Pharmingen). 10.000 lymphocytes positive for CD3 surface marker were counted and the data were analyzed with CELLQUEST software (Becton Dickinson). To facilitate comparison among experiments, $CD4^+$ T-cell depletion was assessed by measuring the ratio of $CD4^+$ to $CD8^+$ T-cells. This value was normalized to the CD4/CD8 ratio of control (uninfected) samples to yield the "mean relative CD4/CD8 ratio". Changes in CD4/CD8 ratio represented $CD4^+$ T-cell depletion since increases in the numbers of $CD3^+$ $CD4^-$ $CD8^-$ cells as a result of virus-induced CD4 downregulation were insignificant in infected cultures.

Assessment of coreceptor utilization of SIVsmm isolates

GHOST cells (N.I.H. AIDS Research and Reference Reagent Program) expressing human CD4 and human chemokine receptors were plated

overnight at 40,000 cell/well per 12-well plates. The following day, the cultures were infected with 20–50 ng p27 of SIVsmm. Four days after infection, the cultures were harvested and assayed for GFP expression by flow cytometry as an indicator of infection.

RESULTS AND DISCUSSION

Coreceptor specificity correlates with the cytopathic phenotype of HIV-2 strains in lymphoid tissue *ex vivo*

In order to elucidate the impact of viral coreceptor specificity on the cytopathic potential of HIV-2, we infected lymphoid histocultures with a panel of HIV-2 isolates that were previously established to exhibit distinct coreceptor utilization patterns *in vitro* (7, 26, 28). As described previously (16, 29, 34), $CD4^+$ T-lymphocyte depletion in infected cultures of human tonsillar or adenoid tissue was monitored as an indicator of virus-induced cytopathicity. The cultures were harvested on day 12 following infection, at which time the tissue was dispersed and immunostained for CD4 and CD8. FACS analysis was used to measure the ratio of $CD4^+$ and $CD8^+$ T-cells in infected versus uninfected cultures, and $CD4^+$ T-cell depletion was detected as a decrease of the CD4/CD8 ratio in infected cultures.

This analysis revealed striking differences regarding the cytopathic potential among different HIV-2 isolates, segregating the HIV-2 strains into two distinct phenotypes. The primary isolate A1958 depleted $CD4^+$ T-cells very mildly as reflected in the slightly depressed CD4/CD8 ratio in infected cultures, whereas the primary isolate 7924A and the T-cell line-adapted strain CBL20 aggressively depleted $CD4^+$ T-cells in these cultures as reflected in severely depressed CD4/CD8 ratios (Fig. 1A). These differences in cytopathicity were observed despite similar replication kinetics of these viruses as

measured by accumulation of HIV-2 p27 antigen in the culture media between successive media changes (Fig. 1A inset).

Interestingly, this marked distinction in cytopathic effect corresponded to the difference in coreceptor preferences among these HIV-2 isolates (Fig. 1A). Specifically, the mildly cytopathic strain A1958 has the R5 phenotype, whereas the highly virulent strains CBL20 and 7924A have the X4 phenotype. These results mirror the pattern typically found for HIV-1 infections in lymphoid tissue (16, 29, 34) which is demonstrated here for comparison purposes with a recombinant isogenic HIV-1 virus pair, NL4-3 and 49-5 differing solely in reciprocal specificity for CXCR4 and CCR5, respectively (9, 29, 38). Whereas the R5 strain (49-5) only mildly depressed the CD4/CD8 ratio compared to uninfected control tissue (Fig. 1A), the X4 isogenic counterpart (NL4-3) aggressively depleted $CD4^+$ T-cells in these cultures, thereby recapitulating the dramatic impact of CXCR4 specificity on cytopathicity of HIV-1.

These results indicate that viral coreceptor specificity significantly influences the cytopathic potential of HIV-2 in lymphoid tissue. Moreover, HIV-2 cytopathicity seems to be controlled in a manner very similar to that of HIV-1, with specificity for CXCR4 linked to an aggressive depletion phenotype. Furthermore, since both the CXCR4-restricted HIV-2 strain (CBL20) and the multitropic primary isolate (7924A) aggressively depleted $CD4^+$ T-cells in these cultures, specificity for CXCR4 appears to be sufficient to confer high cytopathicity to HIV-2 as it does to HIV-1.

X4 HIV-1 and HIV-2 isolates show similar kinetics of $CD4^+$ T-cell depletion

To define further the cytopathic potential of X4 strains of HIV-2, we performed a side-by-side comparison of the kinetics of $CD4^+$ T-cell depletion by HIV-1 and HIV-2 in lymphoid histocultures (Fig. 2). Tonsil tissue was infected *ex vivo* with comparable doses of the primary isolates HIV-2 7924A and HIV-1 12/86. Both isolates were derived from patients with advanced disease and displayed expanded coreceptor usage *in vitro*, including specificity for both CXCR4 and CCR5 (10, 28). Infected and uninfected control tissue was harvested at various time points following infection and $CD4^+$ T-cell depletion was assessed at each time point. This comparison revealed that both the HIV-1 and the HIV-2 strains progressively and profoundly depleted $CD4^+$ T-cells and that they did so with comparable kinetics (Fig. 2), revealing that a lower cytopathicity is not an integral feature of HIV-2.

Coreceptor specificity determines the target cell population for $CD4^+$ T-lymphocyte depletion by HIV-2

To investigate further the mechanism underlying the differential cytopathicity of HIV-2 in lymphoid histocultures, we stratified the depletion analyses into coreceptor-expressing subsets of $CD4^+$ T-cells. Immunostaining of tonsil tissue and FACS analysis revealed that CXCR4 is expressed on the majority of $CD4^+$ T-cells (mean 88.5% ± 1.6%, n=25), whereas CCR5 is expressed on a minor subset (mean 10.4% ± 0.8%, n=25) (see also (17)).

Stratification of the T-cell depletion analysis into $CCR5^+$ ($CXCR4^+$) and $CCR5^-$ ($CXCR4^+$) $CD4^+$ T-cell subsets revealed a distinct depletion pattern for the HIV-2 isolates tested. The R5 isolate A1958 preferentially and efficiently depleted within the $CCR5^+CD4^+$ subset, whereas the X4 strain HIV-2 CBL20 and the multitropic isolate HIV-2 7924A caused profound depletion in both T-cell subsets (Fig. 1B). These results mirror those observed with the R5 (49-5) and X4 (NL4-3) strains of HIV-1 (Fig. 1B); such subset-specific effects are readily evident even despite some degree of overlap between the $CCR5^+$ and $CCR5^-$ cell populations in FACS (17). Thus, R5 strains of HIV-2 are highly cytopathic for CCR5-bearing $CD4^+$ T-cells, and X4 strains of HIV-2 are cytopathic for the larger set of $CD4^+$ T-cells that bear CXCR4, demonstrating that $CD4^+$ T-cell depletion by HIV-2 is highly controlled by coreceptor specificity. Therefore, HIV-2 should be viewed as an intrinsically cytopathic virus, with its overall effects on the T-cell pool determined by the coreceptor properties of the particular viral strain and the cellular expression pattern of CCR5 and CXCR4 within the $CD4^+$ T-cell population.

An attenuated HIV-2 strain displays an R5 depletion phenotype in *ex vivo* lymphoid tissue

Earlier studies described an "attenuated" HIV-2 strain (HIV-2 ST) isolated from an asymptomatic HIV-2-infected individual in Senegal, West Africa, which exhibited a non-cytopathic phenotype *in vitro* as indicated by

the absence of virus-induced cell fusion or cell death in T-cell lines and peripheral blood lymphocytes (PBL) (23). A recent study has shown that the molecular clone of this virus, HIV-2 ST/SXB1, which displays the biological properties of the parent HIV-2 ST isolate (24), uses CCR5, BOB and Bonzo but not CXCR4 as coreceptor (11). Based on our finding that coreceptor preferences highly influence the cytopathic phenotype of HIV-2 in peripheral lymphoid tissue, we hypothesized that the apparent non-cytopathic phenotype of HIV-2-ST/SXB1 as assessed in T-cell lines or PBL is a direct consequence of its inability to use CXCR4.

To test this hypothesis, we infected tonsil cultures with HIV-2 ST/SXB1 as well as the X4 primary HIV-2 isolate 7924A and the R5 primary HIV-2 isolate SLRHC for comparison purposes. 12 days following infection the tissue was harvested and $CD4^+$ T-cell depletion in infected cultures was analyzed. As predicted, both HIV-2 ST/SXB1 and SLRHC depleted $CD4^+$ T-cells only mildly, whereas the X4 strain 7924A depleted cells quite aggressively (Fig. 3A). Furthermore, stratification of the analysis into the $CCR5^+$ and $CCR5^-$ $CD4^+$ T-cell subsets revealed that HIV-2 ST/SXB1 preferentially but potently depleted cells within the $CCR5^+$ $CD4^+$ T-cell pool, as did SLRHC (Fig. 3B), thus mirroring the typical depletion phenotype of other R5 HIV-2 and HIV-1 strains (Fig. 1A, B and (16, 29, 34)). This finding demonstrates that the reported non-cytopathic and non-fusogenic properties of HIV-2 ST/SXB1 in T-cell lines or PBL are likely a consequence of its coreceptor specificity for CCR5 and its inability to use CXCR4, rather than of an intrinsically non-

cytopathic character. It is of interest to note that both HIV-2 ST/SXB-1 and SLRHC (Fig. 3) were derived from an asymptomatic individual, whereas HIV-2 A1958 (Fig. 1) was isolated from a patient who had progressed to AIDS. Therefore, these results also demonstrate that both early and late stage R5 HIV-2 isolates are similarly mildly cytopathic in secondary lymphoid tissue in contrast to X4 HIV-2 isolates, which further highlights the dominant impact of coreceptor specificity on CD4+ T-cell depletion by HIV-2. Together these findings imply that the differential expression of CXCR4 and CCR5 in the lymphoid tissues is a crucial factor for the distinct cytopathic behavior of R5 and X4 HIV-2 strains in our experiments. This principle is consistent with a recent study demonstrating that infection of rhesus monkeys with chimeric SHIV viruses carrying X4 or R5 HIV-1 envelopes led to markedly different pathogenic effects in various lymphoid tissues *in vivo*, which was interpreted as the result of differential expression of CCR5 and CXCR4 in the respective tissues (19).

SIV depletes $CCR5^{+}CD4^{+}$ T-cells in human lymphoid tissue *ex vivo*

SIV is closely related to HIV-2 (8), but exhibits an interesting difference regarding its coreceptor utilization profile. SIV isolates from naturally infected, disease resistant, sooty mangabey monkeys (SIVsmm from *Cercocebus torquatus atys*), as well as SIV strains that cause disease in rhesus macaque monkeys (SIVmac from *Macaca mulatta*), universally use CCR5 as a coreceptor, and most strains exhibit additional capacities to use the orphan

receptors BOB and/or Bonzo (7, 11, 12). In contrast to HIV-1 and HIV-2, however, SIV does not typically evolve to exploit CXCR4 as a coreceptor *in vivo*, although both macaque and sooty mangabey CXCR4 have been demonstrated to be permissive for HIV-1 entry *in vitro* (7). In view of the close relationship of HIV-2 and its simian ancestor SIVsmm, the differences and similarities in coreceptor dependence among HIV-2 and SIV, and the host-dependent pathogenicity of SIV strains, we compared the $CD4^+$ T-cell depletion phenotype of several SIV strains with that of HIV-2 in human lymphoid histocultures. It had been established earlier that human peripheral blood mononuclear cells (PBMC) support SIV infection (13), and the cross species infectivity of SIV was further demonstrated by the productive infection of a laboratory worker following accidental exposure to SIVmac (21). These factors provide a rationale for evaluating these viruses in the human lymphoid tissue system.

We therefore infected tonsil cultures *ex vivo* with two recombinant SIV strains (SIVmac239 and SIVmac316) that are highly virulent in rhesus macaques, and two primary SIVsmm isolates (SIVsmm FKl and SIVsmm FBo) that were isolated from naturally-infected sooty mangabeys. All four SIV strains display specificity for CCR5, BOB and Bonzo as coreceptors ((12, 30) and Fig. 5). In addition we performed infections with SIVmac239PS, a derivative of SIVmac239 that is impaired in BOB-utilization as a result of a single amino acid substitution in the V3 region of the envelope (30). For comparison purposes we also infected cultures with the X4 primary HIV-2 7924A and the

R5 HIV-2 isolate A1958. On day 12 post-infection, infected and uninfected cultures were harvested and analyzed. FACS analysis revealed that all SIVmac strains and the primary SIVsmm isolates mildly depleted $CD4^+$ T-cells in these cultures (Fig. 4A, 5A), despite robust replication (insets Fig. 4A, 5A). Thus, SIVmac and SIVsmm isolates displayed a cytopathic phenotype comparable to that of the R5 HIV-2 A1958 (Fig. 4A) and R5 HIV-1 strains (Fig. 1A and (16, 29, 34)), but contrasting with the aggressive depletion phenotype of the X4 HIV-2 7924A (Fig. 4A). No significant difference was observed for the cytopathic potential of SIVmac239 and SIVmac239PS, demonstrating that coreceptor specificity for BOB does not enhance viral cytopathicity in peripheral lymphoid tissue.

Stratification of the T-cell depletion analysis in the $CCR5^+$ and $CCR5^-$ subsets of $CD4^+$ T-cells further revealed that the SIVmac and SIVsmm strains preferentially and rather potently depleted $CCR5^+$ $CD4^+$ T-lymphocytes (Fig. 4B, 5B). This finding suggests that the mild overall $CD4^+$ T-cell depletion induced by these viruses is a consequence of relative inaccessibility of the overall $CD4^+$ T-cell population for these viruses due to restricted cellular expression of CCR5. Furthermore, the coreceptor specificity for BOB and Bonzo does not significantly increase the target cell pool for depletion and/or enhance viral cytopathicity in human lymphoid tissue compared with that of viruses with restricted specificity for CCR5. The expression patterns for BOB and Bonzo in human tissue have been not well established to date, but they

clearly do not facilitate aggressive $CD4^+$ T-cell depletion by HIV-2 or SIV in human lymphoid tissue *ex vivo*. This failure to augment overall $CD4^+$ T-cell depletion is consistent with the *in vivo* comparison of SIVmav239 and SIVmac239PS that revealed no significant contribution of BOB utilization to viral replication or pathogenesis in the rhesus macaque model (30), and mirrors our finding that alternate coreceptors do not have a major role in T-cell depletion by HIV-1 (34).

In summary, this study demonstrates that primary and T-cell line adapted HIV-2 strains display distinct cytopathic phenotypes in peripheral human lymphoid tissue depending on their coreceptor specificities for CXCR4 or CCR5, respectively. As with HIV-1, specificity of HIV-2 for CCR5 alone or in combination with additional coreceptors such as BOB and Bonzo is linked to restricted overall $CD4^+$ T-cell depletion potential, while specificity for CXCR4 is linked to a highly virulent phenotype. As assessed in *ex vivo* lymphoid cultures, these cytopathic properties of HIV-2 are very similar to these of HIV-1, both quantitatively and qualitatively. We also demonstrate that recombinant and primary strains of SIV, which are genetically closely related to HIV-2 but typically do not exploit CXCR4 as a coreceptor, displayed a rather mild cytopathic phenotype in human peripheral lymphoid tissue mirroring that of R5 HIV-1 and HIV-2. We further observed that an HIV-2 strain that was previously recognized as attenuated *in vitro* displays cytopathic properties in lymphoid tissue that are similar to these of R5 HIV-1, HIV-2 and SIV strains, indicating that viral coreceptor specificity represents a

major regulator of the biological heterogeneity that has been reported for different strains of HIV-2 (1, 5). Finally, we found that coreceptor expression patterns determine the target cell population for $CD4^+$ T-cell depletion by HIV-2 and SIV, which further emphasizes that coreceptor specificity is a key determinant of cytopathicity of HIV-2 in mature lymphoid tissue. Together, our findings strongly suggest that a lower intrinsic cytopathic potential does not underlie the remarkably slower disease progression that is described in many HIV-2-infected individuals (20, 25, 32, 40), since diverse HIV-2 strains exhibited robust $CD4^+$ T-cell depletion potential in lymphoid tissues *ex vivo* that was indistinguishable from that of HIV-1; of course, studies of additional HIV-2 isolates would be useful for establishing how generalizable these findings are relative to other viral strains.

It is particularly notable that the viral load in peripheral blood of HIV-2-infected individuals typically is much lower than that in HIV-1-infected individuals. The viral load *in vivo* is an indicator of viral fitness, or replicative capacity, and viral clearance in the context of the particular host environment and poorly defined viral and/or host features. It is evident that a different dynamic equilibrium of host and virus is established during infection with HIV-2 compared with HIV-1 (3, 31). Therefore, the present results strongly suggest that this equilibrium, rather than the cytopathic character of HIV-2 *per se*, is responsible for the attenuated effects of HIV-2 *in vivo*. We speculate that key host immune responses that are not apparent in short-term *ex vivo* lymphoid cultures operate *in vivo* to control HIV-2

infection relatively efficiently, as has been proposed previously (41). This conclusion should prompt further investigation to elucidate the basis of this distinct virus-host relationship as a possible foundation for strategies to modulate these processes in patients infected with HIV-1 and/or HIV-2.

ACKNOWLEDGEMENTS

The authors thank Beatrice Hahn, Ruth I. Connor and Robin Weiss for kindly providing viruses, Bruce Chesebro for kindly providing plasmids, and Dee J. Holthe, Cecilia Stewart, Claudette Delphis, Ursula Perotti, Sharon Hall, Jaqueline Hylton, Nancy W. Abbey, Mark D. Weinstein, and the surgical staffs at Kaiser hospitals (San Rafael and San Francico, Calif.) and San Francisco General Hospital for generous assistance in obtaining post-tonsillectomy samples. Recombinant IL-2 was the generous gift of Chiron Corporation. The authors acknowledge the technical assistance of Eric Wieder and Lisa Gibson in the conduct of these experiments and the assistance of Heather Gravois, John Carroll and Neile Shea in the preparation of this manuscript.

B.S. is supported by the Boehringer Ingelheim Fond. M.L.P. is supported by the Biomedical Sciences Graduate Program, the California Universitywide AIDS Research Program, and the N.I.H. Medical Scientist Training Program at UCSF. F.K. is supported by the Deutsche Forschungsgemeinschaft (SFB466), and R.M.G. and E.P. are supported by N.I.H. grant P51 RR-0165.This work was supported in part by N.I.H. grant R01-AI43695 (M.A.G.) and the J. David Gladstone Institutes (M.A.G. and R.M.G.).

FIGURE LEGENDS

FIG.1: The cytopathic phenotype of HIV-2 is linked to coreceptor specificity in *ex vivo* lymphoid tissue.

(A) $CD4^+$ T-cell depletion in adenoid cultures by various HIV-2 strains and two HIV-1 recombinants was assessed by FACS analysis on day 12 post-infection. Shown are mean relative CD4/CD8 ratios (n=3) with standard error of the mean (S.E.M.). Previously reported coreceptor specificities are indicated (7, 26, 28, 29). **(inset A)** Viral replication of the HIV-2 isolates was monitored by assessing accumulation of p27 in the culture supernatant between successive medium changes. **(B)** In the same infection, depletion within the $CCR5^+$ and the $CCR5^-$ subset of $CD4^+$ T-cells was analyzed by multiparameter FACS on day 12 post-infection. Shown are mean relative CD4/CD8 ratios (n=3) with S.E.M. for $CCR5^+$ and $CCR5^-$ T-cells.

FIG.2: Comparable and progressive depletion of $CD4^+$ T-cells by multitropic primary isolates of HIV-1 and HIV-2 in *ex vivo* tonsil cultures.

Ex vivo tonsil cultures were inoculated with the primary HIV-1 isolate 12/86 and HIV-2 isolate 7924A, respectively, and $CD4^+$ T-cell depletion was assessed by FACS analysis on subsequent days (3, 6, 9, 13, 16) post-infection. Shown are mean relative CD4/CD8 ratios (n=3) with S.E.M. (* n=2 for indicated samples).

FIG.3: An HIV-2 strain with attenuated cytopathicity *in vitro* displays the typical depletion phenotype of R5 strains.

(A) Tonsil histocultures were inoculated with HIV-2 ST/SXB1, the X4 HIV-2 isolate 7924A and the R5 HIV-2 isolate SLRHC, respectively. $CD4^+$ T-cell depletion was assessed on day 12 post-infection. Previously reported coreceptor specificities are indicated (11, 28). Shown are mean relative CD4/CD8 ratios (n=3) with S.E.M. **(inset A)** Viral replication was monitored by assessing accumulation of p27 in the culture supernatant between successive medium changes. **(B)** In the same infection, depletion within the $CCR5^+$ and the $CCR5^-$ subsets of $CD4^+$ T-cells was analyzed by multiparameter FACS on day 12 post-infection. Shown are mean relative CD4/CD8 ratios (n=3) with S.E.M.

FIG.4: Mild depletion of $CD4^+$ T-cells by recombinant strains of SIVmac in *ex vivo* lymphoid tissue.

(A) *Ex vivo* tonsil cultures were inoculated with recombinant strains SIVmac239, SIVmac316 and SIVmac239PS, and primary HIV-2 isolates A1958 and 7924A, respectively. $CD4^+$ T-cell depletion was assessed by FACS on day 12 post-infection. Shown are mean relative CD4/CD8 ratios (n=3) with S.E.M. Coreceptor preferences are indicated as described previously (7, 12, 28, 30). **(inset A)** Viral replication was monitored by assessing accumulation of p27 in the culture supernatant between successive medium changes. **(B)** In the same infections, T-cell depletion analysis was stratified into $CCR5^+$ and $CCR5^-$

$CD4^+$ T-cell subsets by multiparameter FACS. Shown are mean relative CD4/CD8 ratios (n=3) with S.E.M. for $CCR5^+$ and $CCR5^-$ T-cells.

FIG.5: Mild depletion of $CD4^+$ T-cells by primary SIVsmm isolates in *ex vivo* lymphoid tissue.

(A) *Ex vivo* tonsil cultures were inoculated with primary SIVsmm isolates FKl and FBo, respectively. $CD4^+$ T-cell depletion was assessed by FACS on day 12 post-infection. Shown are mean relative CD4/CD8 ratios (n=3) with S.E.M. **(B)** In the same infections, T-cell depletion analysis was stratified into $CCR5^+$ and $CCR5^-$ $CD4^+$ T-cell subsets by multiparameter FACS. Shown are mean relative CD4/CD8 ratios (n=3) with S.E.M. for $CCR5^+$ and $CCR5^-$ T-cells. **(inset A)** Viral replication was monitored by assessing accumulation of p27 in the culture supernatant between successive medium changes. **(C)** Coreceptor preferences of SIVsmm isolates FKl and FBo were established by infection of GHOST cells stably expressing CD4 together with various coreceptors. Shown is usage of alternative coreceptors relative to CCR5.

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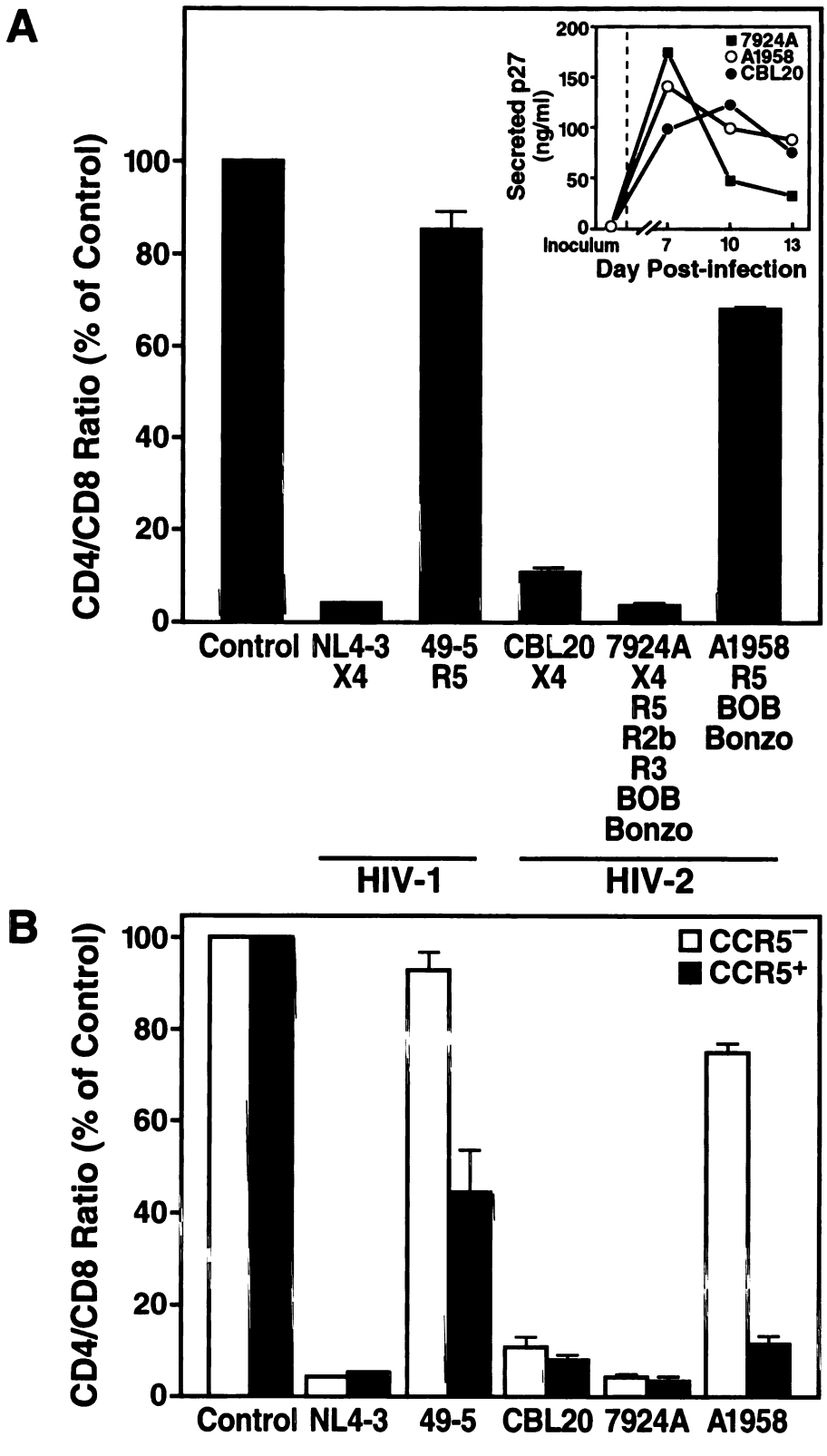
A
CD4/CD8 Ratio (% of Control)
100
80
60
40
20
0
Secreted p27 (ng/ml)
200
150
100
50
0
7924A
A1958
CBL20
Inoculum
7
10
13
Day Post-infection
Control
NL4-3 X4
49-5 R5
CBL20 X4
7924A X4 R5 R2b R3 BOB Bonzo
A1958 R5 BOB Bonzo
HIV-1
HIV-2
B
CD4/CD8 Ratio (% of Control)
100
80
60
40
20
0
CCR5⁻
CCR5⁺
Control
NL4-3
49-5
CBL20
7924A
A1958

Figure 1

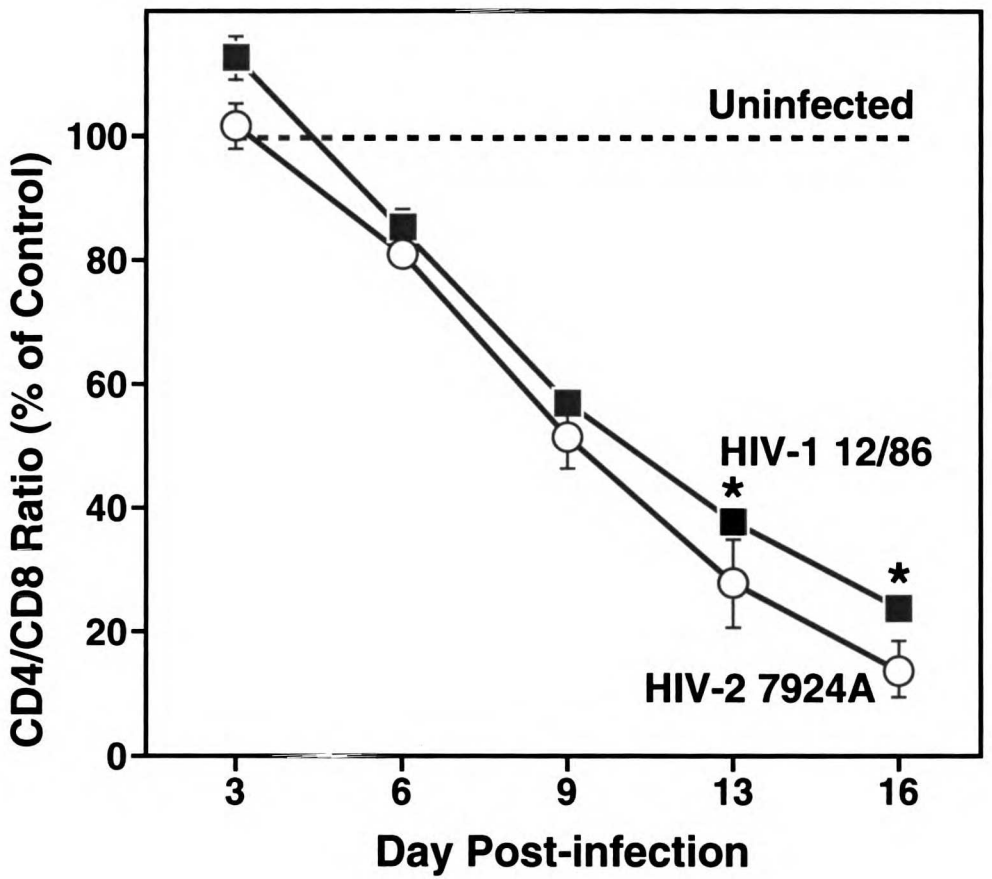
Uninfected
HIV-1 12/86
HIV-2 7924A
*
*
CD4/CD8 Ratio (% of Control)
0
20
40
60
80
100
3
6
9
13
16
Day Post-infection

Figure 2

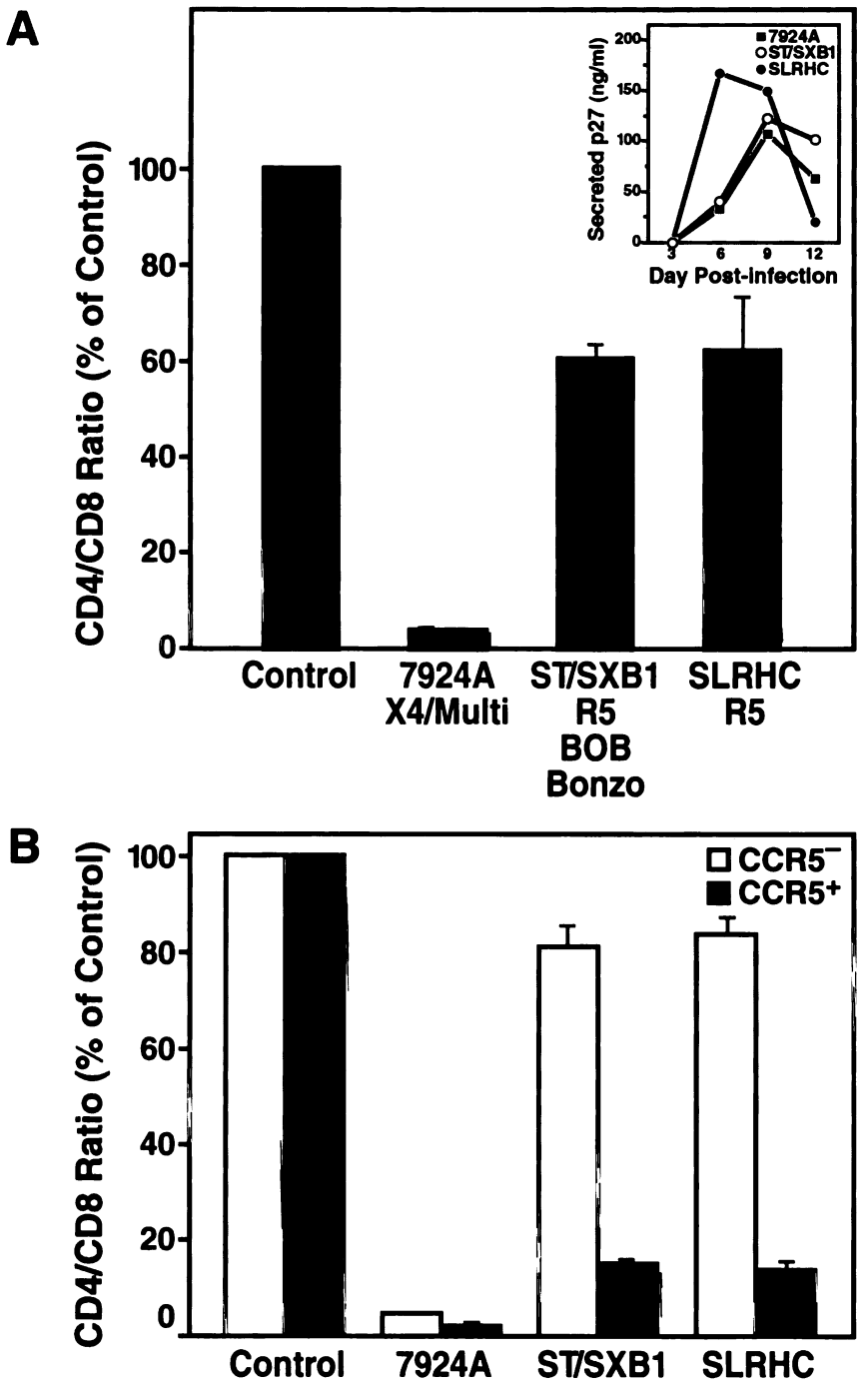
A
CD4/CD8 Ratio (% of Control)
100
80
60
40
20
0
Control
7924A
X4/Multi
ST/SXB1
R5
BOB
Bonzo
SLRHC
R5
Secreted p27 (ng/ml)
200
150
100
50
0
3
6
9
12
Day Post-Infection
7924A
ST/SXB1
SLRHC
B
CD4/CD8 Ratio (% of Control)
100
80
60
40
20
0
CCR5⁻
CCR5⁺
Control
7924A
ST/SXB1
SLRHC

Figure 3

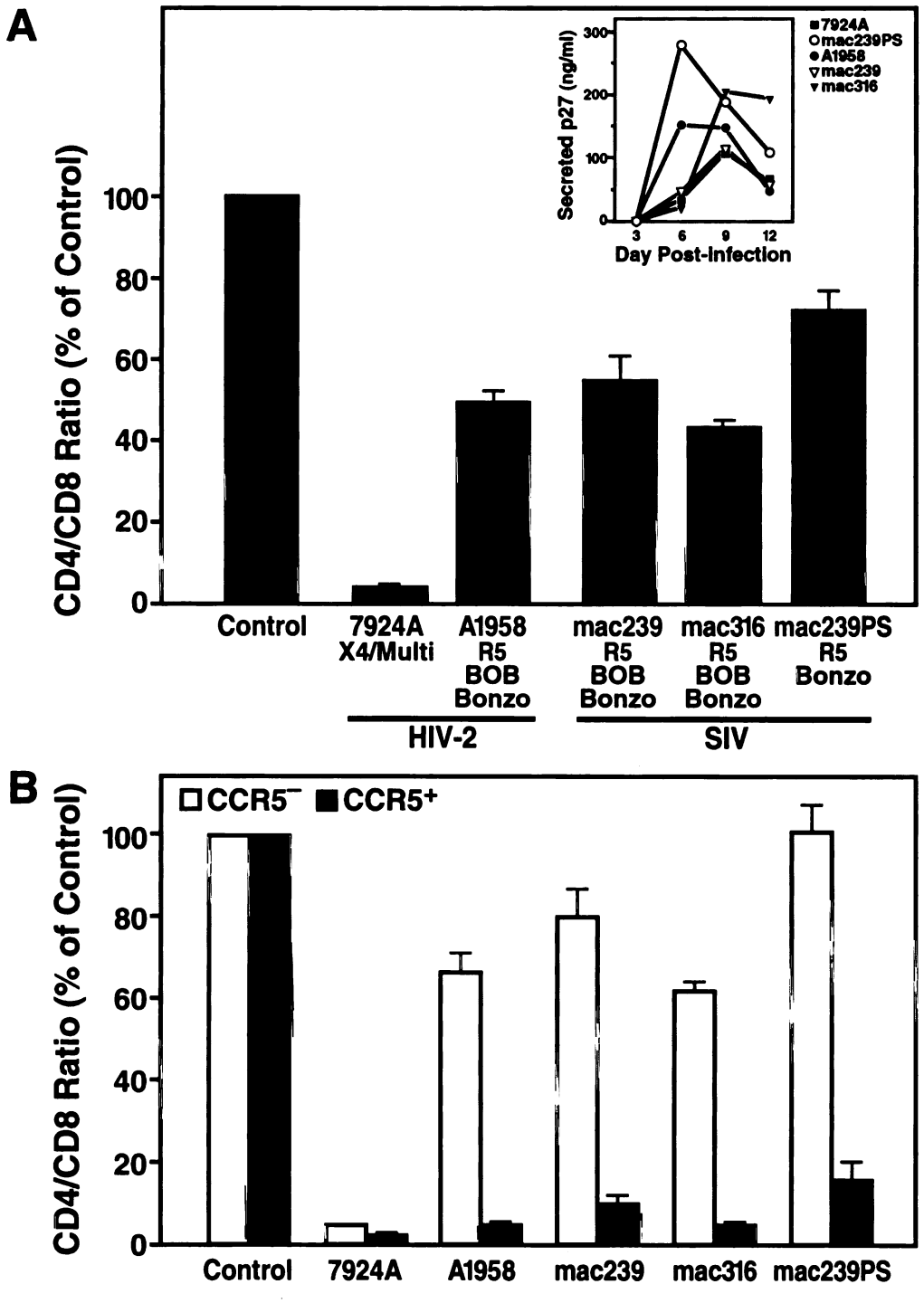
A
CD4/CD8 Ratio (% of Control)
100
80
60
40
20
0
Secreted p27 (ng/ml)
300
200
100
0
3
6
9
12
Day Post-Infection
■7924A
○mac239PS
●A1958
▽mac239
▼mac316
Control
7924A
X4/Multi
A1958
R5
BOB
Bonzo
mac239
R5
BOB
Bonzo
mac316
R5
BOB
Bonzo
mac239PS
R5
Bonzo
HIV-2
SIV
B
CD4/CD8 Ratio (% of Control)
□CCR5⁻ ■CCR5⁺
100
80
60
40
20
0
Control
7924A
A1958
mac239
mac316
mac239PS

Figure 4

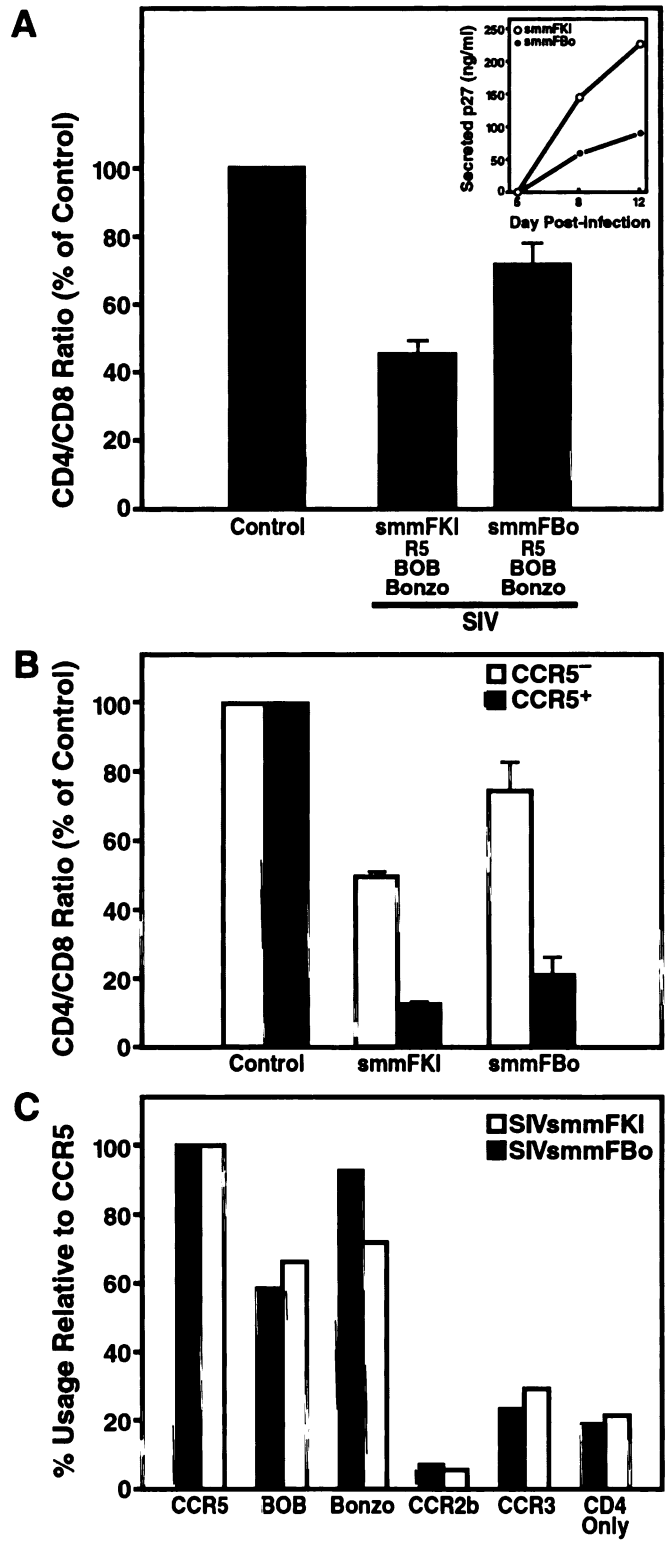
A
CD4/CD8 Ratio (% of Control)
100
80
60
40
20
0
Control
smmFKI
R5
BOB
Bonzo
smmFBo
R5
BOB
Bonzo
SIV
smmFKI
smmFBo
Secreted p27 (ng/ml)
250
200
150
100
50
0
8
12
Day Post-Infection
B
CD4/CD8 Ratio (% of Control)
CCR5⁻
CCR5⁺
Control
smmFKI
smmFBo
C
% Usage Relative to CCR5
SIVsmmFKI
SIVsmmFBo
CCR5
BOB
Bonzo
CCR2b
CCR3
CD4
Only

Figure 5

Chapter 6

Primary and Recombinant HIV-1 Strains Resistant to Protease Inhibitors Are Pathogenic in Mature Human Lymphoid Tissues

Prologue

The earlier chapters have addressed the pathogenic consequences of viruses that emerge during replication within the host as determined by coreceptor preference and cellular tropism. This chapter addresses the pathogenic consequences of viruses that have become drug resistant. Protease inhibitors (PI) represent key components of most clinically effective antiviral regimens because they profoundly inhibit the replication of many HIV-1 strains. Although PI therapy may provide lasting suppression of viral replication in some cases, viral resistance to these drugs often emerges during the course of chronic treatment leading to a rebound of virus production in treated individuals. Often patients exhibit decreasing $CD4^+$ T-cell counts following this rebound of virus production. However, increasingly recognized is a subset of individuals who exhibit stable or increasing $CD4^+$ T-cell counts despite rising viral loads following the development of PI-resistance. One hypothesis to explain this phenomenon is that the resistant viral quasispecies have a reduced capacity to kill target cells despite preserved replication potential. In the following chapter, we tested this hypothesis in human tonsil histocultures. We found that the acquisition of resistance to PI does not attenuate the pathogenic potential of these viruses on mature $CD4^+$ T-cells in lymphoid histocultures. These results suggest that other mechanisms are responsible for the continued accumulation of $CD4^+$ T-cells in patients who develop PI resistance.

Collaborators in this study include M. Myers and D. Eckstein who performed some of the experiments (Figure 4 and 3, respectively), F. Mammano and F. Clavel who provided recombinant viruses, T. Liegler, M. Hayden and B.

Grant who provided the primary isolates, and S. Deeks who provided clinical information on patients from whom the primary isolates were derived. I was the lead author of this study and performed the experiments in Figures 1 and 3 and drafted the manuscript. M. Goldsmith supervised this work.

Primary and Recombinant HIV-1 Strains Resistant to Protease Inhibitors are Pathogenic in Mature Human Lymphoid Tissues

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ABSTRACT

Preserved peripheral $CD4^+$ T-cell counts despite virologic failure in patients on protease inhibitor (PI)-containing antiviral regimens is a frequent occurrence in human immunodeficiency virus (HIV) disease. One hypothesis to explain the relative sparing of $CD4^+$ T-cells is that HIV strains exhibiting PI resistance concomitantly are attenuated in terms of cytopathicity for mature T-cells. To test this hypothesis, we used a three-dimensional human tonsil histoculture microenvironment to assess the pathogenic potential of a panel of primary and recombinant HIV-1 strains derived from patients experiencing PI failure. All of the viruses tested replicated efficiently in these cultures and, in some cases, better than comparable wild-type viral isolates. Furthermore, the PI-resistant strains depleted $CD4^+$ T-cells potently and comparably with wild-type isolates in these *ex vivo* lymphoid tissues. These results demonstrate that PI-resistant viruses are not inherently less pathogenic for mature T-cells. Therefore, the sustained peripheral lymphocyte counts in patients with selective virologic failure may be due to specific defects in viral replication in other cell compartments or to an undefined host adaptation to viral infection during PI therapy.

INTRODUCTION

Maturation of human immunodeficiency virus (HIV) into infectious virions depends upon proteolytic processing of the Gag and Gag-Pol polyprotein precursors by an aspartic protease encoded by the *pol* gene.(18-20, 34) Protease has been effectively targeted for therapeutic intervention by synthetic peptidomimetic molecules that occupy the catalytic site of this enzyme and thereby disrupt enzymatic activity leading to production of non-infectious virions that propagate systemic infection poorly.(2, 9, 23) Protease inhibitors (PI) can thus profoundly inhibit the replication of many HIV type-1 (HIV-1) strains (36) and are now the backbone of highly active antiretroviral therapy (HAART) for many HIV-1-infected patients.(12, 17, 35)

Although PI therapy may provide lasting suppression of viral replication in some cases, viral resistance to these drugs often emerges during the course of chronic treatment leading to a rebound of virus production in treated individuals.(5, 6, 10, 11, 26, 28) The development of PI resistance results from a complex interplay between HIV-1 evolution toward increasing degrees of drug resistance and changes in replicative capacity of the viral quasispecies. The earliest mutants generally are those that harbor single amino acid substitutions that confer low-level resistance to PI, and further replication during drug therapy promotes accumulation of additional drug-selected mutations.(5, 24) This stepwise acquisition of various mutations in the viral protease gene has been observed *in vitro* and *in vivo*.(5, 38) Most of these mutations are not commonly found in PI-naïve HIV-1-infected patients,(3) implying they strongly compromise the fitness of selected variants.

Importantly, such mutations can compromise the catalytic activity of protease and therefore adversely affect the absolute replication potential of HIV-1.(4, 16, 22) Following the stepwise accumulation of resistance mutations in protease, compensatory mutations often arise that can restore viral fitness to varying degrees.(22) Many studies have characterized the effects of drug-selected mutations on the replicative capacities of HIV-1 mutants *in vitro*, including kinetic analyses of virus production and measurements of competitive replication upon co-inoculation with viral variants.(8, 15, 16, 21, 22, 27, 37) Collectively, these *in vitro* studies indicate that mutations conferring PI resistance can indeed impair viral replication fitness. However, the impact of PI resistance *per se* on HIV replication and pathogenesis in the complex *in vivo* environment is not well established. Since viral load and pathogenesis are typically interdependent, it was widely expected that PI-treated individuals harboring resistant viral strains would exhibit decreasing $CD4^+$ T-cell counts when viral loads increased after the emergence of PI resistance. This predicted pattern is indeed observed. However, increasingly recognized is a subset of individuals who exhibit stable or increasing $CD4^+$ T-cell levels despite rising viral loads upon development of resistance.(10, 26) One mechanism potentially underlying this unexpected pattern is that the resistant viral quasispecies have diminished capacity to kill target cells despite preserved replication patterns.

In the present study, we tested this hypothesis by inoculating human tonsil histocultures with a panel of PI-sensitive and PI-resistant, primary and recombinant HIV-1 strains and evaluated the capacity of these viruses to deplete $CD4^+$ T-cells in these cultures. The histoculture system is useful for such analyses of pathogenesis because it represents a three-dimensional, heterogeneous milieu

of lymphoid tissue that retains cell-cell interactions found *in vivo* and does not require exogenous stimuli for maintenance of the cultures. Our findings demonstrate that the acquisition of resistance to PI does not inherently attenuate the cytopathic effects of these viruses on mature $CD4^+$ T-cells in lymphoid histocultures, and suggest that other mechanisms are responsible for continued accumulation of $CD4^+$ T-cells in the face of replication of PI-resistant HIV-1.

RESULTS AND DISCUSSION

As a first step to evaluate the pathogenic impact of PI-resistance mutations in HIV-1 protease, human tonsil histocultures were inoculated in parallel with a previously described isogenic pair of recombinant HIV-1 strains containing PI-resistant or PI-sensitive protease genes. Virus 210ww contains a protease gene isolated from a patient shortly before the initiation of Ritonavir monotherapy (RTV) and 210wm contains a protease gene isolated from the same individual following RTV therapy with mutations in protease, two of which (I54V and V82A) confer PI-resistance.(21) This virus pair was constructed in the genetic background of pNL4-3XC, a CXCR4-dependent (X4) full-length HIV-1 proviral molecular clone.(1) 210wm has been shown to exhibit reduced infectivity and replication potential in single cycle infectivity assays *in vitro* and growth kinetics assays compared to 210ww.(21)

Tonsil histocultures were inoculated with equal doses of 210ww and 210wm (300-400 $TCID_{50}$/tissue block) and virus production in these cultures was monitored by quantifying capsid protein (p24) released into the culture medium over time. Tissues were dispersed into single cell suspensions at 16 days following infection and immunostained with monoclonal antibodies (mAbs) against CD3, CD4, and CD8 to measure selective depletion of $CD4^+$ T-cells using multi-parameter flow cytometry. Previous studies have shown that X4 strains, including parental NL4-3 and various recombinants thereof, aggressively deplete $CD4^+$ lymphocytes in these lymphoid histocultures.(13, 25, 29) Consistent with the X4 phenotype of the parental pNL4-3XC, both 210ww and 210wm severely depressed the CD4/CD8 ratio in tonsil histocultures, indicating selective and marked depletion of $CD4^+$ T-cells compared to the uninfected control (Fig. 1A).

Death of $CD4^+$ T-cells as the principal basis of the decreased CD4/CD8 ratios was verified by counting absolute numbers of $CD4^+$ T-cells. In addition, both viruses replicated to high levels in these cultures as evidenced by their release of significant amounts of p24 into the culture medium (Fig. 1B). Interestingly, despite *in vitro* data suggesting that 210wm had significantly impaired fitness (21, 38), this virus did not exhibit compromised replication or pathogenic potential in tonsil histocultures when compared to its pretherapy partner 210ww. These results demonstrate that PI-resistance mutations that reduce HIV-1 fitness in some culture systems do not, in and of themselves, attenuate the cardinal pathogenic effect of X4 viral infection in tonsil histocultures *ex vivo*.

It is possible that the two protease mutations in the context of a recombinant viral strain (210wm) are not sufficient to compromise the pathogenicity of this virus in lymphoid histocultures, but that additional viral evolution occurring under the selection pressure of PI therapy *in vivo* compromises the pathogenicity of primary PI-resistant viruses. Therefore, primary HIV-1 strains (T. Liegler, manuscript submitted) were isolated from patients experiencing virologic failure to PI therapy with preserved or increasing $CD4^+$ T-cell counts, and analyzed in tonsil explants *ex vivo*. Prior to the initiation of PI-based antiretroviral therapy, Patient 6 had an initial viral load (VL) of 89,000 copies/ml and a CD4 count of 81 cells/μl (Fig. 2A) while Patient 11 had an initial viral load of 35,000 copies/ml and a CD4 count of 97 cells/μl (Fig. 2B). Following treatment regimens that included multiple protease inhibitors (T. Liegler, manuscript submitted), these patients reached viral load nadirs of 1371 (Patient 6) and 736 (Patient 11) (Fig. 2). Despite the subsequent development of

resistance represented by rebound of viral loads and emergence of primary resistance mutations, $CD4^+$ T-cells continued to accumulate in these patients (Fig. 2A,B). For the present study we tested viral isolates from patients with marked viral rebound despite preserved $CD4^+$ T-cells (arrows in Fig. 2). As described elsewhere (T. Liegler, manuscript submitted), these isolates were obtained by coculture of patient peripheral blood mononuclear cells (PBMC) with allogeneic uninfected cells in the presence of RTV to recapitulate selection pressures in treated patients *in vivo* and to expand the dominant PI-resistant viral quasispecies. The sequences of the protease genes from these patient isolates were determined from RT-PCR products amplified from these viral stocks. Each virus tested was confirmed to have primary and secondary genetic markers of protease resistance compared to a clade B consensus sequence (Patient 6: L10I, L24I, M46I, I54V L63P, A71V, V77I, V82T, I84V; Patient 11: L10V, K20R, M36I, M46I, I54V, A71V, V82A, I84V L90M). Importantly, these sequences were consistent with those observed in plasma at the time of virus isolation. MT2 and/or GHOST cell assays demonstrated both isolates to be of the X4 phenotype (data not shown). The specificity of these viruses for CXCR4 further emphasizes the discordance between increasing viral loads and CD4 lymphocyte accumulation in these patients, because the emergence of X4 viruses in untreated HIV-positive patients is strongly associated with significant and rapid loss of $CD4^+$ T-cells.(7, 14, 30-33)

To test the hypothesis that these primary isolates have attenuated pathogenicity, we compared the $CD4^+$ T-cell depletion potential of these viruses to a PI-sensitive X4 primary isolate (7/86) (7, 29) in tonsil explants. We first inoculated tonsil histocultures with equal titers (50 $TCID_{50}$/tissue block) of 7/86

and the isolate from Patient 6 (#6) in the presence and absence of RTV (1 μM), and compared total $CD4^+$ T-cell depletion after 15 days. In the absence of RTV, both viruses markedly depleted $CD4^+$ lymphocytes (Fig. 3A) in tonsil histocultures and replicated to high levels in these tissues (Fig. 3B-C). In the presence of RTV, replication by 7/86 was substantially inhibited (Fig. 3B) as was depletion of $CD4^+$ T-cells (Fig. 3A), thereby confirming its PI-sensitivity. In contrast, #6 replicated to high levels (Fig. 3C) and depleted $CD4^+$ lymphocytes aggressively (Fig. 3A) in the presence and absence of RTV, thereby confirming its PI-resistance. In three independent experiments, no consistent difference in virus production was observed between histocultures infected with 7/86 or #6. These results indicate that the overall pathogenic potential of this primary PI-resistant isolate in mature lymphoid histocultures is not decreased.

Nonetheless, it remained possible that resistant viruses may deplete $CD4^+$ T-cells with slower kinetics than do wild-type viruses and that this difference is sufficient to permit $CD4^+$ T-cell accumulation *in vivo* despite viral replication. To test this possibility, we performed a kinetic analysis of CD4 depletion with the second isolate derived from Patient 11 (#11), described earlier, who also experienced a marked increase in $CD4^+$ T-cells despite viral load rebound (Fig. 2B). Histocultures were inoculated with PI-sensitive 7/86 and PI-resistant #11, and $CD4^+$ T-cell depletion was monitored over time. In the absence of RTV, 7/86 progressively depleted $CD4^+$ T-cells (Fig. 4A), while in the presence of RTV (1 μM), it neither depleted $CD4^+$ T-cells (Fig. 4A) nor replicated (Fig. 4C) during this period. However, #11 replicated efficiently (Fig. 4D) and progressively depleted $CD4^+$ T-cells (Fig. 4B) in the absence or presence of RTV. These results confirm

that this PI-resistant isolate is not intrinsically compromised in its capacity to deplete $CD4^+$ T-cells in mature lymphoid histocultures *ex vivo*.

Human tonsil explants represent an informative model of HIV-1 infection in mature lymphoid tissues.(13, 25, 29) The present results with recombinant viruses evaluated in this system demonstrate that mutations in HIV-1 protease *per se* do not compromise viral pathogenic potential in mature T-cells. Furthermore, they reveal that primary X4 viral isolates obtained from patients during virologic failure to PI therapy in the setting of continued CD4 accumulation retain the capacity to deplete $CD4^+$ T-cells as efficiently as a wild-type isolate. Therefore, unknown host and/or viral factors appear to be responsible for preserving or increasing the CD4 counts in this subset of patients. Further experiments will be necessary to establish the pertinent mechanism(s).

Previous studies described a significant loss of fitness in viruses possessing PI-resistance mutations in protease.(21, 38) The present results suggest that the previously observed decreased replication potential of 210wm *in vitro* did not compromise its ability to replicate and deplete $CD4^+$ T-cells in tonsil histocultures *ex vivo*, demonstrating that the inherent pathogenicity of HIV-1 in secondary lymphoid tissue is not affected by its mutations in protease that confer PI-resistance. Therefore, the mechanism(s) underlying $CD4^+$ T-cell accumulation in patients harboring PI-resistance mutations must act independently of mature T-cell destruction. However, we cannot exclude that there may be differences in viral replication capacity in other contexts. In particular, these mutations may affect replication and pathogenicity in other lymphoid tissues such as thymus or bone marrow that have prodominantly immature populations of lymphocytes. For example, it has been found that 210wm exhibits severely compromised

replication in human thymus (C. Stoddart, personal communication). If PI-resistant HIV-1 strains replicate poorly in specific lymphoid compartments (central or peripheral) that contribute to the growing T-cell pool, exclusion of virus replication from these sites conceivably may explain why patients with PI-resistant viruses can continue to accumulate $CD4^+$ T-cells despite ongoing peripheral T-cell depletion. The finding that HIV-1 strains with PIR mutations remain cytopathic for mature $CD4^+$ T-cells predicts that the discordance between virologic failure and preservation of $CD4^+$ T-cells may be transient and eventually replaced by progressive loss of T-cells in some HIV-infected individuals.

Acknowledgements

The authors thank Dee J. Holthe, Cecilia Stewart, Claudette Delphis, Sharon Hall, Ursula Perotti, Mark Weinstein and the surgical staffs at Kaiser Hospitals (San Rafael, San Francisco) and San Francisco General Hospital for generous assistance in obtaining post-tonsillectomy samples and to Abbott Laboratories for a generous gift of RTV. The authors acknowledge the technical assistance of Dr. Eric Wieder and Lisa Gibson, Sam Lee and other members of the Gladstone/UCSF Laboratory of Clinical Virology, and of Heather Gravois, John Carroll, and Neile Shea in the preparation of this manuscript. M.L.P. was supported by the UCSF Chancellor's Fellowship, the Biomedical Sciences Graduate Program, the National Institutes of Health Medical Scientist Training Program at UCSF, and the California Universitywide AIDS Research Program. M.M. and D.A.E. were supported by the Biomedical Sciences Graduate Program. D.A.E. was also supported by the National Institutes of Health Medical Scientist Training Program at UCSF. This work was supported in part by N.I.H. R01-AI43695 & R01-CA86814 (M.A.G.), N.I.H. P30-MH59037 (RMG), and the J. David Gladstone Institutes.

FIGURE LEGENDS

Fig. 1. $CD4^+$ T cell depletion and viral replication in human tonsil histocultures infected by recombinant PI-sensitive and PI-resistant HIV-1 strains. (A) $CD4^+$ T cell depletion as indicated by mean CD4/CD8 ratio relative to an uninfected control on day 16 after infection by recombinant viruses 210ww and 210wm. For each replicate, cells were pooled from 6–10 tissue blocks (mean ± SEM, n=3) and analyzed by multi-parameter flow cytometry. One representative experiment is shown (n=3). (B) Viral replication was monitored by assessing the accumulation of p24 in the culture medium between successive medium changes on days 3, 6, 9, 12, and 15.

Fig. 2. $CD4^+$ T-cell accumulation in HIV-1-infected patients experiencing virologic failure to PI therapy. Shown are the CD4 counts (left axis) and viral loads (right axis) of two patients (A, Patient 6; B, patient 11) with selective virologic failure while on HAART. (A) Patient 6, (B) Patient 11. Beginning of graphs indicate time point of HAART initiation. Arrows indicate time of virus isolation.

Fig. 3. $CD4^+$ T cell depletion and viral replication in human tonsil histocultures infected by a primary PI-resistant HIV-1 isolate derived during sustained $CD4^+$ T-cell increases. (A) $CD4^+$ T cell depletion as indicated by mean relative CD4/CD8 ratio on day 15 after infection by PI-sensitive 7/86 and PI-resistant isolate #6. (B) HIV-1 p24 concentration in the culture medium of 7/86-infected histocultures sampled at various time points. (C) HIV-1 p24

concentration in the culture medium of isolate #6-infected histocultures sampled at various time points. A representative experiment is presented (n=3).

Fig. 4. Kinetic analysis of $CD4^+$ T-cell depletion and replication of a protease inhibitor-resistant virus obtained during sustained $CD4^+$ T-cell increases. Overall $CD4^+$ T-cell depletion over 12 days in duplicate samples of one donor tissue infected with 7/86 (A) or #11 (B) is shown along with corresponding HIV-1 p24 concentration in cultures infected with 7/86 (C) and isolate #11 (D).

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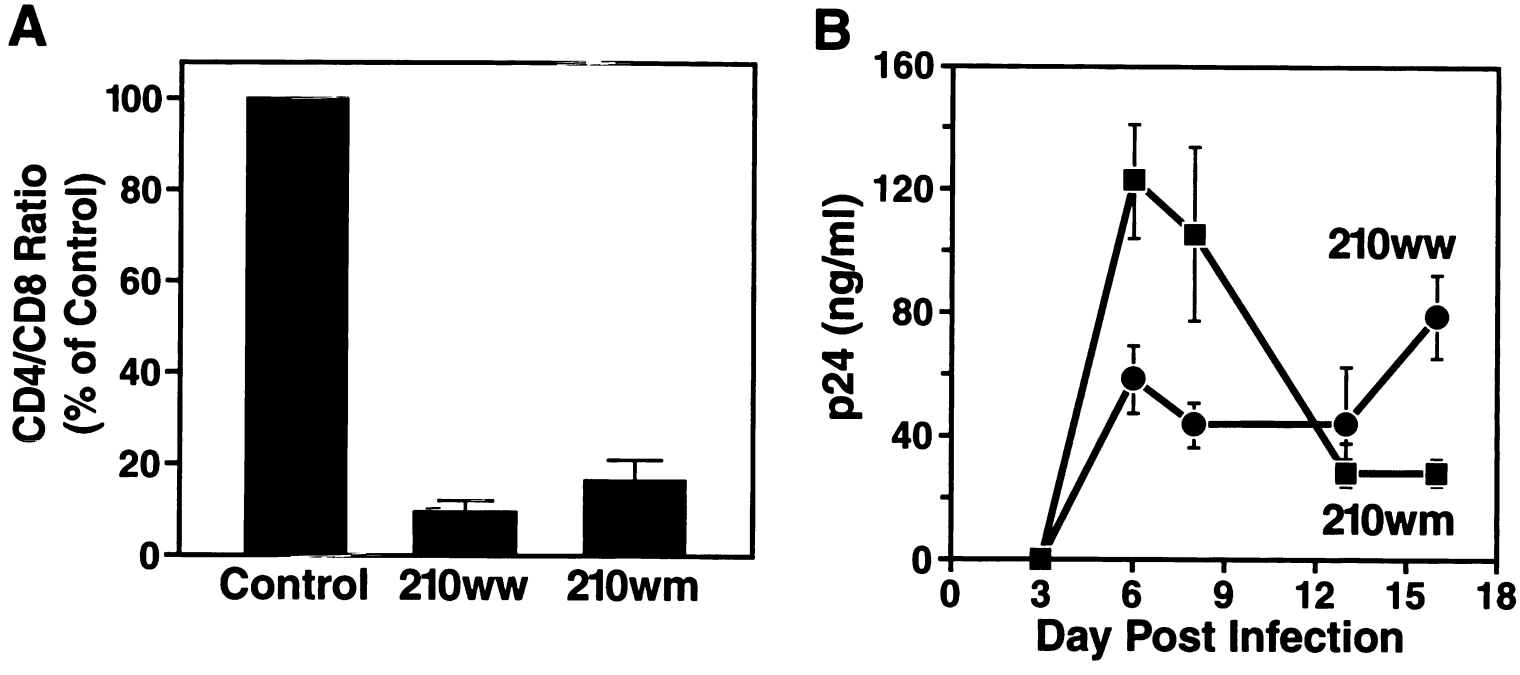
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CD4/CD8 Ratio
(% of Control)
100
80
60
40
20
0
Control
210ww
210wm
B
p24 (ng/ml)
160
120
80
40
0
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3
6
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18
Day Post Infection
210ww
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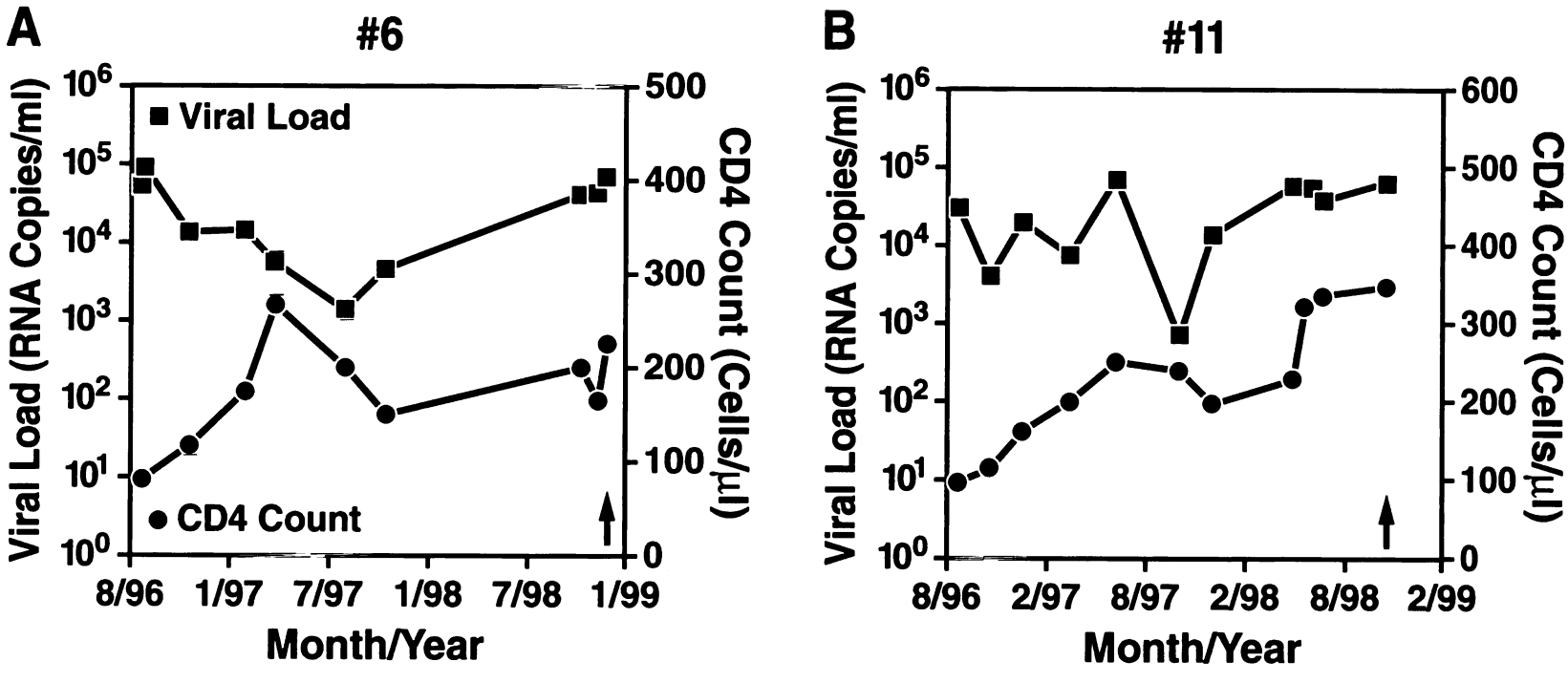
Figure 1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CD4 Count
Viral Load (RNA Copies/ml)
CD4 Count (Cells/μl)
Month/Year
8/96
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7/97
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#11
Viral Load (RNA Copies/ml)
CD4 Count (Cells/μl)
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8/96
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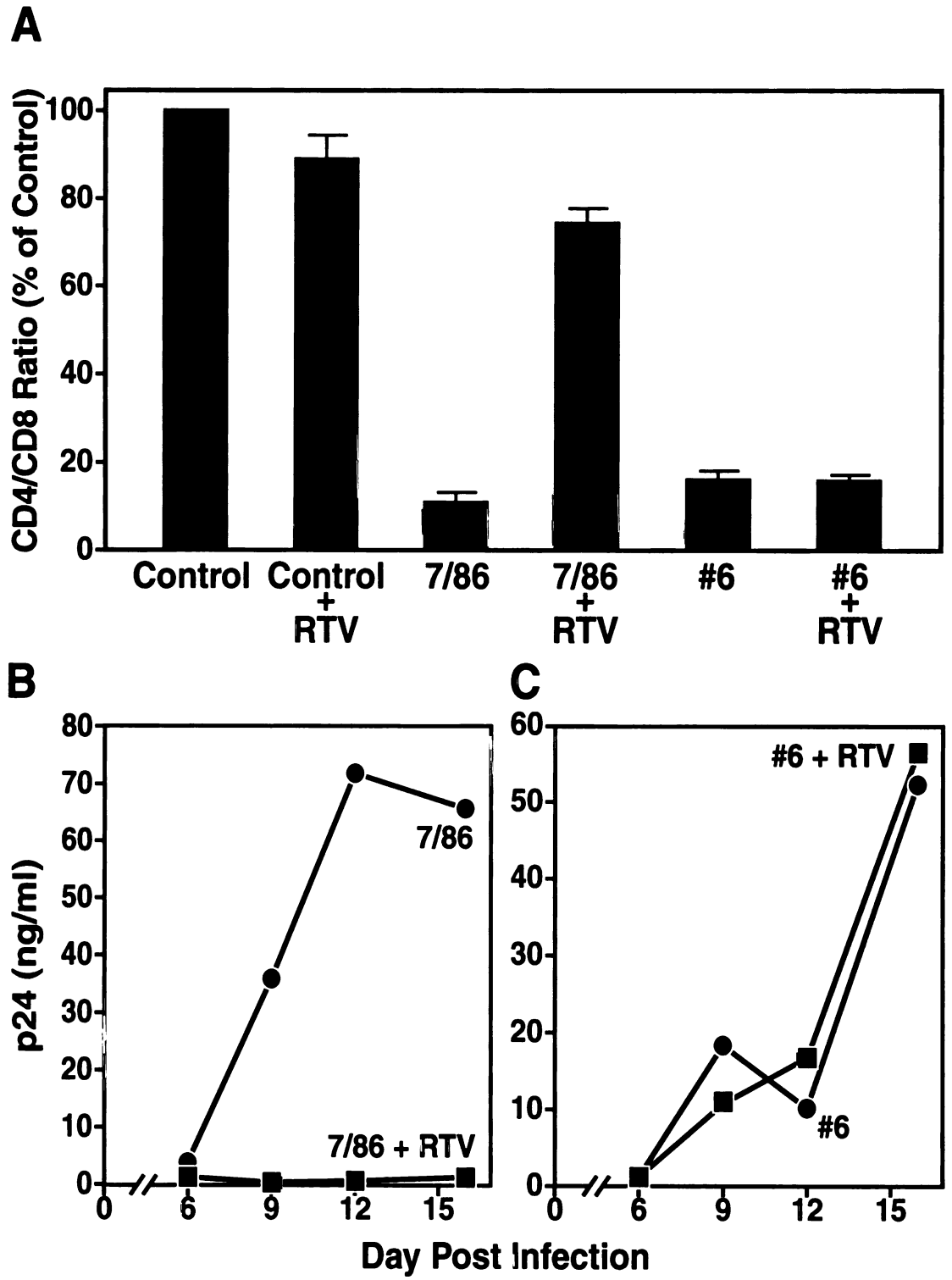
2/99

Figure 2


A
CD4/CD8 Ratio (% of Control)
100
80
60
40
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Control
Control + RTV
7/86
7/86 + RTV
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B
p24 (ng/ml)
80
70
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#6 + RTV
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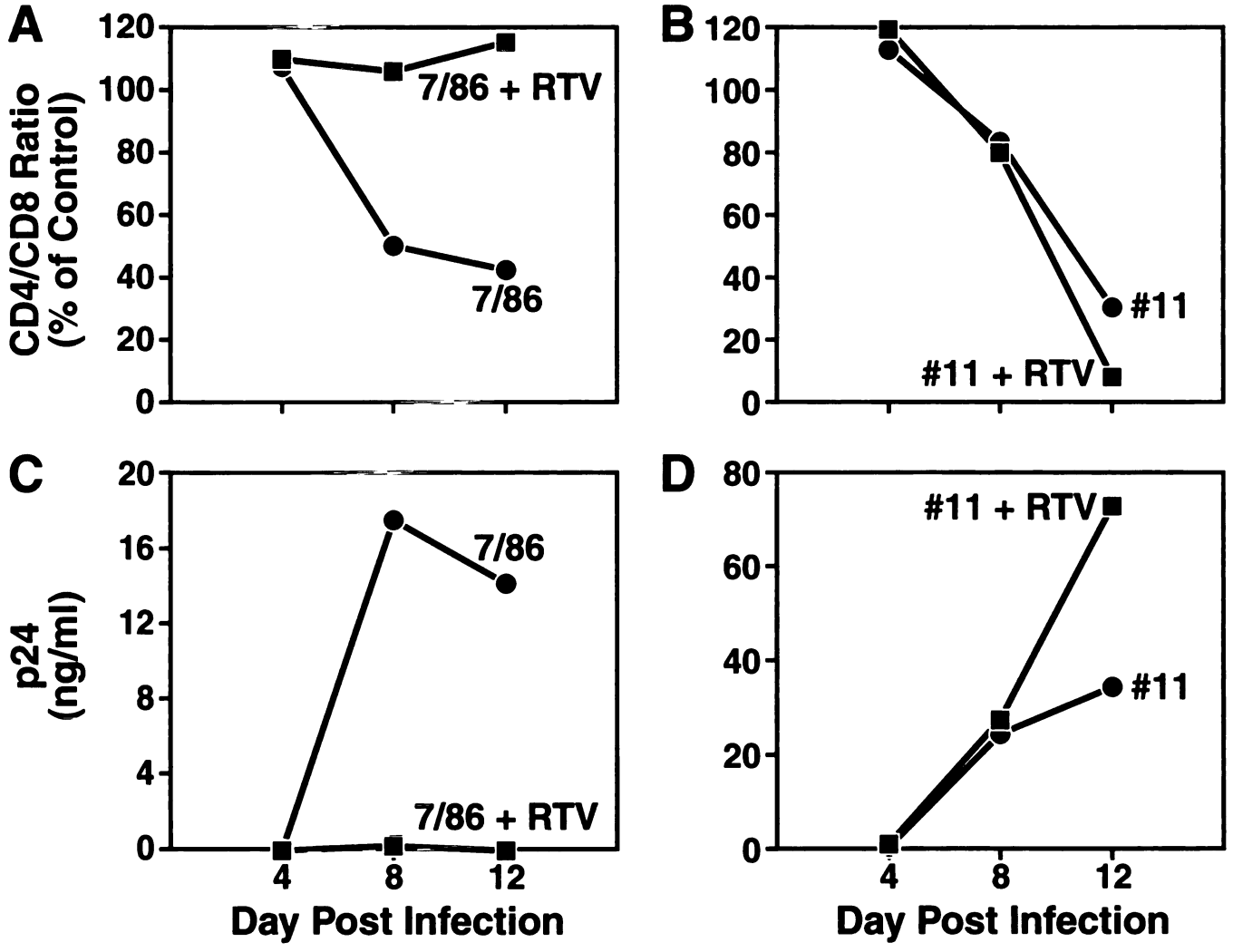
Day Post Infection

Figure 3


A
CD4/CD8 Ratio (% of Control)
120
100
80
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7/86 + RTV
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16
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Figure 4

Chapter 7

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

Prologue

The pathogenesis of HIV disease remains a subject of substantial interest among immunologists, virologists and clinicians. The role of infection of specific T-cell subsets is both poorly understood and the subject of controversy. Knowledge of the molecular and cellular events underlying the global deterioration of CD4 T-cells has important implications regarding efforts to halt progressive immunodeficiency through antiviral treatment and/or to reverse it through immune reconstitution efforts. These studies seek to understand why HIV viral gene expression is detected in non-activated, naïve, and/or resting T-cells *in vivo* while such cells are resistant to productive infection *in vitro*. In particular, we sought to determine whether the presence of HIV within naïve cells represents *de novo* infection or instead is a marker of prior infection of cycling T-cell precursors. The findings indicate that all subsets of CD4 cells in lymphoid tissues are substantially permissive for productive infection and elimination by HIV. Quiescent, naïve T-cells in these tissues exhibit significant permissivity for productive infection. Therefore, these cells are not exclusively a reservoir for latent virus. Instead, they also support active replication following infection *de novo*, which leads to cytotoxicity and acceleration of the overall decline of CD4 T-cells.

This work was performed in close collaboration with co-first author, D. Eckstein who provided experimental data presented in Figures 1B, and 8, invaluable intellectual contributions to the experimental design, and critical editing of the final document. Y. Korin, D. Scripture-Adams, and J. Zack generously adapted their assay for the histoculture system and together we

generated the data presented in Figure 9, P. Chin provided technical and experimental support, M. Roederer gave valuable technical advice and intellectual input, and M. Sherman helped generate the data presented in Figure 6A and made significant intellectual contributions. M. Goldsmith supervised this project.

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

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ABSTRACT

A paradox in HIV-1 biology is the observation that viral gene expression is detected in naïve, resting T-cells in vivo while such cells are resistant to productive infection in vitro. We tested the hypothesis that the endogenous microenvironment of human lymphoid tissues supports de novo infection of quiescent T-cells. In acute infection experiments, we found that HIV-1 substantially infected and depleted resting, naïve CD4+ lymphocytes. Cell cycle analysis and DNA labeling experiments established that these cells were definitively quiescent and thus infected *de novo* by HIV-1. These findings provide direct evidence 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 which likely targets them for direct elimination by HIV-1.

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 leading to severe immunodeficiency. Several clinical studies have suggested that particular cellular subsets are differentially susceptible to the pathogenic effects of HIV-1 and thus are lost selectively during the course of HIV disease(3, 15, 22, 26, 36, 50, 55) implying that certain cellular characteristics regulate susceptibility to productive infection and depletion by HIV-1. This hypothesis remains controversial, however, because other studies have failed to detect preferential loss of individual subsets as total numbers of $CD4^+$ T-cells decline *in vivo* (6, 10, 19, 23, 30, 35).

Many *in vitro* studies with peripheral blood mononuclear cells (PBMC) have also suggested that specific cellular characteristics govern the susceptibility of cells to infection by HIV-1. These characteristics fall into three general categories: activation, maturation (i.e., naïve vs. memory), and proliferation. First, cellular activation has been shown to be required for the completion of reverse transcription and/or proviral integration in cultured cells (8, 44, 46, 53, 54). In particular, the expression of select cell surface molecules such as CD25 and HLA-DR coincides with cellular activation and appears to define cells that are susceptible to lentivirus infection (11, 34, 51). Second, cell maturation has also been shown to be an important factor regulating HIV-1 infection of $CD4^+$ T-cells *in vitro*. Specifically, cultured memory cells are preferentially infected (25, 40, 45, 52) while resting and/or naïve cells are highly resistant to infection (11, 37, 53, 54). Third, cellular proliferation status has been observed to regulate HIV-

1 infection in PBMC, with reverse transcription and viral integration being incomplete or inefficient in the absence of cellular proliferation (7, 8, 44, 47, 54). However, despite this extensive evidence of multiple cellular restrictions in the *in vitro* context, recent work has revealed that non-activated, naïve, and/or non-proliferating lymphocytes in HIV-1 infected patients exhibit active HIV-1 gene expression (4, 32, 56). 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 these seemingly resistant cell populations were virally infected *de novo* or acquired virus 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 independently of exogenous stimulation (20), thus preserving the endogenous cytokine milieu and cellular heterogeneity. We therefore used human tonsil or spleen 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. The results provide strong evidence of productive *de novo* infection and depletion of virtually all classes of $CD4^+$ T-cells within the tissue context regardless of the activation, maturation, or proliferation status of these cells. Therefore, such cells are not solely reservoirs of latent virus, but also are permissive hosts for HIV-1 replication. Loss of these cells due to HIV-mediated cytopathic effects likely contributes directly to the overall collapse of the immune system.

MATERIALS AND METHODS

Preparation of viral stocks.

NL4-3 was obtained from 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 generous gift from Bruce Chesebro. Infectious virus stocks were prepared by transfecting 293T cells with proviral DNA as previously described(2). The p24 Gag concentration of viral stocks was assessed by enzyme linked immunosorbent assay (NEN Life Sciences, Boston, Mass.).

Culture and infection of human lymphoid tissues *ex vivo*.

Human noninflammatory spleen tissue derived from surgical specimens (provided by National Disease Research Institute, Philadelphia, Pa.) and 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(20). Tissues were inoculated with HIV-1 strains at approximately 50 TCID50/tissue block, as determined by terminal dilution of the virus stocks in quadruplicate on heterologous phytohemagglutinin-activated PBMC as described previously (20, 33). Following 12-day infections, cells were mechanically isolated from infected and uninfected tissue and analyzed by flow cytometry (see below).

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 (33, 42). 5,000 to 10,000 $CD3^+$ lymphocytes were counted and the data were analyzed with CellQuest software (Becton Dickinson, San Jose, Ca.). $CD4^+$ T-cell depletion was assessed in two ways. First, to facilitate comparison among experiments, $CD4^+$ T-cell depletion was expressed as the ratio of $CD4^+$ to $CD8^+$ T-cells in infected relative to uninfected tissues as described previously (20). Second, to determine $CD4^+$ depletion in more complex subsets we enumerated absolute cell numbers. Total cell numbers were determined by inoculating dispersed cells with Fluorospheres (1:10 dilution) (Coulter Immunotech, Miami, Fl.) and calculated per manufacturer instructions. To identify activated, non-activated, naïve and memory subsets the following monoclonal antibodies (mAbs) were used from Beckton Dickinson: 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 (phycoerythrin conjugated, 1:20 dilution), anti-HLA-DR (phycoerythrin conjugated, 1:20 dilution), and anti-CD62L (fluoroscein isothiocyanate, 1:20 dilution). The following antibodies from Pharmingen (San Jose, Ca.) were also used: anti-CD45RA (cychrome conjugated, 1:2.5 dilution) and anti-CD45RO (phycoerythrin conjugated, 1:10 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).

Flow cytometric analysis of cellular proliferation.

For propidium iodine staining, cells were first stained with an anti-CD4 mAb (Becton Dickinson, allophycocyanin conjugated, 1:40 dilution) on ice for 30 minutes. Cells were then washed and fixed with 2% paraformaldehyde for 30 minutes at room temperature. Following a final wash, cells were resuspended in a solution of RNase A (1μg/ml, Sigma, St. Louis, Mo.) and propidium iodide (10 μg /ml, Molecular Probes, Eugene, Or.) for 30 minutes and analyzed by flow cytometry. For BrdU labeling, lymphoid histocultures were cultured for 12-hours in standard histoculture media supplemented with 50 μM BrdU (Sigma). Following this incubation, cells were dispersed and fixed and permeabilized overnight in a solution of 1% paraformaldehyde and 0.01% Tween 20. Cells were washed and then treated with DNase (10 mg/ml, Sigma, St. Louis, Mo.) in phosphate buffered saline (with calcium and magnesium) at 37° C. After washing, cells were immunostained with a combination of mAbs recognizing CD4 (Becton Dickinson,clone SK3, allophycocyanin conjugated, 1:40 dilution), CD45RA (Pharmingen, cychrome conjugated, 1:2.5 dilution), CD45RO (Pharmingen, phycoerythrin conjugated, 1:10 dilution), and BrdU (Pharmingen, fluoroscein isothiocyanate conjugated, 1:2.5 dilution) in 10% fetal bovine serum in PBS for 30 minutes on ice. For both propidium iodide and BrdU analysis, 30,000 to 60,000 lymphocytes were collected and analyzed by CellQuest.

Four color cell cycle. Four color cell cycle analysis was performed using the method of Schmid et al. (39). Briefly, PBS washed cells were surface stained with a biotinylated monoclonal antibody specific for CD4, and an APC conjugated antibody specific for CD45RA (Becton Dickinson). After washing in PBS supplemented with 2% FCS (FACS PBS), cells were stained in 100 μl of FACS

PBS containing 2 μg of streptavidin Alexa 488 (Molecular Probes), incubated 20 minutes at 4 degrees, then washed in FACS PBS. For detection of DNA and RNA, cells were resuspended in 0.5 ml of phosphate-citrate buffer, PH 4.8, containing 0.02% saponin (PCBS) and 10 μg/ml of seven-aminoactinomycin D (Calbiochem, San Diego, Ca.), and incubated 20 min at room temperature. PBS washed cells were resuspended in 0.5ml of PCBS containing 10 μg/ml of actinomycin D (Roche Molecular Biosystems (Indianapolis, In.) and placed on ice for 5 minutes prior to addition of 5 μl of a 0.1 mg/ml stock of pyronin Y (Polysciences, Warringon, Pa.) for RNA staining. The cell mixture was vortexed and kept on ice protected from light for at least 10 minutes before anlaysis on a FACSCalibur flow cytometer. Analysis of surface staining and DNA/RNA content was performed using Cellquest software.

RESULTS

The heterogeneous cellular milieu within human lymphoid histocultures supports the replication of human immunodeficiency virus type 1 (HIV-1) and the selective depletion of $CD4^+$ T-cells, thereby recapitulating key aspects of HIV-1 infection *in vivo*. To elucidate in detail the roles of individual cell types in HIV-1 replication and pathogenesis in these tissues, we used flow cytometry to define specific subpopulations of cells within histocultures derived from human tonsil or spleen. Using conventional forward and side scatter analysis, we first delineated several distinct populations in tonsil samples based on size and granularity (Fig. 1A). One population, which was identifiable by low forward and side scatter signals (Fig. 1A), contained principally T-cells as evidenced by substantial reactivity with anti-CD3 antibodies (Fig. 1A, left histogram), and is hereafter termed "lymphocytes." A second, distinct population of cells characterized by more pronounced forward and side scatter properties (Fig. 1A) also contained primarily $CD3^+$ cells (Fig. 1A, right histogram). We termed these larger, more granular cells "blasts" because of their resemblance to cells found in lectin-activated PBMC cultures. Blasts typically represented a minor fraction in relation to lymphocytes in these samples. Dual-parameter light scatter analysis that distinguishes doublets from single cells confirmed that the majority of these larger blasts were indeed single cells (data not shown). The remaining cells in the culture had extremely pronounced side scatter and a broad range of forward scatter signals (Fig. 1A). Definitive classification of the cells comprising this mixed population was confounded by high levels of autofluorescence, but immunostaining for various surface markers revealed a mixture of diverse cell

types within this population including dendritic cells ($CD21^+$ or $CD83^+$), macrophages ($CD14^+$ and $CD68^+$), large $CD3^+$ cells, and multicellular conjugates of T-cells and dendritic cells or macrophages (Fig. 1A and data not shown). Light scatter and immunostaining profiles of spleen samples were indistinguishable from these tonsil samples, with similar populations of lymphocytes, blasts and other cell types described above (data not shown). Therefore, lymphoid tissues cultured *ex vivo* are heterogeneous and composed of several distinct and identifiable cell populations.

We next determined the relative susceptibility of $CD4^+$ lymphocytes and blasts to HIV-mediated depletion by measuring the selective loss of either subset following inoculation of histocultures with the CXCR4-dependent (X4) HIV-1 strain NL4-3 (1, 33, 41, 43) or the CCR5-dependent (R5) strain 49-5 (9, 33). Consistent with previously published results (21, 33, 42), NL4-3 caused marked and selective loss of $CD4^+$ T-cells following a 12-day infection (Fig. 1B). Significant depletion of both lymphocytes and blasts was observed, although blasts typically exhibited greater susceptibility to a given virus inoculum than did lymphocytes (Fig. 1B). As expected from previous observations that restricted CCR5 expression among T-cells leads to decreased target cell frequencies for R5 viruses (21, 42), we observed less overall depletion in the 49-5-infected cultures (Fig. 1B). Nonetheless, the same pattern of enhanced depletion of blasts compared with lymphocytes was also evident in these cultures (Fig. 1B). These results demonstrate that HIV-1 potently depletes both lymphocytes and blasts in human lymphoid tissues, and that blasts exhibit somewhat greater susceptibility to both X4 and R5 strains.

We also sought to determine the biologic characteristics of lymphoid blasts that account for their increased susceptibility to depletion by HIV-1. Based on extensive prior studies using PBMC cultures, it is widely held that resting lymphocytes are non-permissive for HIV-1 infection. To determine which biologic parameters influence susceptibility to cellular depletion within the lymphoid tissue context, we used multicolor flow cytometry to define the activation and maturation phenotypes of lymphocytes and blasts within lymphoid histocultures. First, CD25 and HLA-DR were used as conventional markers of cellular activation. The levels of cellular activation did not markedly differ between tonsil (typically excised secondary to tonsillitis) and spleen (typically excised secondary to non-inflammatory conditions) specimens, and the cellular activation profile of a given tissue did not change consistently during the culture periods (data not shown). Typically, CD25 was found on a minority of lymphocytes within tonsil tissue and on a somewhat greater proportion of blasts (Fig. 2A,C). Likewise, HLA-DR was found on a larger proportion of blasts than lymphocytes (Fig. 2B,D). The increased expression of activation markers in the blast population suggests that activation status may indeed be a factor that contributes to the increased susceptibility of this population to depletion by HIV-1.

To test this hypothesis directly, we inoculated tonsillar explants with NL4-3 or 49-5 and quantitated the activated and non-activated T-cell subsets by flow cytometry following a 12-day infection. Surprisingly, marked and comparable depletion of both activated and non-activated $CD4^+$ lymphocytes and blasts was observed (Fig. 3). The X4 strain NL4-3 caused a profound loss of the overall $CD4^+$ T-cell population with no preference for activated cells, depleting both

$CD25^+$ and $CD25^-$ cells (Fig. 3A) as well as $HLA\text{-}DR^+$ and $HLA\text{-}DR^-$ cells (Fig. 3C) by greater than 90%. Similarly, the R5 strain 49-5 exhibited no significant preference for activated cells, depleting both activated and non-activated cells modestly and comparably (Fig. 3B,D) in accordance with the restricted expression of CCR5. A kinetic experiment examining activation marker expression over time revealed no significant differences in CD25 or HLA-DR expression in HIV-infected histocultures compared to an uninfected culture at any time point excluding that HIV-induced changes in the expression of activation markers confounds these results. Thus, based on stratification with these conventional cell surface markers, the apparent activation status of $CD4^+$ cells in human lymphoid histocultures does not substantially influence susceptibility to the cytopathic effects of HIV-1.

Second, to distinguish states of lymphocyte maturation, CD45RA was used in combination with either CD45RO or CD62L to identify naïve or memory cells. The majority of $CD4^+$ lymphocytes and blasts were found to be memory cells (non-$CD45RA^+/CD62L^+$) (Fig. 4A,C); the blast fraction contained somewhat fewer memory cells (Fig. 4C) than did the lymphocyte fraction (Fig. 4A). The majority of both lymphocytes and blasts had a memory phenotype as determined by reactivity to anti-CD45RO antibodies (Fig. 4B,D). Interestingly, a population of RA^+/RO^+ cells was found exclusively within the blast population (Fig. 4D); cells with this surface phenotype may represent an intermediate stage in the development of naïve T-cells into memory cells (24, 28). That blasts are enriched for naïve cells—a population considered to be resistant to HIV-1 infection—suggested that differences in maturation status between lymphocytes

and blasts do not account for their differential susceptibility to HIV-1-mediated depletion.

To determine whether maturation phenotype influences susceptibility of T-cells to depletion, we measured the specific depletion of naïve and memory $CD4^+$ lymphocytes and blasts following 12-day infections with HIV-1 strains NL4-3 or 49-5. We found that NL4-3 severely depleted both naïve and memory $CD4^+$ lymphocytes, but preferential depletion of memory cells was nonetheless evident (Fig. 5A). Similarly, memory lymphocytes were significantly and preferentially depleted by 49-5 relative to naïve cells (Fig. 5B), although the overall efficiency of CD4 depletion caused by this R5 virus was lower. As in earlier experiments (Fig. 1B), blasts typically exhibited overall greater susceptibility to depletion than lymphocytes, but the preferential depletion of memory cells within either the lymphocyte or blast population was most striking and consistent (Fig. 5). These results demonstrate that diverse subsets of $CD4^+$ T-cells within the lymphoid tissue environment are highly susceptible to the cytopathic effects of HIV-1, and that no recognized $CD4^+$ T-cell subset is invulnerable to direct elimination by HIV-1.

Third, we compared the proliferative status of these cellular pools by measuring either propidium iodide (PI) staining or bromodeoxyuridine (BrdU) incorporation in conjunction with flow cytometry. Both methods revealed that lymphocytes exhibited negligible proliferative activity while a substantial fraction of blasts were cycling (Fig. 6A). Thus, cellular proliferation status, rather than activation or maturation phenotypes, distinguishes blasts from lymphocytes in lymphoid histocultures. The significant virus-induced depletion of non-cycling lymphocytes within lymphoid tissues, particularly by X4 HIV-1 (Fig. 1B),

suggests that these cells may be productively infected by HIV-1 at significant levels. We used flow cytometry to measure kinetically the frequency of lymphocytes and blasts expressing intracellular Gag (p24) as a marker of productive infection following inoculation of histocultures with HIV-1 strains NL4-3 or 49-5. Substantial rates of infection were observed in both cell populations (Fig. 6B). In the NL4-3 infected cultures, nearly 12% of $CD3^+$ lymphocytes were p24-positive at peak time points, while infection rates in blasts were approximately twice these levels at all time points (Fig. 6B). As expected, overall lower rates of productive infection were observed in the 49-5-infected cultures, but a significant fraction of both lymphocytes and blasts were $p24^+$ (Fig. 6B). These results demonstrate that lymphocytes in human lymphoid tissues are productively infected by HIV-1 at substantial frequencies despite their resting status.

Next we sought to determine the basis of preferential depletion of memory cells over naïve cells, by comparing levels of BrdU incorporation within the memory and naïve subsets of lymphocytes and blasts. Consistent with the earlier analysis of non-stratified cells, negligible proportions of either naïve ($CD45RA^+/RO^-$) or memory ($CD45RA^-/RO^+$) lymphocytes exhibited significant BrdU incorporation (Fig. 7A). Notably, in all tissue samples tested less than 0.2% of naïve lymphocytes incorporated BrdU, while significant proportions of naïve ($CD45RA^+/RO^-$), memory ($CD45RA^-/RO^+$) or transitional ($CD45RA^+/RO^+$) blasts incorporated BrdU (Fig. 7A). The minimal evidence of proliferation among naïve and memory lymphocytes is particularly striking since we detected significant HIV-1-induced depletion of both subsets (Fig. 5). An important question is whether depletion is associated with direct infection of these subsets, since naïve

cells are resistant to *de novo* infection by HIV-1 *in vitro* (37). To determine directly if resting naïve and/or memory cells in lymphoid tissues are infected *de novo* by HIV-1, we immunostained NL4-3- and 49-5-infected tonsil explants for intracellular p24. Naïve lymphocytes were infected at significant frequencies (Fig. 7B) despite their minimal proliferative activity (Fig. 7A). In particular, naïve $CD4^+$ lymphocytes were productively infected by NL4-3 at significant levels whether these cells were defined as $CD45RA^+$/RO- (Fig. 7B) or $CD45RA^+/CD62L^+$ (data not shown). Non-proliferating, memory cells defined by the $CD45RO^+$ phenotype or non-$CD45RA^+/CD62L^+$ phenotype were also productively infected by NL4-3 at very high levels (Fig. 7B, and data not shown). As expected, blast cells of all maturation phenotypes were also measurably infected by NL4-3, with the highest levels being observed in the memory fraction (Fig. 7B). Interestingly, blasts with a naïve ($CD45RA^+$) or intermediate phenotype ($CD45RA^+/CD45RO^+$) (Fig. 4) were consistently infected at lower levels than were non-cycling lymphocytes (Fig. 7A,B). The R5 virus 49-5 also demonstrated a strong preference for memory cell targets within either the non-blast or blast fraction (Fig. 7B). Collectively, these results based on cell subsetting provide strong evidence that cellular proliferation is not an absolute requirement for infection and depletion of $CD4^+$ T-cells within lymphoid tissues.

Another important consideration is the history of a given cell since cycling blasts and/or memory effector cells have the capacity to return to a non-cycling state (29). To determine the fraction of proliferating blasts that return to the non-proliferating lymphocyte population within tonsil histocultures, we performed pulse-chase experiments to follow the potential trafficking of BrdU-labeled cells. Histocultures were labeled for 12 hours with BrdU and then chased in the

absence of BrdU for an additional 16-24 hrs. Following the initial labeling period, approximately 19% of $CD4^+$ blasts and only 1% of $CD4^+$ lymphocytes had incorporated BrdU (Fig. 8). After the chase intervals, no significant change in the percentage of $BrdU^+$ blasts was observed (Fig. 8A), suggesting that residual labeling during the chase period was minimal. In contrast, the percentage of labeled lymphocytes increased modestly (3.5-fold) during the chase period to a maximum of nearly 4% (Fig. 8A). Interestingly, the majority of these $BrdU^+$ lymphocytes were memory cells because the naïve T-cell pool increased by only 0.1% during the chase period (Fig. 8A). The increased frequency of $BrdU^+$ lymphocytes detected during the chase period likely represented a very small pool of memory/effector blasts that entered S phase during the labeling period and subsequently returned to a non-cycling state (characterized by non-blast morphology) prior to the end of the chase interval. These results imply that a small fraction of non-proliferating lymphocytes productively infected by HIV-1 may be derived from cells that were infected as blasts. Nevertheless, the negligible rate of recirculation into the $CD4^+$ naïve pool indicates that cells within this population are likely infected *de novo*.

To determine directly whether the proliferating fraction of naïve lymphocytes can account for the surprisingly high frequency of productively infected cells observed in this population, the cumulative fraction of proliferating naïve T-cells and blasts trafficking into this population was measured during a typical HIV-1 infection of tonsil histocultures. Parallel histocultures were infected with NL4-3 or cultured continuously in medium containing BrdU (25 _M) for seven days. Cells were then dispersed, immunostained for BrdU or intracellular p24, and analyzed by flow cytometry. While approximately 11.3%

of naïve CD4$^+$ lymphocytes were p24-positive, only 1.2% of these cells had incorporated BrdU over the seven-day period, representing approximately an 8-fold difference (Fig. 8B). In contrast, 16.5% of CD4$^+$ blasts had incorporated BrdU during the same time period, thus confirming the ability of immunostaining and flow cytometry to detect proliferating cells in these cultures (Fig. 8B). Together with the pulse-chase experiments, these data provide direct evidence that non-proliferating, naïve CD4$^+$ lymphocytes are infected *de novo* by HIV-1 in human lymphoid histocultures.

Finally, recent studies have suggested productive HIV infection depends upon a particular stage of the cell cycle that precedes cell division rather than upon cell division *per se* (27). In particular, unstimulated, non-cycling PBMC in G0/G1a produce only partial reverse transcripts upon HIV-1 infection while stimulated cells reaching the G1b phase or beyond support the completion of reverse transcription(27). Cells in G1b have committed to DNA synthesis and subsequent mitosis and display very high RNA levels but have not yet synthesized DNA (14, 18). To determine whether lymphocytes that are permissive to HIV-1 infection in lymphoid tissues have progressed to G1b, we used flow cytometry to compare the cell cycle status of histoculture lymphocyte and blast subsets. We stained these cells with a combination of the nucleic acid dyes (7-aminoactinomycin D for DNA content and pyronin Y for RNA content) and monoclonal antibodies (anti-CD4, anti-CD45RA). As expected, stimulated PBMC cultures exhibited substantial progression to G1b or S/G2/M (Fig. 9A), while unstimulated PBMC resided nearly exclusively in G0/G1a. Likewise, the majority of histoculture blasts were found in either G1b or S/G2/M (Fig. 9C), while virtually all lymphocytes were in arrested G0/G1a (Fig. 9D); naïve

lymphocytes also rested nearly exclusively in G0/G1a with only a negligible fraction in S/G2/M (Fig. 9E). These results demonstrate that the resting, non-cycling lymphocytes in these lymphoid tissues have not progressed beyond G0/G1a, providing verification that these cells are fully quiescent. Therefore, *de novo* HIV-1 infection of resting and naïve $CD4^+$ lymphocytes within tissues occurs in a G1b-independent manner.

DISCUSSION

Recent studies have demonstrated that resting and/or naïve $CD4^+$ lymphocytes are productively infected within peripheral blood and lymphoid tissues from HIV-positive individuals (4, 32, 56). An important question is whether these resting, naive cells were infected *de novo*, because numerous experiments using PBMC cultures have shown these cells to be highly resistant to productive infection by HIV-1*in vitro* (8, 11, 37, 44, 46, 53, 54). Human lymphoid histocultures represent an attractive model system to address this question as lymphoid organs are the primary sites for HIV-1 replication *in vivo*. In this study, we identified distinct subsets of activated, non-activated, memory, naïve, proliferating and non-proliferating subsets and demonstrated that each of these subsets is susceptible to infection and depletion by HIV-1. In particular, we provide substantive evidence that resting and naïve $CD4^+$ lymphocytes are infected *de novo* within these tissues. Collectively, our results demonstrate that the heterogeneous and expansive $CD4^+$ host cell pool contained within human lymphoid tissues is highly vulnerable to infection and killing by HIV-1, processes that likely accelerate the collapse of the immune system in HIV-1 disease.

The endogenous heterogeneity found within lymphoid histocultures provided a highly relevant setting in which to study the cellular characteristics that regulate HIV-1 infection. In contrast to studies based on PBMC cultures, we found no absolute restrictions for either HIV-1 replication or target cell depletion within lymphoid tissues based on stratification by state of cellular activation, maturation, or proliferation. First, endogenously activated T-cells from lymphoid histocultures were just as susceptible to viral pathogenic effects as

were non-activated cells. Second, naïve cells within these tissues were not resistant to infection or depletion by HIV-1, despite their apparent resistance *in vitro* (37). Finally, both proliferating and non-proliferating $CD4^+$ T-cells were infected and eliminated by HIV-1 in these cultures. Furthermore, we observed no correlation between proliferative activity and the degree of susceptibility to infection. Strikingly, non-proliferating naïve lymphocytes were infected by HIV-1 at higher frequencies than were actively proliferating naïve blasts, demonstrating that cellular proliferation is not a dominant determinant of productive viral replication within target cells. Recent work also demonstrated that productive HIV-1 infection of PBMC 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 (27). We found here that non-dividing $CD4^+$ lymphocytes from lymphoid histocultures resided nearly exclusively in the G0/G1a phases that precede G1b, thus revealing that these cells were infected by HIV-1 in a G1b-independent manner. Therefore, despite considerable *in vitro* evidence that cellular activation, maturation, and proliferation regulate HIV-1 replication, 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.

Because experiments using lymphoid histocultures are prospective, the question of *de novo* infection can be addressed directly. That non-proliferating, naïve T-cells were infected and depleted by HIV-1 in histocultures implies that these cells were indeed infected *de novo*. Pulse-chase DNA labeling experiments uncovered an exceedingly low rate of conversion of blasts into cells within the resting, naive lymphocyte compartment. Therefore, this small fraction of cells

potentially infected during an earlier, replication-active developmental stage cannot account for the substantial levels of productive infection detected within the naive T-cell pool. Collectively, these experiments provide strong evidence that lymphoid tissues support *de novo* HIV-1 infection of quiescent and naïve $CD4^+$ T-cells.

Although both PBMC and lymphoid histocultures support HIV replication, exhibit similar pathogenic effects by X4 and R5 HIV-1 strains, and contain various lymphocyte subsets, they also have some fundamental differences that may have important implications regarding key aspects of pathogenesis. First, PBMC are inherently resistant to infection without stimulation by soluble factors (8, 11, 37, 44, 46, 53, 54) or coculture with antigen presenting cells (17, 31, 48), while histocultures support HIV-1 infection and replication without exogenous stimulation (20). Second, PBMC cultures contain principally lymphocytes, whereas lymphoid histocultures also contain dendritic cells, macrophages, and stromal cells (20, 21) that may mediate critical cell-cell interactions or release natural cytokines affecting permissivity to viral infection. Finally, histocultures are solid organ explants that may preserve three-dimensional cellular arrangements influencing the response to HIV-1. Interestingly, one study found that $CD4^+$ lymphocytes from tonsils from HIV-infected patients contained 40-fold more replication-competent virus than did cells from peripheral blood (38), indicating that lymphoid tissues are more permissive for HIV-1 infection *in vivo* and likely play a central role in pathogenesis.

What then is the importance of cellular activation, maturation status, and proliferation in the spread or pathologic effects of HIV-1 *in vivo*? None of these

attributes alone are essential for viral replication within lymphoid tissues *ex vivo*. We hypothesize that the microenvironment within lymphoid tissues contains cytokines or other factors that act locally to increase the activity of intracellular factors that promote HIV-1 replication without markedly altering the overall cellular phenotype. It has been shown that particular cytokines permit infection of non-activated and non-proliferating $CD4^+$ cells by HIV-1 or HIV-based vectors (13, 49). Therefore the cytokine milieu found within lymphoid tissues may support the full HIV-1 replication cycle within otherwise resistant populations like resting naïve T-cells, thus allowing productive infection and depletion of these cells. It is also possible that cellular events triggered by virus-associated factors, such as auxillary proteins, might induce a replication-permissive state in this tissue environment.

These findings suggest that no identifiable $CD4^+$ T-cell subset is entirely invulnerable to infection by HIV-1. Active viral replication in nearly every $CD4^+$ T-cell subset within lymphoid organs would be expected to contribute significantly to the gradual attrition of all such cells . In this context, resting, naïve T-cells should not be considered exclusively as reservoirs for latent HIV-1 (5, 8, 12, 13, 16, 44, 54). It is nonetheless likely that individual cells have varying degrees of permissivity to HIV-1 infection depending upon their immediate environment. For example, while naïve T-cells that circulate through peripheral blood or other locations may be resistant to HIV-1 infection, their trafficking into lymphoid tissues may convert them into permissive hosts. 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.

In conclusion, the data presented here demonstrate that virtually every recognized $CD4^+$ T-cell subset is susceptible to HIV-1 infection when present within lymphoid tissue, where greater than 98% of T-cells reside *in vivo* (12) and HIV-1 replication is very active. In particular, resting and naïve $CD4^+$ lymphocytes in these sites may serve not only as a reservoir of latent virus, but also as permissive hosts for viral replication leading to cellular elimination by HIV-1. An intriguing possibility is that a limiting factor of HIV-1 disease progression is the ability to infect this pool of quiescent cells. The evolution of HIV-1 strains (e.g. X4 viruses) with an enhanced ability to target these cells may be an important step in accelerating the overall decline of the $CD4^+$ T-cell repertoire leading to advanced HIV disease.

ACKNOWLEDGEMENTS

The authors thank Bruce Chesebro and Malcolm Martin for kindly providing plasmids and Dee J. Holthe, Cecilia Stewart, Claudette Delphis, Ursula Perotti, Mark Weinstein, Nancy Abbey and the surgical staffs at Kaiser Hospitals (San Rafael, San Francisco) and San Francisco General Hospital for generous assistance in obtaining post-tonsillectomy samples. The authors acknowledge Eric Wieder, Lisa Gibson, Jean-Charles Grivel, Laura Napolitano, and Elizabeth Sinclair for technical advice and the assistance of Heather Gravois and John Carroll in the preparation of this manuscript.

M.L.P. was supported by the Biomedical Sciences Graduate Program, the California Universitywide AIDS Research Program, and the N.I.H. Medical Scientist Training Program at UCSF. D.A.E. was supported by the Biomedical Sciences Graduate Program and the National Institutes of Health Medical Scientist Training Program at UCSF. This work was supported in part by N.I.H. grant R01-AI43695 (M.A.G.), UCSF-California AIDS Research Center (#CC99-SF-001) and the J. David Gladstone Institutes (M.A.G.).

FIGURE LEGENDS

Figure 1. Flow cytometric analysis of lymphocytes and blasts from human lymphoid histocultures, and their depletion by HIV-1. (A) Forward and side scatter properties (note log scale on y-axis) of cells dispersed and pooled from six uninfected tissue blocks of human spleen following culture *ex vivo* for 15 days post-splenectomy. Anti-CD3 mAb immunostaining of dispersed cells demostrated significant reactivity in the lymphocyte (left inset) and blast populations (right inset). Presented are data typical of spleen and tonsil tissue specimens (n>10). (B) Depletion of $CD4^+$ lymphocytes (open bars) and blasts (closed bars) from human tonsil histocultures as indicated by mean CD4/CD8 ratio relative to uninfected control tissue after a 12-day infection by recombinant viruses NL4-3 (X4) and 49-5 (R5). These data are representative of experiments performed with several donor tissues (n=5).

Figure 2. Flow cytometric identification of activated and non-activated $CD4^+$ T-cell subsets within human tonsil histocultures. Lymphocytes (A,B) and blasts (C,D) were identified by light scatter (see Fig. 1) and subsetted by immunostaining for CD25 (A and C) and HLA-DR (B and D) within 5 hrs. post-tonsillectomy. Presented are dot plots from a single donor tissue. Percentages indicated in the upper right quadrants represent the mean values for $CD25^+$ and $HLA\text{-}DR^+$ subsets from nine tissue specimens. Standard error of the mean (SEM) for each value was less than 20% of the mean.

Figure 3. Depletion of activated and non-activated $CD4^+$ T-cell subsets from human tonsil histocultures by HIV-1. Following a 12-day infection of tonsil histocultures with NL4-3 (A,C) and 49-5 (B,D), the absolute cell numbers remaining in the indicated subsets were measured by flow cytometry. Each data point shown is the mean with SEM (n=3) and the total number of $CD4^+$ T-cells are expressed as a percentage of the uninfected control. These results are representative of experiments in two donor tissues.

Figure 4. Flow cytometric identification of memory and naïve $CD4^+$ T-cell subsets from human tonsil histocultures. Cells from uncultured tonsil specimens were dispersed and immunostained with anti-CD45RA and CD62L mAbs (A and C) or anti-CD45RA and CD45RO mAbs (B and D). 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 (n=8-14);SEM for each subset was less than 20% of the mean.

Figure 5. Depletion of naïve and memory $CD4^+$ T-cell subsets by HIV-1. $CD4^+$ naïve and memory subsets were measured by flow cytometry with cells dispersed from tonsil histocultures and co-immunostained with anti-CD4, anti-CD45RA, and anti-CD62L mAbs following a 12-day infection with NL4-3 (A) or 49-5 (B). Shown is a typical experiment (n=6).

Figure 6. Analysis of cellular proliferation and HIV-1 infection of lymphocytes and blasts in lymphoid tissues. (A) Propidium iodide staining (upper) and anti-BrdU reactivity (lower) of $CD4^+$ lymphocytes and blasts from tonsil histocultures.

(B) Kinetic analysis of frequencies of productively infected cells in tonsil histocultures inoculated with NL4-3 (left) or 49-5 (right) and co-immunostained with mAbs recognizing CD3 and p24. Each point represents the mean plus SEM values (n=3) typical of experiments performed using 2-6 donor tissues.

Figure 7. Cellular proliferation and HIV-1 infection of naïve and memory $CD4^+$ T-cell subsets. (A) BrdU incorporation in naïve and memory (identified by anti-CD45RA and CD45RO mAbs) lymphocytes and blasts from tonsil histocultures following a 12-hour labeling with BrdU. (B) Productively infected cell subsets in these tissues following an 8-day infection with NL4-3 (open bars) or 49-5 (closed bars). Presented are representative data from experiments in 3-5 tissue specimens.

Figure 8. Kinetic analysis of BrdU incorporation in tonsil histoculture lymphocytes and blasts following a 12-hour labeling period. (A) Histocultures were labeled with BrdU for 12-hours and washed extensively. Tissues were cultured for an additional 16 or 24 hours, 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, immunostained for BrdU or intracellular p24, CD4, and CD45RA and analyzed by flow cytometry. Productively infected (open bars) or BrdU-positive (solid bars), $CD4^+/CD45RA^+$ lymphocytes and $CD4^+$ blasts are presented in parallel. Presented are mean values with SEM from a representative experiment (n=4).

Figure 9. Cell cycle analysis of $CD4^+$ lymphocytes and blasts in PBMC and 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 analysis of anti-CD3-, anti-CD28-stimulated (A) and unstimulated (B) PBMC, and histoculture blasts (C), lymphocytes (D), and $CD45RA^+$ lymphocytes (E). Presented is a typical experiment (n=3).

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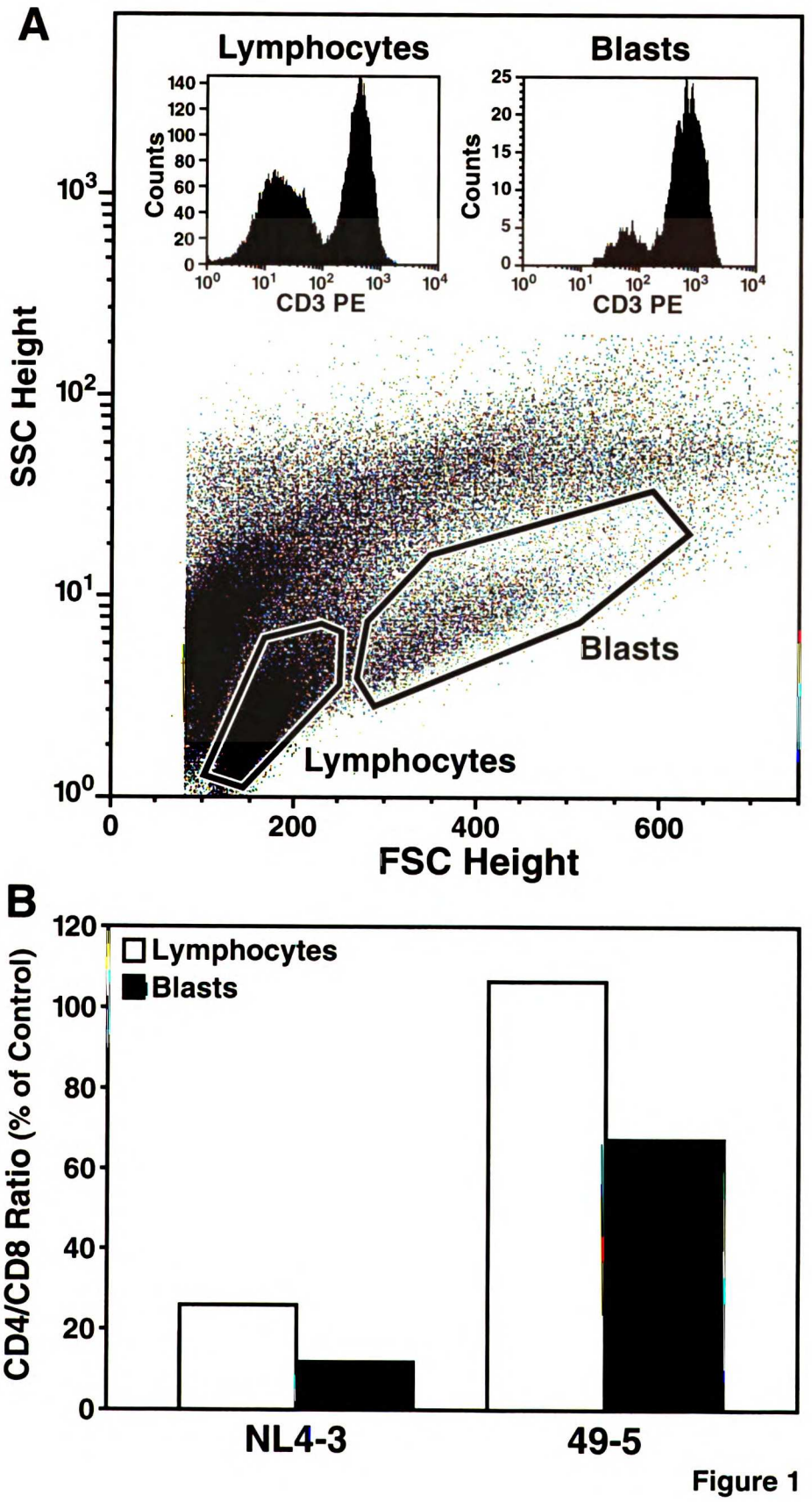
A
Lymphocytes
Blasts
Counts
CD3 PE
SSC Height
FSC Height
Blasts
Lymphocytes
B
Lymphocytes
Blasts
CD4/CD8 Ratio (% of Control)
NL4-3
49-5

Figure 1

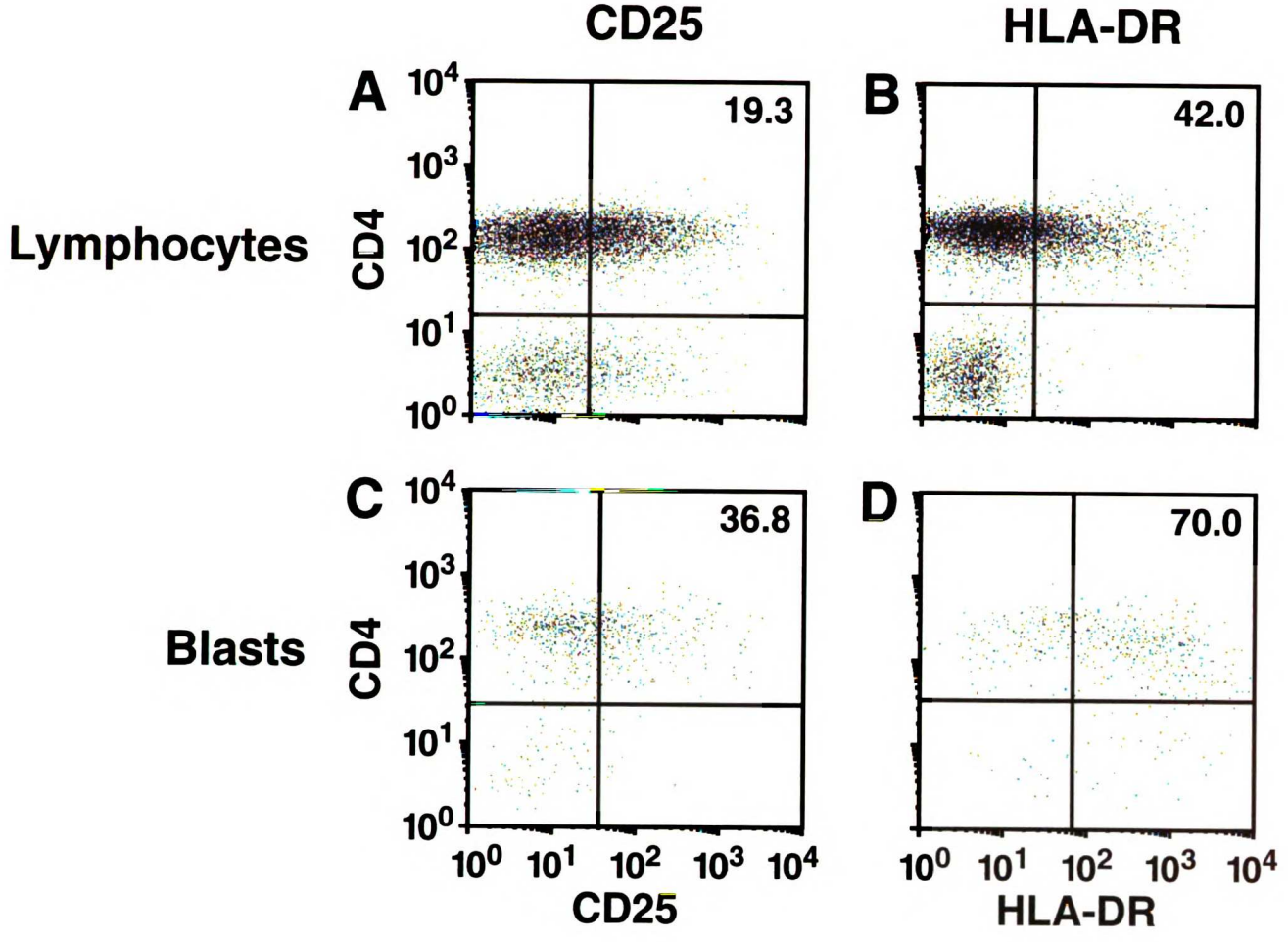
CD25
HLA-DR
A
B
C
D
19.3
42.0
36.8
70.0
Lymphocytes
Blasts
CD4
CD4
CD25
HLA-DR
10^0 10^1 10^2 10^3 10^4

Figure 2

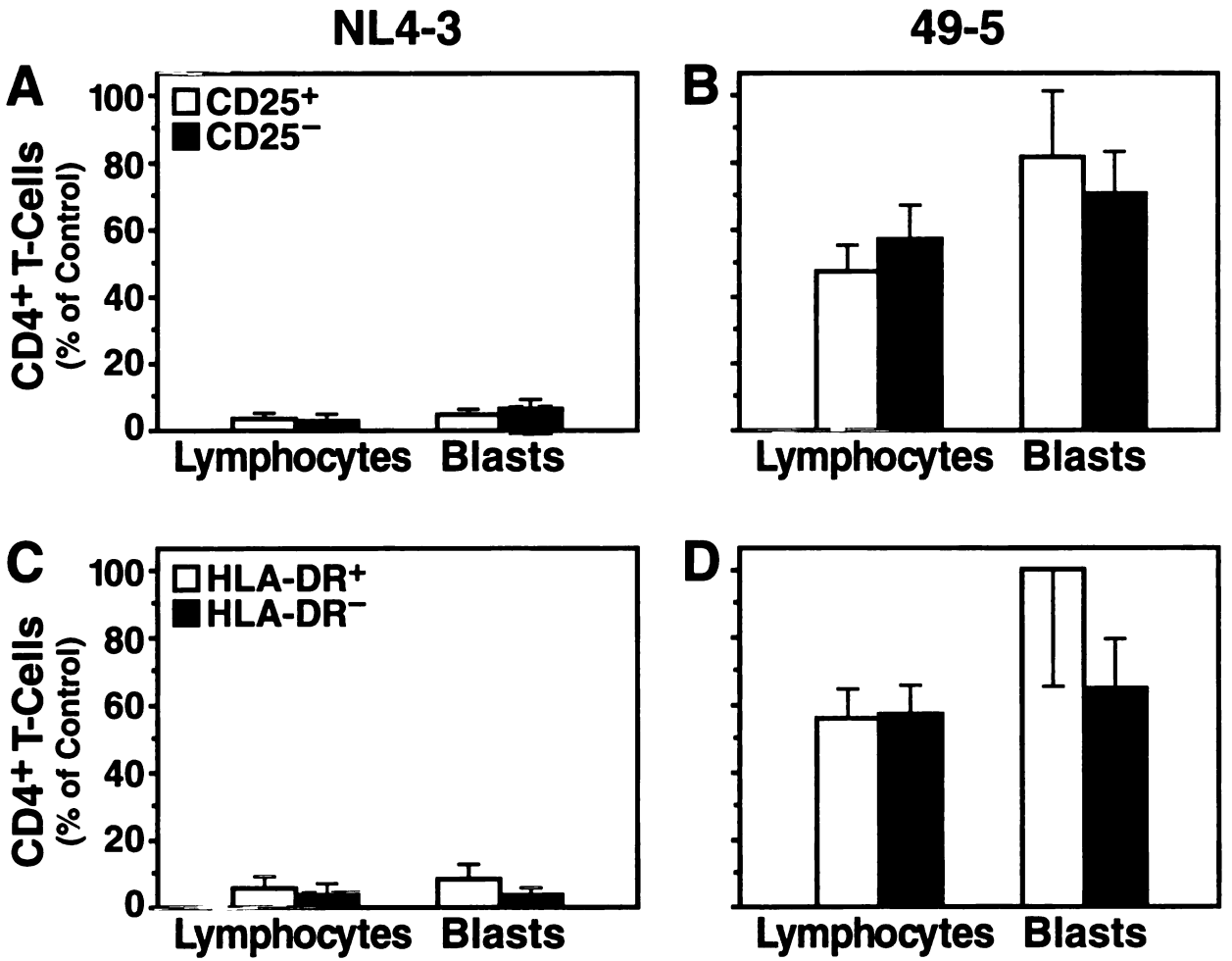
NL4-3
49-5
A
B
C
D
CD4+ T-Cells (% of Control)
CD25+
CD25−
HLA-DR+
HLA-DR−
100
80
60
40
20
0
Lymphocytes
Blasts

Figure 3

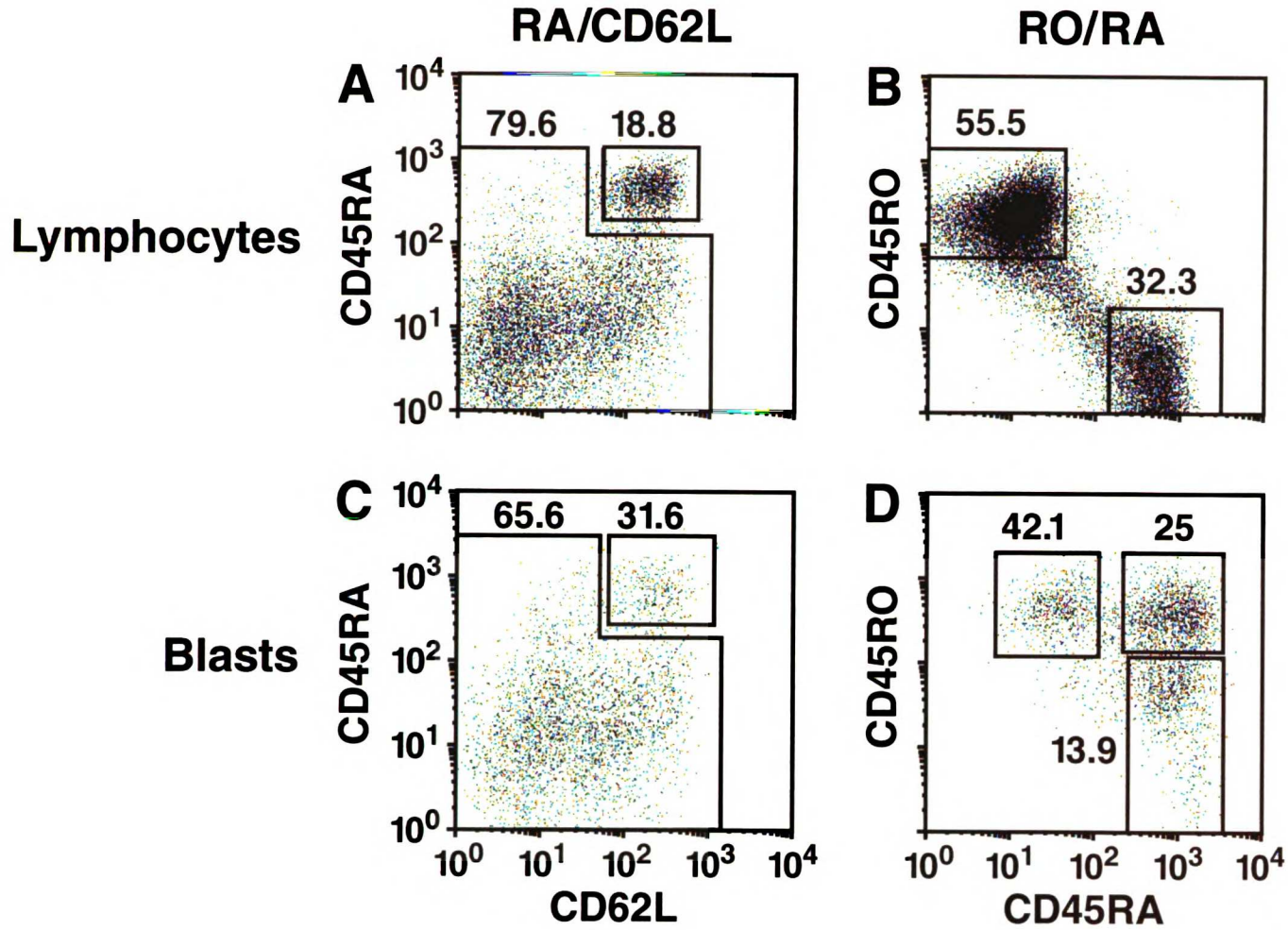
RA/CD62L
RO/RA
A
B
C
D
Lymphocytes
Blasts
79.6
18.8
55.5
32.3
65.6
31.6
42.1
25
13.9
CD45RA
CD45RO
CD62L
10^0
10^1
10^2
10^3
10^4

Figure 4

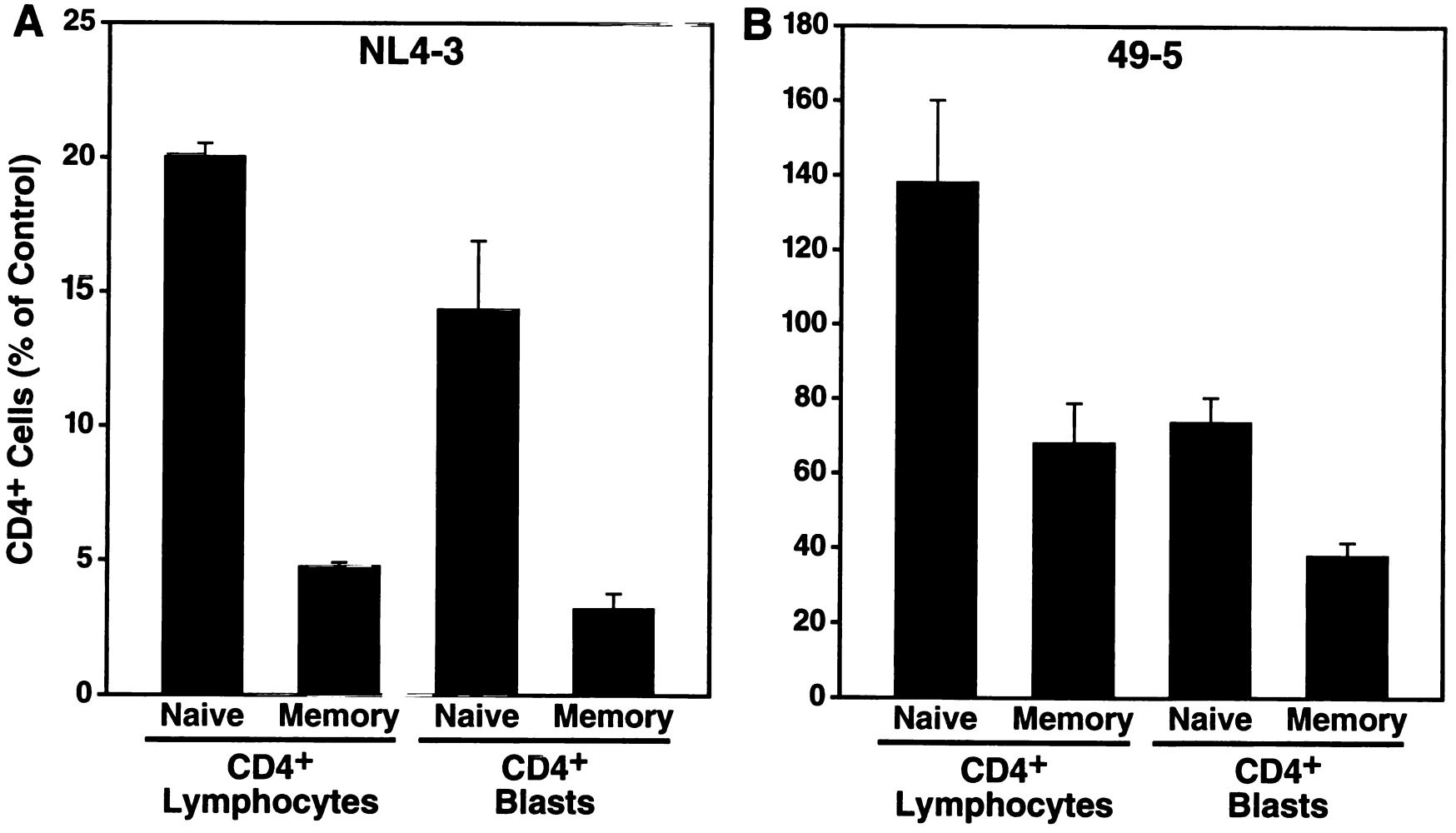
A
NL4-3
CD4+ Cells (% of Control)
25
20
15
10
5
0
Naive
Memory
Naive
Memory
CD4+ Lymphocytes
CD4+ Blasts
B
49-5
180
160
140
120
100
80
60
40
20
0
Naive
Memory
Naive
Memory
CD4+ Lymphocytes
CD4+ Blasts

Figure 5

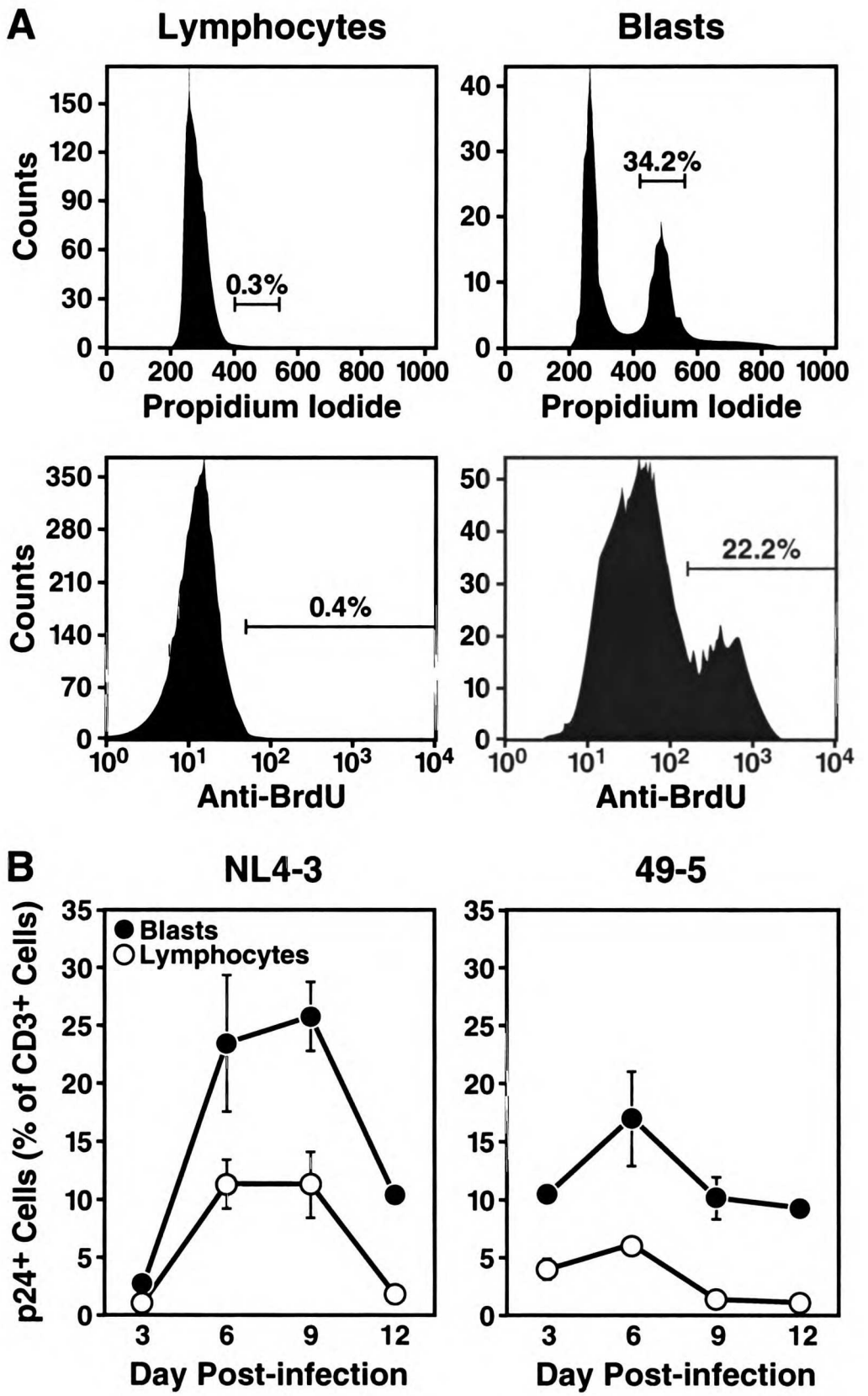
A
Lymphocytes
Blasts
Counts
0.3%
34.2%
Propidium Iodide
0.4%
22.2%
Anti-BrdU
B
NL4-3
49-5
Blasts
Lymphocytes
p24+ Cells (% of CD3+ Cells)
Day Post-infection

Figure 6

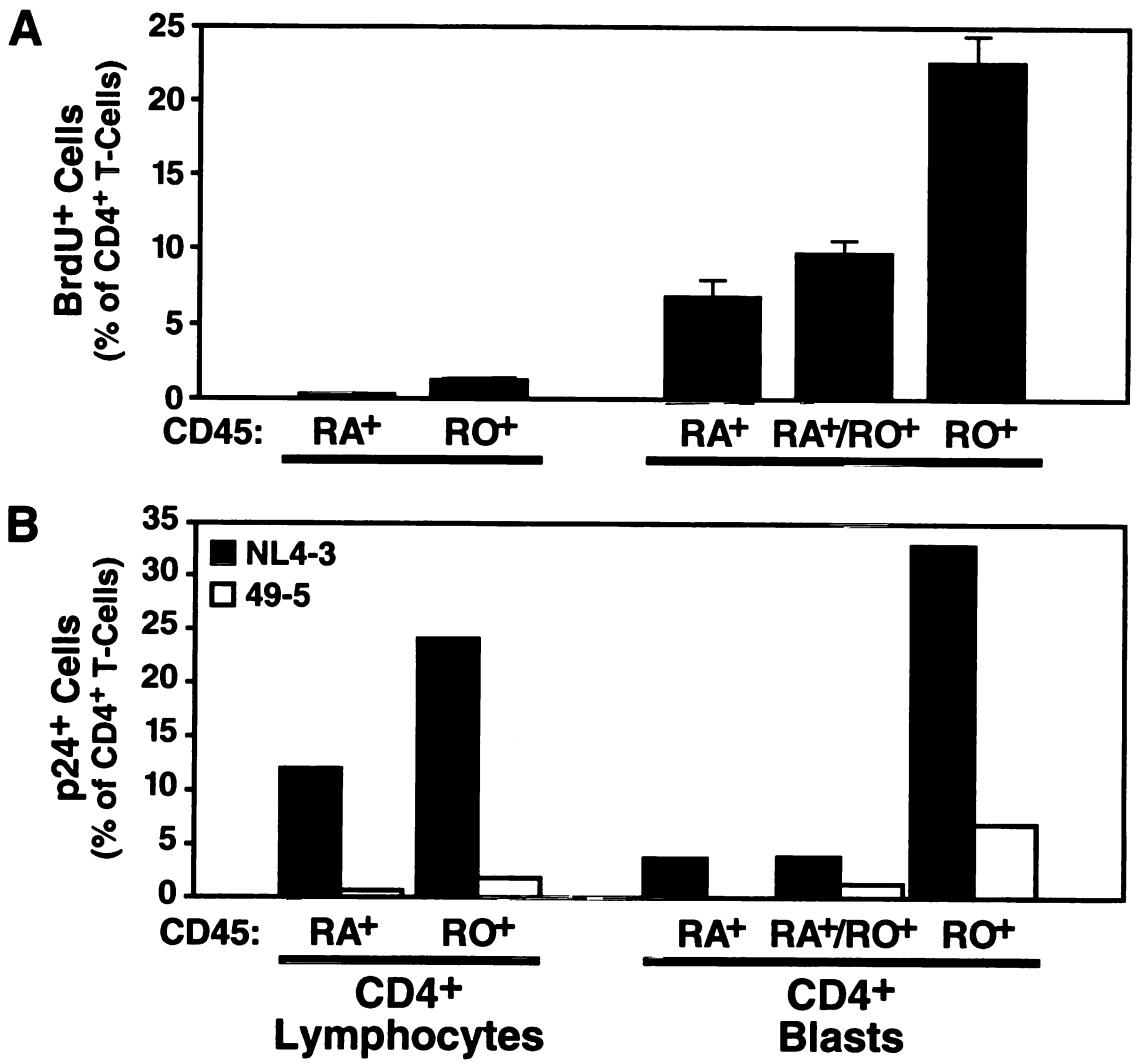
A
BrdU+ Cells
(% of CD4+ T-Cells)
25
20
15
10
5
0
CD45: RA+ RO+ RA+ RA+/RO+ RO+
B
p24+ Cells
(% of CD4+ T-Cells)
NL4-3
49-5
35
30
25
20
15
10
5
0
CD45: RA+ RO+ RA+ RA+/RO+ RO+
CD4+
Lymphocytes
CD4+
Blasts

Figure 7

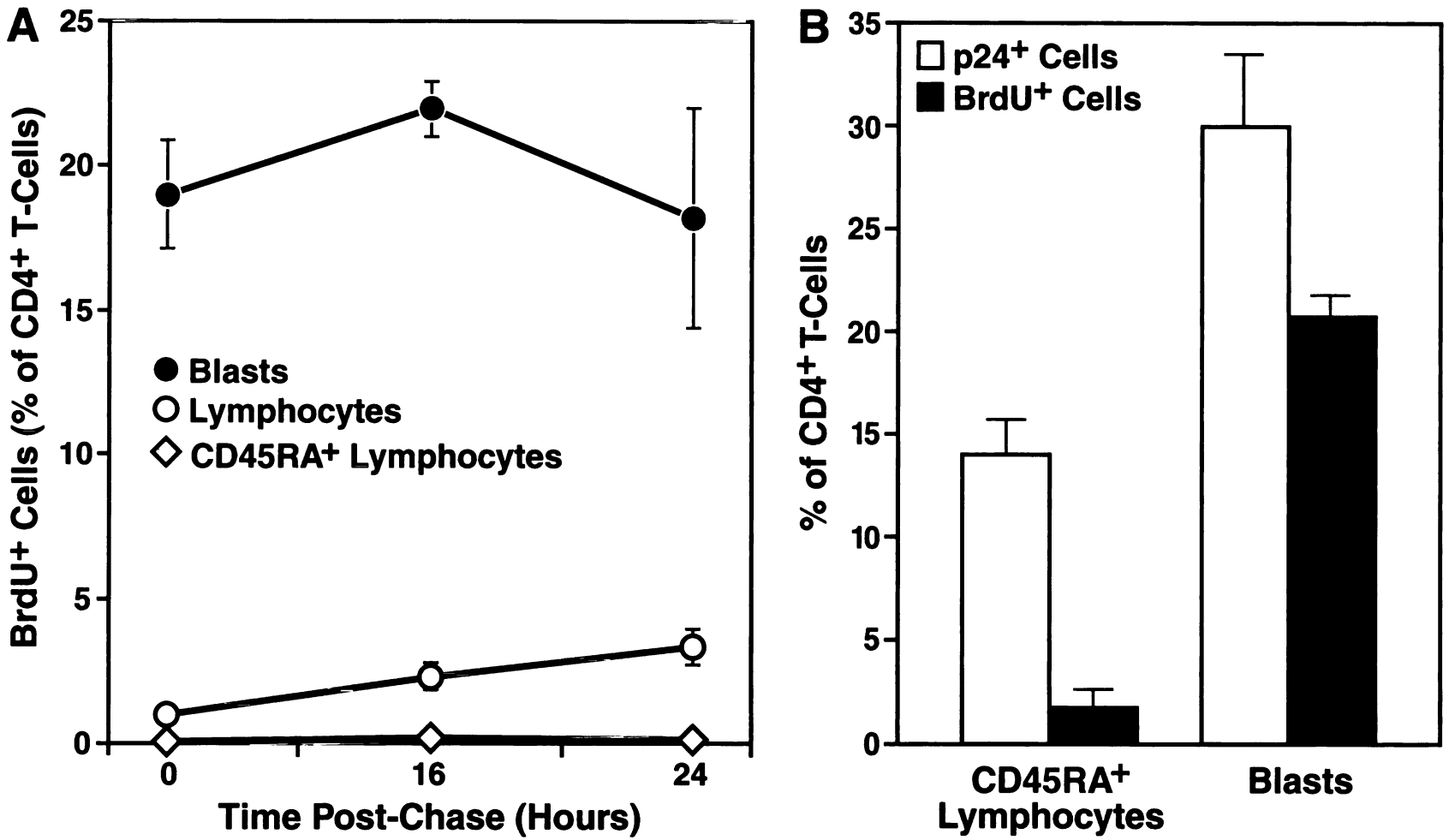
A
BrdU+ Cells (% of CD4+ T-Cells)
25
20
15
10
5
0
Blasts
Lymphocytes
CD45RA+ Lymphocytes
0
16
24
Time Post-Chase (Hours)
B
% of CD4+ T-Cells
35
30
25
20
15
10
5
0
p24+ Cells
BrdU+ Cells
CD45RA+ Lymphocytes
Blasts

Figure 8

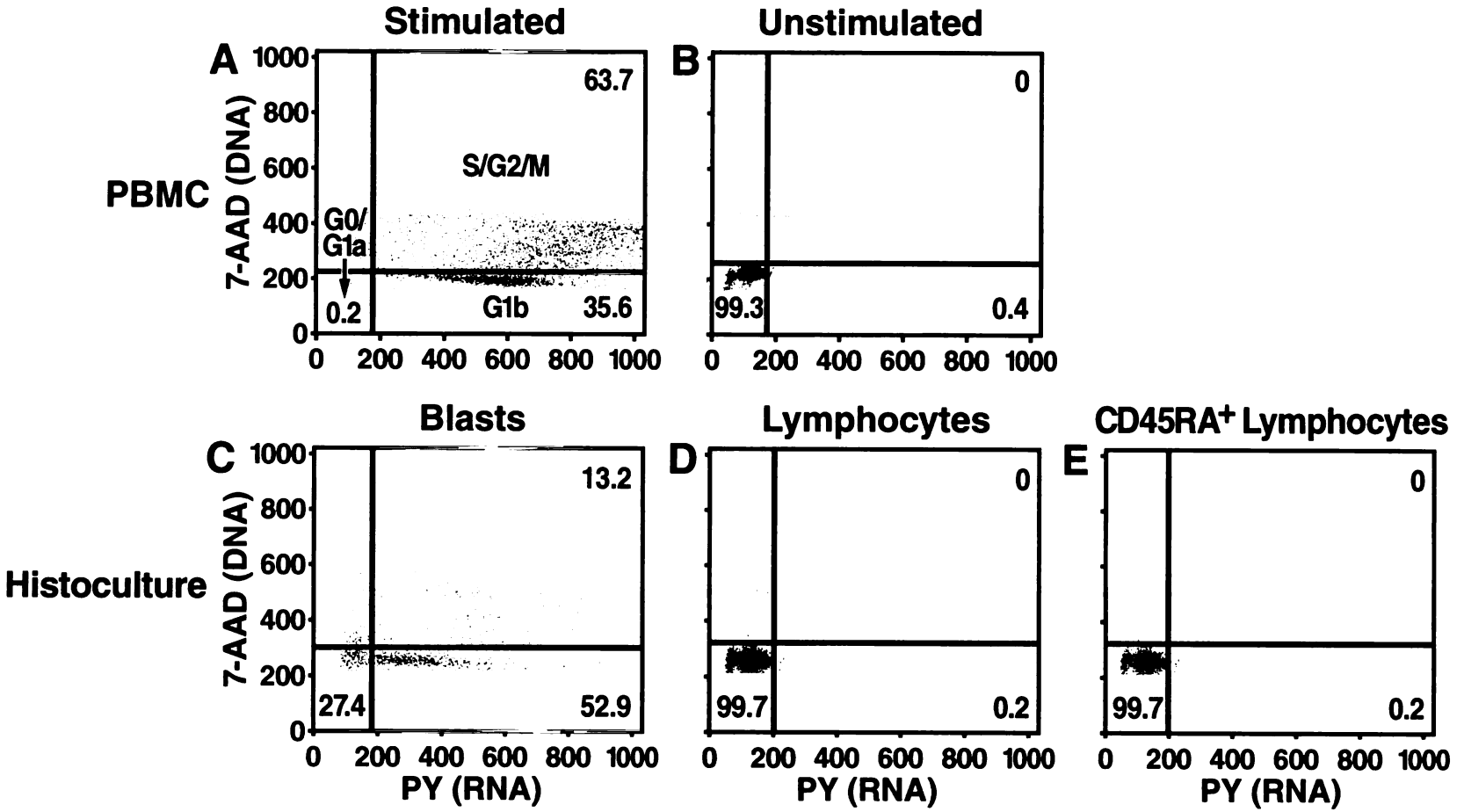
Stimulated
Unstimulated
A
B
PBMC
7-AAD (DNA)
1000
800
600
400
200
0
63.7
S/G2/M
G0/
G1a
0.2
G1b
35.6
0
99.3
0.4
0 200 400 600 800 1000
Blasts
Lymphocytes
CD45RA+ Lymphocytes
C
D
E
Histoculture
13.2
27.4
52.9
99.7
0.2
PY (RNA)

Figure 9

Chapter 8

Conclusions

I. THE CORECEPTOR PARADIGM FOR HIV-1-SPECIFIC DEPLETION OF CD4$^+$ T-CELLS

The primary goal of this study was to determine the impact of coreceptor preference on HIV-1-mediated CD4$^+$ T-lymphocyte depletion. Chapters 1, 2, and 3 firmly establish that selection of either CCR5 or CXCR4 can have dramatic effects on the extent of T-cell depletion. X4 HIV-1 strains deplete CD4$^+$ T-cells aggressively while R5 strains deplete only mildly, thus recapitulating, in part, their clinical properties in human infection.

A critical design feature of these experiments was the utilization of isogenic virus pairs that differ only in select regions of the envelope glycoprotein that control coreceptor preference. Because other HIV-1 gene products can influence viral replication and/or pathogenicity, it was important to exclude these possible contributions to CD4$^+$ T-cell depletion. By isolating coreceptor preference, its direct contribution to the cytopathic potential of HIV-1 could be assessed. By testing various pairs of isogenic viruses we found that all X4 strains depleted CD4$^+$ T-cells severely while R5 strains depleted these cells only modestly. Furthermore, in the setting of dual-tropic envelopes (R5/X4), the aggressive X4 phenotype dominated over the mild R5 depletion phenotype. This cytopathic behavior of X4 and R5 HIV-1 strains was consistent among different recombinant contexts harboring various CCR5- and CXCR4-dependent envelopes, suggesting that T-cell depletion dictated by coreceptor preference is not significantly affected by genomic environment. Moreover, subtle differences in the V3 loop sequence (e.g. single amino acid substitution) that switch coreceptor preference from CCR5 to CXCR4 dramatically augmented the

cytopathic potential of a given strain. Therefore, coreceptor preferences dictate severe or mild $CD4^+$ T-cell depletion for all tested HIV-1 strains in this study.

The most likely mechanism that might account for the differential depletion of $CD4^+$ lymphocytes by X4 and R5 viral strains is that the pools of target cells available to each virus are quantitatively different thus resulting in distinct pathogenic outcomes. Immunostaining of resting lymphoid tissues for CXCR4 and CCR5 demonstrated that CXCR4 was expressed on the vast majority of $CD4^+$ T-lymphocytes whereas CCR5 was expressed on a minority of these cells. Analysis of coreceptor specific depletion of these recombinant strains demonstrated preferential depletion of coreceptor-expressing $CD4^+$ T-cells corresponding to gp120-directed viral coreceptor preference. Therefore, the quantitative differences in depletion between X4 and R5 strains is largely due to differences in the relative abundance of their respective target cells.

Interestingly, not all CCR5-bearing cells appear to be targets for R5 viral infections. Chapter 3 described some experiments where only partial loss of $CCR5^+$ cells was detected, suggesting that expression of CCR5 is not always sufficient for $CD4^+$ T-cell depletion by R5 viruses. Other cellular properties may influence susceptibility to depletion or R5 viruses may themselves modulate cellular proliferation. The latter possibility is supported by our unpublished observations that infection of some donor tissues with R5 HIV-1 strains can induce a significant proliferation of T-cells. This expansion of cells thus diminishes the apparent pathogenic potential of these viruses when depletion is assayed at the end of a typical experiment. Further experiments will be necessary to determine whether these effects are mediated by gp120-signaling and/or viral replication.

Conversely, some $CCR5^-$ cells were depleted by some R5 strains. We speculate, but cannot prove, that the partial depletion of $CCR5^-$ cells by R5 viruses is explained by levels of CCR5 expression on some cells that are below the limit of detection by flow cytometry. Previous work has established that certain $CD4^+$ lymphocytes that do not express CCR5 at levels detectable by flow cytometric methods were nevertheless infectable by R5 viruses (33). However, an alternate possibility is that $CCR5^-$ cells are killed indirectly as a consequence of HIV-1 infection. Although experiments presented in this thesis suggest that lymphocyte killing in lymphoid histocultures represents the cumulative attrition of infected cells, the contribution of indirect killing cannot be formally excluded. As many HIV-1 infected patients progress to AIDS without the emergence of X4 viruses (1, 10, 39, 40), depletion of $CCR5^-$ cells by R5 strains may be a critical step necessary for disease progression in this setting. An important next step in this system should be to determine the contribution of HIV-1-mediated indirect killing to overall $CD4^+$ T-cell depletion.

We also tested whether results obtained with recombinant HIV-1 strains also reflected the phenotypic evolution of HIV-1 *in vivo* by measuring the depletion of primary HIV-1 isolates derived sequentially from an infected individual that progressed to AIDS. This panel of sequential clinical isolates reflected the typical broadening of target cell specificity associated with disease progression. Again, the X4 viruses depleted $CD4^+$ T-cells broadly and severely while the R5 viruses depleted only within the minor CCR5-bearing subset of T-cells. These results strongly suggest that the emergence of viral strains *in vivo* with specificity for CXCR4 likely promotes disease progression and rapid $CD4^+$ T-cell loss.

The interaction between HIV-1 and its coreceptor is an attractive target for antiviral therapy, since it represents an opportunity to disable viral infection at the initial level of cellular entry. Both the remarkable resistance to HIV-1 infection found for individuals homozygous for a CCR5-null allele and the inhibitory effects of the natural receptor ligands or modified chemokines *in vitro* emphasize the potential therapeutic value of targeting virus-coreceptor interactions. One study demonstrated remarkable HIV-1 inhibition with modified forms of RANTES in a xenotransplant model, but chronic administration of these agents selected for variants with CXCR4 specificity (27). Our observations that CXCR4 utilization is a key factor promoting HIV-1 virulence emphasizes the need to consider this receptor as an additional target for antiviral therapy. We found that blocking viral entry through CXCR4 dramatically reduces the pathogenicity of primary and recombinant X4 strains in lymphoid tissues. Not only do these results underscore the causative relationship between specificity for CXCR4 and accelerated $CD4^+$ T-cell decline *in vivo*, but they also emphasize the possible therapeutic value of blocking CXCR4-mediated entry in efforts to limit HIV-1 disease progression *in vivo*. Early clinical trials of both AMD3100 and T22 are underway and may eventually be potent additions to existing HAART regimens. Preliminary studies with T20, another inhibitor of HIV-1 entry, provided potent suppression of viral replication in a small group of infected individuals providing proof-of-concept that viral entry can be effectively blocked in vivo (25).

In addition to CCR5 and CXCR4, more than 10 chemokine receptors and related orphan receptors have been shown to confer HIV-1 entry *in vitro*. In view of the high mutation rate of HIV-1, high viral turnover *in vivo*, and the resulting

potential of HIV-1 to escape coreceptor antagonism by simply changing its coreceptor specificities, it is critical to elucidate the *in vivo* relevance of alternative coreceptors to the natural history of disease. We therefore used a panel of isogenic viruses differing only in their envelope-specified *in vitro* coreceptor utilization patterns to assess the impact of additional coreceptor specificity on the depletion of $CD4^+$ lymphocytes. Although the viruses tested in this study displayed usage of CCR3, CCR8, BOB, or BONZO in addition to the primary HIV-1 coreceptors, no evidence was found that coreceptors other than CXCR4 and CCR5 contribute to HIV-1 pathogenicity in lymphoid tissue cultured *ex vivo*. Therefore, CCR5 and CXCR4 appear to be the dominant coreceptors influencing killing of T-cells in these tissues.

Despite the ability of X4 and R5 HIV-1 viruses to infect and deplete quantitatively different $CD4^+$ target T-cell populations, these viruses paradoxically replicated with similar kinetics in lymphoid histocultures. A likely explanation for these findings is that the overall viral output in these cultures reflects the accumulation of virus produced from both T-cells and other cell types. In support of this hypothesis, we found that macrophages represented an additional cellular target for HIV-1 infection in these tissues. X4 viruses demonstrated the strongest preference for T-cells and to a lesser extent macrophages while R5 viruses demonstrated a strong preference for macrophages and to a lesser extent T-cells. Thus, productive infection of both lymphocytes and macrophages may contribute to the total output of virus in HIV-1-infected histocultures. These data provide compelling evidence 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 in not only preventing $CD4^+$ T-cell depletion but also in limiting viral replication in alternative non-T-cell reservoirs.

II. HIV-2 AND SIV DEPLETION POTENTIAL IN HUMAN LYMPHOID HISTOCULTURES

Since both HIV-2 and SIV use chemokine receptors in concert with CD4 in cellular entry, we determined whether the paradigm of differential $CD4^+$ T-cell depletion mediated by gp120-directed coreceptor preferences established by Chapters 2-4 was also applicable to histocultures inoculated with these viruses. Despite the association of HIV-2 with decreased virulence and the difference in host cell specificity of SIV, these viruses depleted $CD4^+$ lymphocytes in a manner that was completely predictable by their coreceptor preferences. Furthermore, the ability of these viruses to use coreceptors in addition to CCR5 and CXCR4 was also not associated with increased pathogenicity in lymphoid histocultures. Thus, the cytopathic properties of HIV-2 and SIV are very similar to those of HIV-1, both quantitatively and qualitatively. These experiments further establish the dominance of viral coreceptor preference in determining the fate of $CD4^+$ T-cells in mature lymphoid tissues.

Moreover, these findings suggest that a lower intrinsic cytopathic potential does not underlie the remarkably slower disease progression that is described in many HIV-2-infected individuals, since diverse HIV-2 strains exhibited robust depletion phenotypes in lymphoid tissues *ex vivo* that were indistinguishable from that of HIV-1. Despite these observations, the viral load in peripheral blood of HIV-2 infected individuals typically is much lower than

loads observed in HIV-1-infected individuals (3, 32). The viral load *in vivo* is determined by many factors including viral fitness, immune clearance, and additional poorly defined viral and/or host features. Clearly, a different dynamic equilibrium between host and viral factors is established during infection with HIV-2 compared with HIV-1 (3, 32). Therefore, the present results imply that this equilibrium, rather than the cytopathic character of HIV-2 *per se*, is responsible for the attenuated effects of HIV-2 *in vivo*. We speculate that key host immune responses that are not apparent in short-term lymphoid tissues cultured *ex vivo* operate *in vivo* to control HIV-2 infection more efficiently, as has been proposed previously. This conclusion should prompt further investigation to elucidate the basis of this distinct virus-host relationship as a possible foundation for strategies to modulate these processes in patients infected with HIV-1 and/or HIV-2.

III. PROTEASE INHIBITOR-RESISTANT VIRUSES ARE PATHOGENIC IN HUMAN LYMPHOID HISTOCULTURES

The error-prone nature of HIV-1 replication creates new quasispecies with each round of replication. The selection pressures present during viral replication determines which variants in this pool will survive. Inhibitors of the viral protease gene, or PI, are the backbone of HAART for many HIV-1-infected patients. While HAART treatment regimens can provide lasting suppression of viral replication, these regimens also provide a potent selection pressure for the emergence of resistant quasispecies. Unfortunately, viral resistance to these drugs is now a frequent occurrence (15, 16, 18, 31, 34). Although *in vitro* and *in vivo* data suggests that PI resistance might impair viral replication and/or

pathogenicity *in vivo*, both recombinant and primary HIV-1 isolates with PI resistance-conferring mutations were fully capable of infecting and depleting $CD4^+$ T-cells in mature lymphoid tissues. Therefore, unknown host and/or viral factors appear to be responsible for preserving or increasing the CD4 counts in this subset of patients. Further experiments will be necessary to establish the pertinent mechanism(s).

We cannot exclude, however, that there may be differences in viral replication capacity and pathogenic potential in other lymphoid tissues such as thymus or bone marrow that have predominantly immature populations of lymphocytes. For example, some of the viruses tested in this study exhibited severely compromised replication and depletion in human thymus (C. Stoddart, personal communication). If PI-resistant HIV-1 strains replicate poorly in specific lymphoid compartments (central or peripheral) that contribute to the growing T-cell pool, exclusion of virus replication from these sites may explain why patients with PI-resistant viruses can continue to accumulate $CD4^+$ T-cells despite ongoing peripheral T-cell depletion. The finding that HIV-1 strains with PIR mutations remain cytopathic for mature $CD4^+$ T-cells predicts that the discordance between virologic failure and preservation of $CD4^+$ T-cells may be transient and eventually replaced by progressive loss of T-cells is some individuals.

IV. HOST DETERMINANTS FOR HIV-1 INFECTION AND DEPLETION: ROLE OF CELLULAR PHENOTYPE

Recent studies have demonstrated that resting and/or naïve $CD4^+$ lymphocytes are productively infected within peripheral blood and lymphoid tissues from HIV-positive individuals (5, 30, 46). Because numerous experiments using PBMC cultures have shown resting, naive cells to be highly resistant to productive infection by HIV-1 *in vitro* (7, 11, 35, 37, 38, 44, 45), we asked whether such cells could be infected *de novo* within the cellular milieu of lymphoid tissue. In this study, we identified distinct subsets of activated, non-activated, memory, naïve, proliferating and non-proliferating subsets and demonstrated that each of these subsets is infected *de novo* within these tissues. Although cellular proliferation was thought to be a cellular characteristic that promoted viral replication, we observed no correlation between proliferative activity and the degree of susceptibility to infection. Moreover, we found that non-dividing $CD4^+$ lymphocytes from lymphoid histocultures resided nearly exclusively in the G0/G1a phases of the cell cycle, thus confirming their quiescent status.

Importantly, this study highlighted some important differences between PBMC and lymphoid histocultures. First, PBMC are inherently resistant to infection without stimulation by soluble factors (7, 11, 35, 37, 38, 44, 45) or coculture with antigen presenting cells (20, 26, 41), while histocultures support HIV-1 infection and replication without exogenous stimulation (21). Second, PBMC cultures contain principally lymphocytes, whereas lymphoid histocultures also contain dendritic cells, macrophages, and stromal cells (21, 23) that may mediate critical cell-cell interactions or release natural cytokines affecting permissivity to viral infection. Finally, histocultures are solid organ explants that may preserve three-dimensional cellular arrangements influencing the response to HIV-1. All of these differences point to histocultures as being

inherently more permissive for viral replication than are PBMC cultures. This possibility is supported by the recent observation that $CD4^+$ lymphocytes from tonsils from HIV-infected patients contained 40-fold more replication-competent virus than did cells from peripheral blood (36).

Although it is unknown how lymphoid histocultures support viral replication in quiescent cell populations, we hypothesize that the microenvironment within these tissues contains cytokines or other factors that act locally to facilitate HIV-1 replication without significant changes in detectable cellular phenotypes. Studies using HIV-1 or HIV-based vectors have shown that particular cytokines permit infection of non-activated and non-proliferating $CD4^+$ cells without substantially changing the cellular phenotype (13, 42). Therefore the cytokine milieu found within lymphoid tissues provides a permissive environment for the completion of the full HIV-1 replication cycle within populations predicted to be resistant to viral infection like resting naïve T-cells. It is also possible that cellular events triggered by virus-associated factors, such as auxillary proteins, might contribute to the permissiveness of the lymphoid tissue environment. Studies aimed at better understanding the parameters that dictate viral replication within lymphoid tissues will be necessary to determine the mechanism by which resting cells are rendered permissive for productive HIV-1 infection.

Thus, the data presented in Chapter 7 demonstrate that every $CD4^+$ T-cell subset identified in this study is susceptible to HIV-1 infection when present within lymphoid tissue, where greater than 98% of T-cells reside *in vivo* (12) and active HIV-1 replication is very active. In particular, resting and naïve $CD4^+$ lymphocytes in these sites may not only store latent virus, but also actively

produce virions that contribute to the overall viral burden *in vivo*. An intriguing speculation is that is the ability to infect this pool of quiescent cells may be a factor that regulates the rate of HIV-1 disease progression. Thus, the evolution of HIV-1 strains (e.g. X4 viruses) with an enhanced ability to target these cells may be an important step in accelerating the overall decline of the $CD4^+$ T-cell repertoire leading to AIDS.

V. LIMITATIONS OF THE LYMPHOID HISTOCULTURE MODEL

Although the experiments outlined in this study provide compelling evidence that HIV-1 coreceptor preference determines cellular tropism and the extent of $CD4^+$ T-cell depletion in lymphoid tissues, there a number of limitations to the histoculture system that are worthy of mention. First, experiments were performed only in human tonsil and spleen specimens, tissues that are highly permissive for HIV-1 replication. Based on our experience, cells that are resistant to HIV-1-infection in other contexts appear to be fully permissive in mature lymphoid histocultures; subtle differences in viral fitness that may be evident in other culture systems or immature cell types may not be detected in this system. For example, the PI resistant viruses described in Chapter 6 replicate well in tonsil and spleen histocultures but not in explants of human thymus (C. Stoddart, personal communication). Inherent differences in tissue activation or maturation status, coreceptor expression, and/or other undefined factors may account for such differences in viral pathogenicity between different lymphoid tissues. Therefore, to thoroughly characterize HIV-1 pathogenesis and form a more complete understanding of the HIV-1 disease process *in vivo*, experiments

should be performed in multiple lymphocyte culture systems and the results integrated and interpreted according to the different roles and functions of these different lymphoid compartments.

Second, there may be aspects of HIV-1 disease that are not recapitulated *ex vivo*. Lymphoid tissues cultured *ex vivo* do not have access to circulating cytokines and/or growth factors from blood plasma or other more distant sources all of which may impact HIV-1 replication. Histocultures may be able to maintain their endogenous milieu, but this environment is disconnected from the dynamic *in vivo* environment following surgical excision. Furthermore, lymphocyte recirculation does not take place in this system. Therefore, studies designed to measure HIV-1-mediated immune responses or the systemic aspects of viral spread would be severely limited in this context. In addition, the histoculture system may not recapitulate fully the process of HIV-1 evolution *in vivo*, because the intact immune system is an important host factor that imposes selection pressures on viral quasispecies,.

Finally, lymphoid histocultures are cultured for a period of weeks while HIV-1 disease is chronic and manifests over many months to years. Thus, these tissues likely recapitulate only the acute phase of HIV-1 infection. HIV-1 disease *in vivo* is not simply a manifestation of the depletion of $CD4^+$ T-cells but a systemic immune dysregulation mediated at many different levels. Collectively, these limitations point to the persisting need for the development of better, immunologically competent models in which to study both acute and chronic HIV-1 disease (e.g., animal models). Nevertheless, human lymphoid histocultures have been, and will continue to be, useful for studying specific aspects of HIV-1 biology.

VI. FUTURE DIRECTIONS

The studies presented in this thesis have provided important foundations for future experiments by advancing knowledge and raising new questions. The following section will discuss new, provocative experiments and important next steps that were inspired by this study.

Perhaps the most important question not addressed by this study is why do X4 viruses evolve and become dominant *in vivo* and, what factors limits their emergence to later stages of infection. X4 viruses clearly have a significant capacity to cause significant damage to the host immune system due to their expanded target cell range. As previously suggested, the immune system plays a significant role in this process. One hypothesis uses mathematical modeling to propose that the emergence of X4 viruses represents the waning ability of the immune system to control their replication (8). It posits that because these viruses target a broad spectrum of cells, they are likely to attract the most attention from the immune system. Initially, the immune system is able to control their replication, however due to indolent immune destruction, eventually the immune system becomes compromised and X4 viruses no longer are effectively controlled and become dominant.

An alternate, though not mutually exclusive, hypothesis is that HIV-1 has evolved strategically to infect macrophages and other long-lived cells to establish infection in the host. Because T-cells are rapidly killed by the virus and do not sustain viral replication they were not a component of this strategy. In this scenario, R5 viruses are preferentially transmitted because they possess a selective advantage over X4 viruses by efficiently targeting both T-cells and

macrophages for infection. Under this hypothesis, macrophage infection is the essential step necessary for sustaining this initial infection which in and of itself causes relatively little harm to the host. However, by chance R5 viruses can also infect the small $CCR5^+$ subset of $CD4^+$ T-cells in a gradual process of attrition. Once the infection is established by R5 strains, X4 variants may evolve from infected $CCR5^+$ macrophages or $CD4^+$ T-cells and kill a larger proportion of T-cells leading to progressive disease. Therefore, infection and depletion of $CD4^+$ T-cells by these two mechanisms may be unfortunate "byproducts" of coreceptor expression on these cells.

In any event, understanding the mechanism by which X4 viruses are selected may help in developing therapeutic interventions that may prevent their emergence. Experiments that are designed to address the relative fitness of X4 and R5 viruses *in vivo* would be the gold standard in addressing this fundamental question. Technical challenges currently do not permit this level of analysis, but experiments in the histoculture model may be an important intermediate step. For example, fitness could be inferred by co-inoculating lymphoid histocultures with well-defined recombinant HIV-1 strains and quantifying specifically the resulting replication of each strain. Additionally, understanding the mechanisms by which the immune system controls viral replication would also contribute significantly to understanding what forces govern the selection for X4 viruses *in vivo*. However, studies of immune function are limited in the histoculture system, which further underscores the need for a tractable animal model in which to assess immune system-virus interactions.

Although X4 viruses appear to be a causative event in the progression to AIDS, a substantial fraction of HIV-1-infected patients harboring only R5 viruses

also progress to AIDS (1, 10, 39, 40), raising the possibility that viruses with an increased cytopathic potential may be selected for during the course of disease progression. Indeed, a recent study demonstrated that such variants can be selected and have increased cytopathicity in a xenotransplant model of immature lymphocytes (27). We are currently testing these viruses in mature lymphoid tissues. Although essentially all R5 HIV-1 strains tested in the histoculture model to date have identical mild depletion phenotypes, the possibility that R5 viruses that possess increased pathogenicity may be selected *in vivo* will continue to be investigated.

Although loss of $CD4^+$ T-cells by HIV-1 is a well characterized clinical event in HIV-1 infected patients, the mechanism(s) by which these cells are depleted remains to be established. Infected cells may be killed by immune clearance or by direct and/or indirect viral cytopathic effects. While the mechanisms of immune system clearance of HIV-1-infected cells are well understood, the specific process by which HIV-1 kills T-cells is poorly understood. Some studies propose a role for cellular apoptosis, possibly induced by gp120 signaling through the chemokine receptors (2, 4, 6, 14, 17, 24, 43), while others propose that cellular necrosis or apoptosis due to viral replication and/or budding accounts for T-cell death (19, 22). An important area of future investigation will be to validate the occurrence of these proposed phenomena and determine the relative contributions of each of these processes to the overall depletion of $CD4^+$ T-cells.

In addition to the killing of virally-infected cells, uninfected cells are also mysteriously killed in the setting of HIV-1 disease, a phenomenon known as "bystander killing." An increase in cellular susceptibility to apoptosis through

upregulated signaling through the Fas receptor has been proposed as a potential cause for bystander killing (9). Additionally, HIV-1 gp120-mediated signaling also may contribute to bystander killing through circulation of replication-defective virions or soluble gp120 released by virion shedding (2, 4, 6, 14). Bystander killing appears to be an important process *in vivo* as resting lymphocytes from HIV-1-infected patients have substantiallly elevated rates of spontaneous apoptosis. Moreover, significant levels of apoptosis in uninfected cells have been observed in lymphoid tissues from HIV-1 infected individuals implying that bystander killing is not limited to peripheral blood (19, 28, 36). Unfortunately, these studies could not determine whether such killing was due to widespread immune activation, characteristic of HIV-1 infection or to circulating soluble gp120 or replication-defective virions. It has also been hypothesized that bystander killing may coincide with focal areas of HIV-1 replication within lymphoid tissues. With recent technical advances in reagents that detect apoptosis and fluorescent cytometry on tissue sections, prospective experiments using lymphoid tissue *ex vivo* can now be designed that can detect both infected and uninfected, apoptotic and non-apoptotic cells (22), and the geographical relationships between them. These experiments should be helpful in not only elucidating the relative proportions of direct and indirect T-cell killing, but also how these two processes might be related.

While the majority of experiments presented in this study focused on the ability of HIV-1 to infect and deplete T-cells, another important area for future study is determining how other cell types present within lymphoid tissue contribute to HIV-1 replication in this setting. Chapter 4 began to address this issue by demonstrating that HIV-1 can also target macrophages in lymphoid

histocultures. However, an important question raised, but not answered by this study, was whether cell types other than T-cells could contribute significantly to total viral output. Though not conclusive, this study suggested that a fraction of the total viral output from infected histocultures originated in macrophages and possibly other related cell types (e.g. dendritic cells). Histocultures inoculated with strains of HIV-1 deficient in the *vpr* gene, and having a decreased ability to infect macrophage-like cells, produced significantly lower amounts of virus compared to histocultures infected with wild-type viruses (data not shown). These experiments suggested that macrophage-like cells in lymphoid histocultures can produce substantial amounts of virus. Additional experiments are being planned that will determine the specific viral burst size for individual HIV-1 target cell populations purified from infected lymphoid tissues. This information will allow a more comprehensive understanding of HIV-1 replication dynamics *in vivo*.

In addition to macrophages, dendritic cells are found in significant quantities within lymphoid tissues cultured *ex vivo*. Dendritic cells have become increasingly recognized for their ability to promote T-cell activation and to potentiate HIV-1 replication in T-cells (reviewed in (29, 47). Experiments that sorted dendritic cells by flow cytometry followed by electron microscopy suggested that these cells were biologically active in these tissues as multicellular complexes were observed with T-cells (data not shown). These dendritic-T-cell contacts are critically important for T-cell activation and the enhancement of HIV-1 replication. It is not known to what extent these cellular interactions contribute to HIV-1 infection and depletion in the histoculture model.

Experiments that disrupt the dendritic cell-T-cell interaction will be helpful in dissecting out the biological role(s) of dendritic cells in infected histocultures.

Finally, Chapter 7 established that resting, naïve T-cells are important targets for HIV-1 infection in lymphoid tissue. Because naïve T-cells are precursors that give rise to the T-cell repertoire, we speculate that the ability of viral populations to infect this cell population is a critical factor in determining the rate of HIV-1 disease progression. In this context, it will be important to evaluate primary HIV-1 strains for their ability to infect this subset. Additionally, the cellular environment and/or factors that support HIV-1 infection of resting cells are completely unknown. Understanding these parameters in detail may help in the design of additional therapeutics that inhibit HIV-1 replication.

In conclusion, this dissertation has addressed important questions regarding HIV-1 pathogenesis. We found that CXCR4 and CCR5 are the primary coreceptors dictating HIV-1-specific depletion of $CD4^+$ T-cells and we also demonstrated that resting naïve T-cells are targeted for infection and depletion by HIV-1. Hopefully, this dissertation will provide a foundation for new studies that will significantly advance both clinical and basic research regarding HIV-1 pathogenesis.

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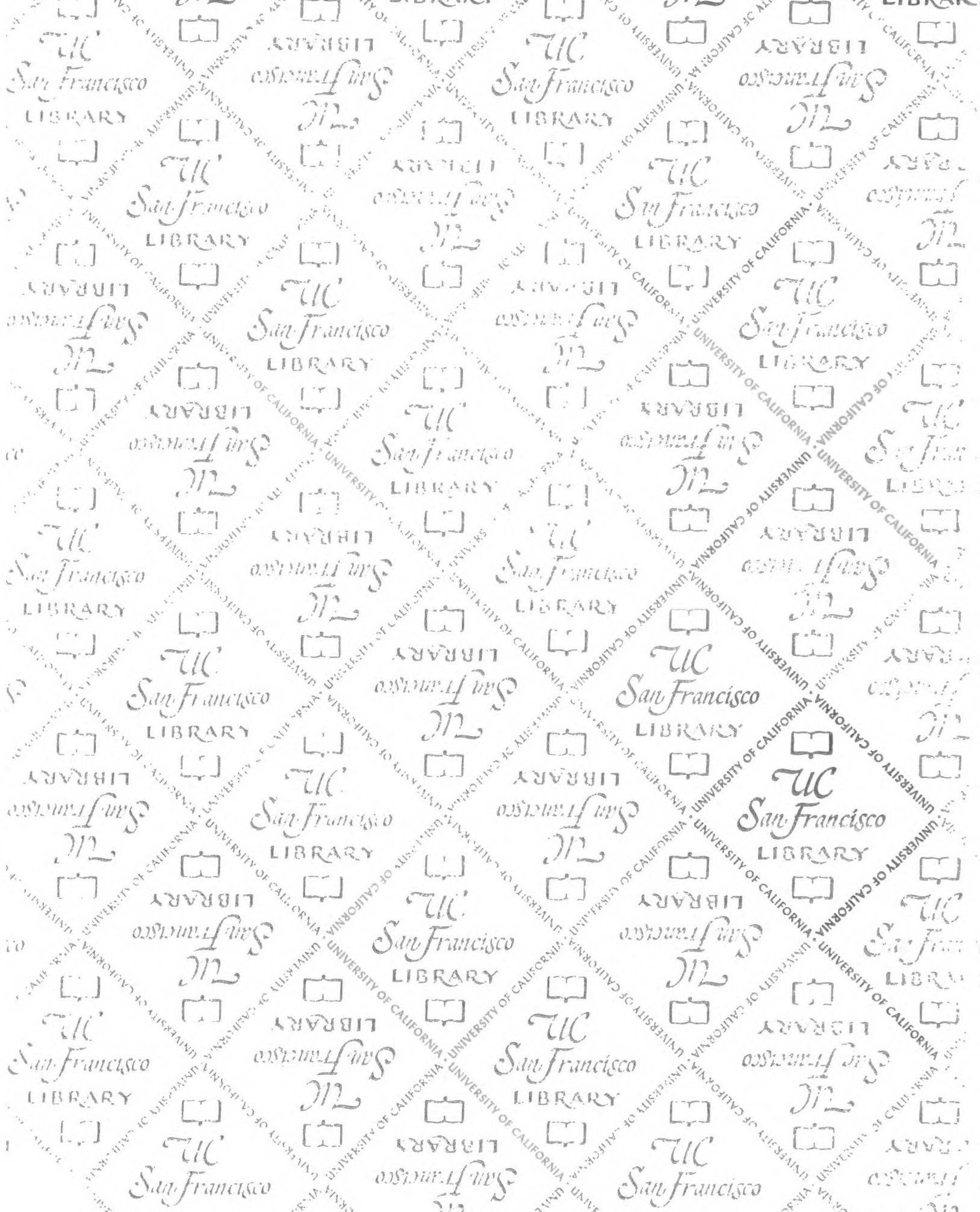
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