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Impact of co-receptor tropism on human immunodeficiency virus type 1 (HIV-1) viral output of primary cells

Author
Cruz, Ann-Marie Michelle

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Impact of Co-Receptor Tropism on Human Immunodeficiency Virus Type 1 (HIV-1) Viral Output of Primary Cells

by

Ann-Marie Michelle Cruz

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

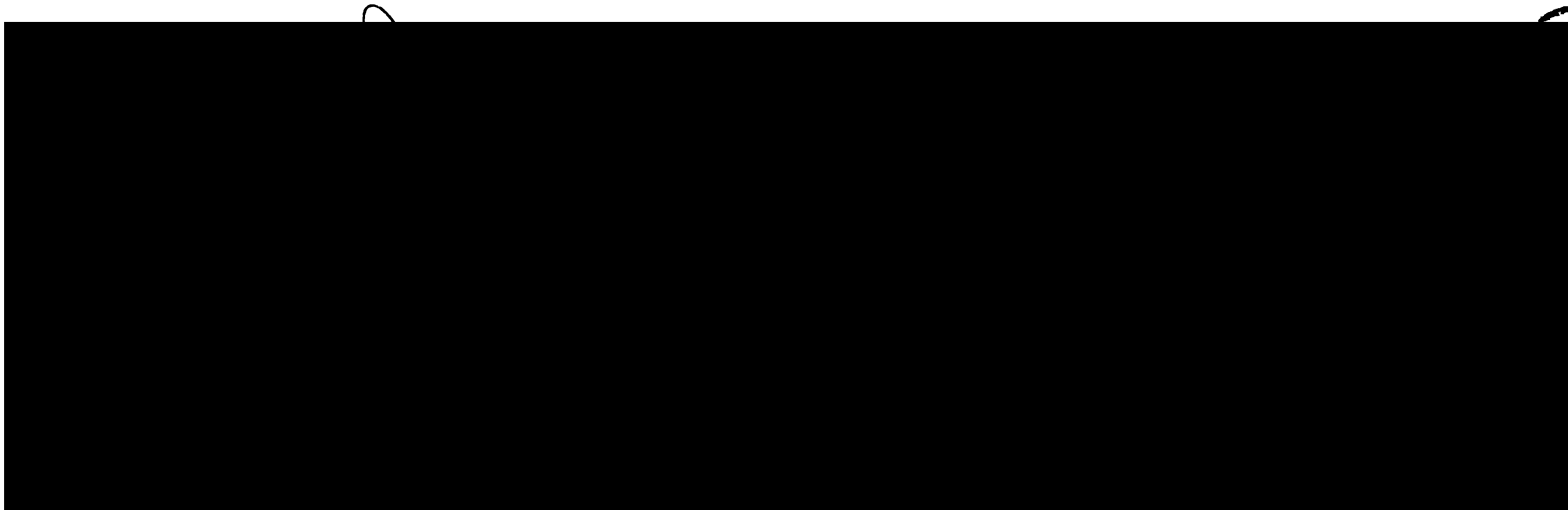
Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO



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Acknowledgements

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Part of the text of this dissertation (Chapters 2 and 3) is a reprint of the material as it appears in Journal of Virology and Journal of AIDS,

respectively. The senior author listed in the publication directed and supervised the research that forms the basis for the thesis.

Note by Joseph McCune: "The published work referenced in this thesis was conceived, planned, carried out, interpreted, and written up by the dissertation candidate. It is of a quality and depth comparable to that of a standard thesis or dissertation."

Impact of Co-Receptor Tropism on Human Immunodeficiency Virus Type 1 (HIV-1) Viral Output of Primary Cells

Ann-Marie Cruz

Abstract

HIV-1 most often utilizes CD4 and one of two co-receptors, CCR5 and CXCR4, in order to enter host cells. Interestingly, many patients in early and in late disease maintain high circulating levels of CCR5 tropic (R5) viruses, even though the target population of CCR5+ cells appears to be small. Such viral loads may be sustained because, relative to CXCR4 tropic (X4) infection, R5 infection of permissive CD4+ T-cells results in the production of significantly more infectious viral particles per cell. To investigate this possibility, the average amount of virus produced per infected cell by R5 and X4 HIV-1 in isolated CD4+ T-cell populations (PBMC) was determined, as well as the average amount of virus released per infected target CD4+ T-cell following isogenic R5 and X4 HIV-1 infections of dispersed human tonsil tissue. Prior stimulation using CD3 and CD28 antibodies was performed in the case of PBMC. Virus production was measured by flow cytometry and by p24 ELISA to quantitate both cell associated and cell released viral capsid protein, and

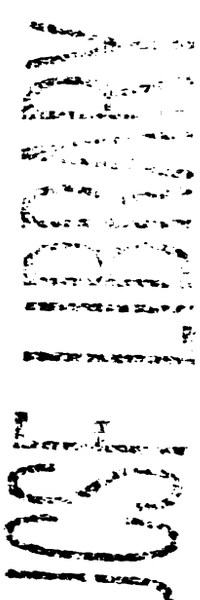
infectious virus was quantitated via TCID50 assay. We provide evidence that R5 HIV-1 compensates for a small target cell population by producing up to 100-fold more infectious virus per CCR5+ target cell than X4 HIV-1. This phenomenon was observed in both of the infection models that were used, evidence that this mechanism of R5 HIV-1 predominance is not restricted to lymphoid tissue, but relevant to blood lymphocyte populations as well. This phenomenon may contribute to the predominance of R5 HIV-1 in early *in vivo* infection.

Table of Contents

List of Figures

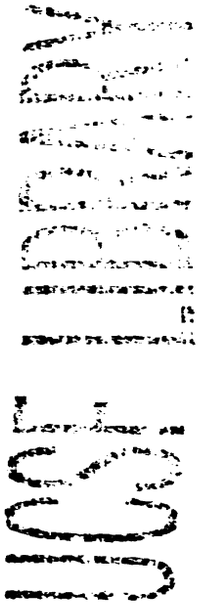
Chapter 1

Introduction: History of HIV-1 Research, Review of HIV Replication Cycle and Clinical Progression



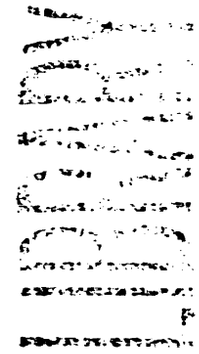
History of HIV-1 Research

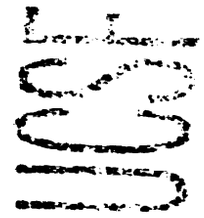
Human Immunodeficiency Virus Type 1 (HIV-1) related disease first came to light after sporadic cases of unexplained immunodeficiency were diagnosed amongst homosexual males in parts of the United States. The syndrome was first referred to as Gay Related Intestinal Disease (GRID) (1, 2) and then as the Acquired Immune Deficiency Syndrome (AIDS). Immunocompromised individuals succumbed to opportunistic infections and the cause of the immune system's failure to clear these infections was unknown. In the early 1980s, several discoveries linked AIDS with the presence of a retrovirus. This virus, first isolated in a Caucasian patient with AIDS, belonged to the retrovirus family and was dubbed LAV (6). In the next two years, publications by several other groups reported the presence of the virus in AIDS patients, referring to the infectious agent as HTLV-III or ARV (27, 35, 48, 51). In 1986, driven by the formation of a federal committee, several of the labs involved agreed to the virus' current designation, Human Immunodeficiency Virus, Type 1 (HIV-1) (14).



In 2005 alone, HIV/AIDS claimed the lives of 3.1 million individuals, 2.4 million of them in Sub-Saharan Africa (UNAIDS/WHO AIDS Epidemic Update, December 2005).(shouldn't this be listed in the reference list instead?) The cost in the form of both human suffering and economic collapse is staggering. There are currently 40.3 million known HIV-1 infected individuals living throughout the world, and the number is rising

every day. Although there is a growing number of therapeutic drugs that can drastically improve the quality of life and life expectancy of HIV-1 infected individuals, these drugs are unavailable in many Third World countries due to high cost. In addition, they cause a number of severe side effects. Despite the tireless efforts of hundreds of scientists, there is no cure or vaccine available for HIV-1. Most likely, there will not be one in the immediate future: HIV-1 has a remarkable ability to mutate its genome so that it can escape both the host immune system and anti-retroviral drugs, and such extensive variation renders the creation of a universal vaccine effective on all HIV-1 strains extremely difficult. The study of HIV-1 therefore continues, with scientists and clinicians exploring every aspect of HIV-1 infection and of the virus itself.

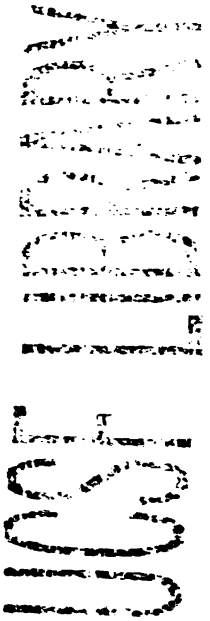




HIV Genetics and Viral Life Cycle

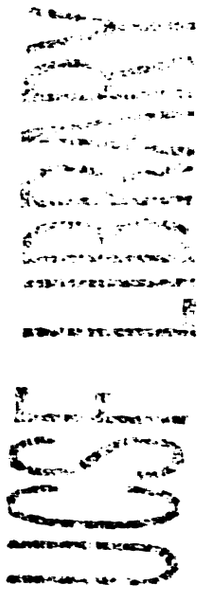
HIV-1 is a member of the family Retroviridae, consisting of viruses with a diploid RNA genome (note: some viruses, e.g., rotaviruses, have dsRNA genomes with single + and - strands that are complementary to one another; in the case of HIV, the strands of the diploid genome are separate and usually non-identical to one another) which utilize reverse transcriptase to transform their RNA genomes into DNA. The viral DNA can then be either integrated into the host cell genome or transcribed directly by host enzymes (can you double-check on this last clause? I know that this has been speculated but I'm not sure that it's been proven

at high frequency, if at all. There should be mention of it in any
k. The questions are: can viruses that are deficient in integration
replicate and spread? Does this happen with any frequency in
HIV-1 is a member of the lentivirus sub-group, distinguished
by the fact that these viruses are capable of infecting non-
ng cells. Hence, cellular mitosis is not necessary for the viral
to be transported into the host cell nucleus and integrated into
ome. The HIV-1 genome encodes nine open reading frames
by 5' and 3' long terminal repeats. The encoded polyproteins are
ently cleaved post-translationally to generate a series of discrete
teins with defined functions.



an enveloped virus that enters a host cell through fusion of the
cellular membranes, a process that is mediated by interaction
the viral envelope protein and cellular membrane proteins
ed in greater detail in the following section). Upon entry into the
, the viral reverse transcriptase, packaged in the virion, is
along with the diploid viral RNA genome. Reverse transcription
ace in the host cell cytoplasm, generating a double stranded DNA
The resulting viral cDNA is transported into the nucleus in a
otein complex and integrated into the host cell genome.
er, the provirus enters a period of replicative latency, remaining
until triggered, e.g., by cellular activation induced host cell

transcription factors such as NFκB. Transcription then generates a series of multiply-spliced transcripts encoding the regulatory gene products Tat and Rev, which serve to amplify transcription and to transport RNA into the cytoplasm, respectively. The remaining viral proteins are translated in the cytoplasm, in several cases in the form of polyproteins. As viral maturation proceeds, these polyproteins are cleaved, the capsid forms to incorporate the diploid viral RNA genome, and virus budding at the plasma membrane leads to release from the cell and further viral maturation, finally generating mature virions that can begin the life cycle again (26).

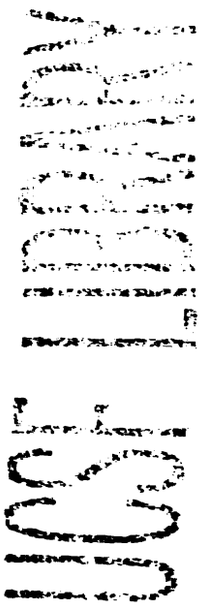


HIV Entry and Receptor Biology

A membrane fusion process between the host cell plasma membrane and the virion envelope mediates HIV-1 entry. The primary receptor for HIV-1 is CD4, although there have been reports of rare CD4 independent viral strains (9, 17). After binding of the viral gp120 envelope protein to CD4, a conformational change is triggered in gp120, allowing it to bind at a separate site to a co-receptor on the surface of the target cell (30). Several chemokine receptors can theoretically serve as the viral co-receptor, including CCR5, CXCR4, CCR2b, CCR3, CCR8, BOB (gpr15), Bonzo (CXCR6), V28 (CX_3CR1), gpr1, APJ, ChemR23, US28, BLTR and CCBP2 (41, 43). However, the two utilized by the vast majority of viral strains *in vivo* are CXCR4 and CCR5 (4, 19, 24, 61, 62). In the case of HIV-1, interaction of CXCR4 or CCR5 with the viral gp120 protein allows

for an additional conformational change in the viral envelope, exposing critical domains necessary for fusion on the trans-membrane viral envelope protein, gp41. Gp41 then mediates fusion of the viral and target cell envelopes, completing the entry process and allowing for the release of the viral genome and associated proteins into the cytoplasm of the target cell (20).

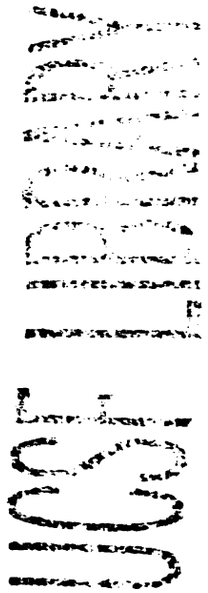
As with virtually all other viruses, the receptors used by HIV-1 determine the cell types that the virus is able to enter and, given favorable intracellular conditions, to replicate and spread. In the case of HIV-1, tropism for CD4 and CXCR4 enables the virus to infect certain subpopulations of CD4+ T cells (e.g., intrathymic T progenitor cells, T-helper cells) and, in the case of CD4 and CCR5, subpopulations of T cells, monocytes, macrophages, and dendritic cells (49, 62). These important mediators of immune function circulate through the peripheral blood and are segregated preferentially within organs of the hematolymphoid system, including the thymus, lymph nodes, spleen, and gut associated lymphoid tissue (GALT). As a large proportion of immune cells are constantly circulating through this network, especially during the course of chronic infection, HIV-1 is able to establish a systemic presence very quickly. In addition, it is this specificity for target cells that causes the characteristic immunodeficiency associated with HIV-1 disease. As target cells are eliminated from the immune system, the infected individual is



progressively less able to resist opportunistic infections (e.g., *Pneumocystis carinii* pneumonia or toxoplasmosis) and certain cancers (e.g., Kaposi's sarcoma).

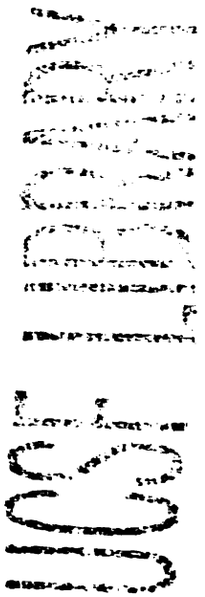
HIV Transmission and Clinical Disease Progression

HIV-1 can be transmitted between individuals through either blood or genital secretions. Routes of infection include sexual contact, vertical transmission from mother to neonate, the use of non-sterile needles by multiple individuals (through drug use or medical procedures), and blood transfusion. Target cells at the site of infection, most often dendritic cells and/or macrophages, are exposed to the virus, and if successfully infected, can carry the virus back to draining lymph nodes where other target cells are very highly concentrated. Viral spread at the site of infection is often very rapid: over half of CD4+ cells in the lamina propria of the gut are apparently lost during the earliest stages of infection (12, 36).



In the first weeks after transmission, the virus replicates extremely rapidly, reaching levels as high as 10^{6-8} copies/ml in many cases. Such replication is often accompanied clinically by flu-like symptoms. After this initial spike in viral load, viral replication is partially suppressed (possibly

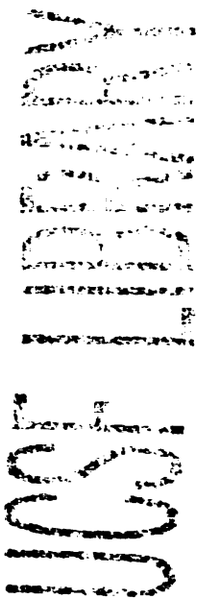
by the host immune system), a lower "set point" of circulating virus is reached, and a period of clinical quiescence ensues. This can last from weeks to years and, during this time, the virus is thought to be slowly depleting the CD4+ T cell population. This period is then often followed by a resurgence of viral replication, a rapid loss of target CD4+ T cells, and direct and indirect negative effects on multiple other cell subpopulations (e.g., CD8+ T cells, antigen presenting cells, and certain categories of neurons). The infected individual is then susceptible to opportunistic infections and progresses to AIDS (15).



Co-receptor Tropism in Disease Progression

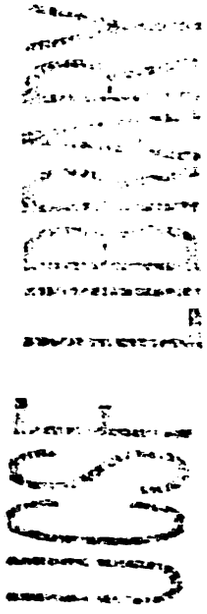
Many factors influence the rate of HIV-1 disease progression. Genetic variations in either the host or the virus can make a significant difference in the severity of disease (44). Changes in the sequence of accessory or envelope proteins of HIV-1 represent an excellent example of this. Co-receptor tropism is mainly determined by the variable regions of the HIV-1 gp120 protein (V1-V3). Replacing the V3 loop of a CXCR4 tropic (X4) HIV-1 strain with that of a CCR5 tropic (R5) strain effectively changes the co-receptor preference of the virus from CXCR4 to CCR5 (13, 40). In fact, this change in co-receptor preference can be triggered by changing as few as 1-3 amino acid residues in V3 (55, 56), especially if the changes are focused in "hotspots" (18, 25, 32). Overall, a shift from CCR5 to CXCR4 tropism is associated with an increase in the net positive

charge of V3 (25, 28, 56). These changes in charge can be induced either by amino acid substitutions (32, 34, 46) or by changes in post-translational modification, specifically glycosylation (5, 31, 38, 47). The impact of V3's net charge on tropism may be due to the difference in overall charge of the extracellular domains of CCR5 and CXCR4 (11, 23, 37)(positive and negative, respectively), but the exact mechanism is unclear. Less is known about the variations in V1 and V2, which contribute to tropism, but sequence changes in these regions can also have a significant impact (10, 42). It should be noted that other regions of gp120, including a structural domain composed of residues of regions C1-C4 of gp120 (7, 50, 60) and in gp41 (8), have been associated with co-receptor interactions, and therefore may influence tropism.



CCR5 and CXCR4 are beta chemokine receptors expressed on several subsets of cells in the innate and adaptive immune system. The role of these two receptors is normally to direct immune cell movement to sites of inflammation via attraction to a gradient of their specific ligands, SDF-1 for CXCR4 and MIP-1α, MIP-1β, or RANTES for CCR5. Interaction of receptor and ligand results in an intracellular signaling pathway that is unique to each, although they often overlap (57). CCR5 and CXCR4 belong to a large family of receptors known as G-protein coupled receptors, or GPCRs. All GPCRs have seven trans-membrane domains spanning the plasma membrane, with each domain consisting of 20-27

amino acids. Each also carries three extracellular and three intracellular loops, but these, like the N- and C- terminal domains, are extremely variable (33). This allows for members of the GPCR family to interact with a wide variety of ligands. Following contact with the appropriate ligand, the GPCR activates trimeric G-proteins in the interior of the cell by allowing the exchange of bound GDP for GTP. These trimers then separate into two subsets, GαGTP and Gβγ, and each triggers further signaling events. The G proteins are extremely diverse and interact with a variety of downstream effectors, eventually leading to regulation of gene expression.

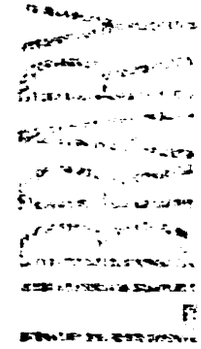


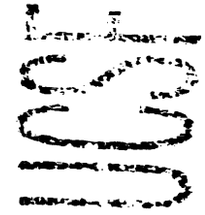
The chemokine receptors within the GPCR family are divided into four major groups depending on the number and spacing of conserved cysteine residues: C, CC, CXC, and CX_3C. These groups are also referred to as α, β,γ, and δ, respectively. Some chemokine receptors are fairly promiscuous, binding several different ligands. CCR5, for instance, utilizes the ligands RANTES, MIP-1α and MIP-1β. CXCR4, on the other hand, binds only SDF-1. In the case of CCR5 and CXCR4, chemokine binding is followed almost immediately by receptor dimerization and association with members of the JAK family of tyrosine kinases, which in turn activate STAT transcriptional factors (57). There is some evidence that CCR5 signaling may lead to the activation of different JAK/STAT partners than

CXCR4 and subsequently cause different changes in gene expression (58, 59).

Co-receptor use during *in vivo* infection follows one of two patterns. (I can't tell from the paragraph below what the two patterns are. This is an important paragraph for your hypothesis - it should be clear!) During the first stages of infection *in vivo*, interaction of virus with CD4+ T cells expressing CCR5 are highly favored over those in which CXCR4 is used instead, even if the virus is dual tropic for both co-receptors (54, 63). Thereafter, about 50% of patients show conversion of the original viral isolate to an X4 variant. In approximately 50% of infected individuals studied, however, a transition from CCR5 tropic (R5) to CXCR4 tropic (X4) HIV-1 was not detected (16, 52). This phenomenon is quite striking, and several hypotheses have been presented to account for the observed predominance of R5 viruses in these patients. First, it has been suggested that R5 HIV-1 may have preferential access to cells with the appropriate co-receptors. For instance, it has been observed that there are differential expression patterns of CCR5 and CXCR4. CXCR4 is expressed on most CD4+ T-cells and virtually all naïve, non-activated CD4+ T-cells (62). CCR5, on the other hand, is expressed on activated CD4+ T-cells, macrophages, and dendritic cells (49). These latter cell types are prevalent in mucosal tissues, the most common sites of HIV-1 transmission (45, 53). This differential localization could give R5 HIV-1 a

replicative advantage soon after transmission. Alternatively, R5 HIV-1 may have an advantage *in vivo* due to differential expression of ligands for CCR5 and CXCR4. SDF-1 and other CXCR4 ligands are highly expressed at mucosal transmission sites, which may result in a selective block of CXCR4 tropic (X4) HIV-1 infection (3). A final hypothesis is based not on target cell or co-receptor availability, but instead on transport of virus to target cells. Recent evidence has indicated that cell types such as mucosal epithelial cells, which are not productively infected by HIV-1 but instead serve to transport virus to targets, may selectively take up and deliver R5 HIV-1 (39).



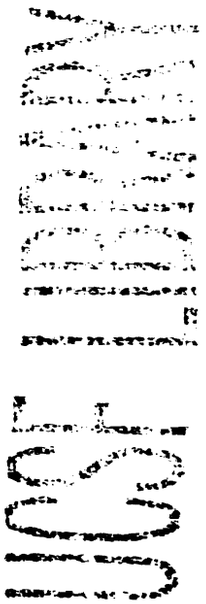


Overview of Thesis Work

Alone or in concert, these mechanisms and others may account for the predominance of R5 HIV-1 in early *in vivo* infection. Through the experimental plan that follows, a further potential explanation for R5 HIV-1 predominance has been investigated. Previous evidence has shown that a variety of factors, including both HIV-1 and host cell states, can impact the quantity of HIV-1 virions released from a target cell. Variables such as the state of disease progression (29), target cell activation state (21), and the presence or absence of particular viral proteins (22) have been investigated, and significant differences in viral production detected.

Eckstein at al. (21) demonstrated that Vpr is essential for HIV-1

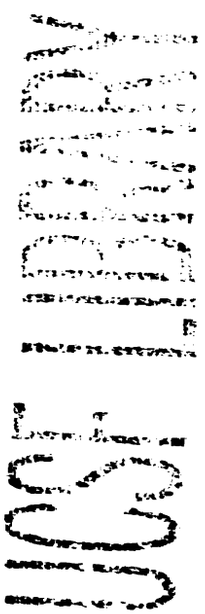
replication in macrophages, but not in primary T-cells. This study also demonstrated that, in an infection of *ex vivo* peripheral lymphoid tissue, R5 and X4 HIV-1 produce approximately equivalent amounts of virus over time. Infection of CD4+ T-cells accounted for approximately 50% of virus produced in an R5 HIV-1 infection over time, the rest being produced by macrophages. This was surprising, since CCR5+ CD4+ T-cells represent only a small (20% or less) fraction of the total CD4+ T-cell population. Yet, the amount of virus produced after R5 infection of this subpopulation appeared to be greater than expected, prompting the hypothesis: in an infection of CCR5+ CXCR4+ CD4+ T-cells, a cell type permissive for both R5 and X4 HIV-1 infection, R5 HIV-1 infection may result in the eventual production of significantly more infectious virions per target cell than X4 HIV-1. The R5 "burst size," in other words, would be larger. If so, this differential virion production could contribute significantly to the predominance of R5 HIV-1 in early infection *in vivo*.



To pursue this hypothesis, it was necessary to choose experimental systems appropriate for the comparison of R5 and X4 HIV-1 infection in a biologically relevant way. To this end, primary human cell culture models were used. As laboratory-adapted strains of HIV-1 are incapable of infecting directly isolated peripheral blood mononuclear cells (PBMCs) effectively, several methods of PBMC stimulation were tested. Stimulation of isolated CD4+ T-cells through the TCR with antibodies

against CD3 and CD28 was chosen, and used to study the replication kinetics of several laboratory adapted and primary HIV-1 strains (Chapter 2). Isogenic pairs differing only in the V3 loop of gp120 were used to isolate the impact of co-receptor preference from other factors influencing viral replication. These studies indicated that R5 HIV-1 is in fact capable of producing a greater amount of viral protein over time.

However, these experiments offered only indirect evidence that a greater number of virions were being produced per infected cell. To ascertain the average viral output of cells susceptible to both R5 and X4 HIV-1, CCR5+CXCR4+ cells were sorted away from their CCR5-CXCR4+ counterparts via flow cytometry following infection of TCR stimulated CD4+ T cells (Chapter 3). Measurements of cell number, percent of infected cells, and viral protein/infectious virion production were used to determine the average viral output per infected cell for both populations. R5 HIV-1 infection of CCR5+CXCR4+ cells generated 5-10 fold more viral protein and infectious virus per infected cell than X4 HIV-1 in these studies. While these data were encouraging, it was then necessary to ask these questions in a more biologically relevant model. It has been shown that a large part of HIV-1 infection *in vivo* occurs in the lymphoid tissues. Therefore, a tonsil histoculture model was employed to examine both the role of co-receptor preference in infection of lymphoid tissues and the impact of the presence of additional cell types that normally interact with



:arget cells in vivo (macrophages, dendritic cells, CD8+ T-cells,
Chapter 3). In this system, the difference between R5 and X4 HIV-
even more prominent, with R5 HIV-1 producing 5-100 fold viral
and infectious particles than X4 HIV-1. This may indicate that
:ell types in lymphoid tissues influence the extent of HIV-1 infection
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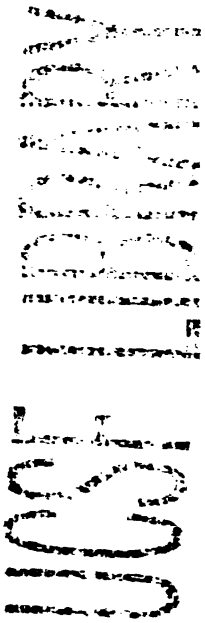

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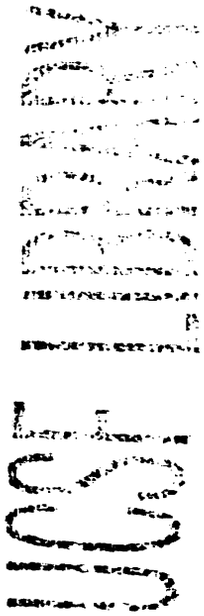

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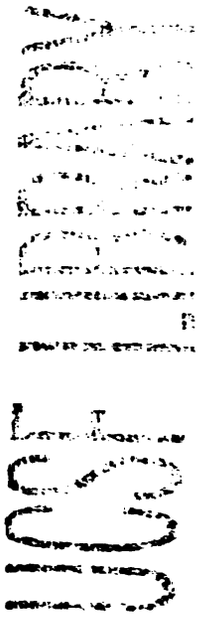

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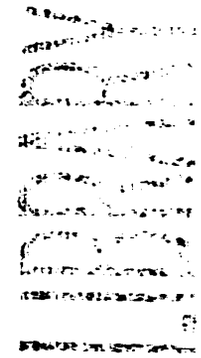

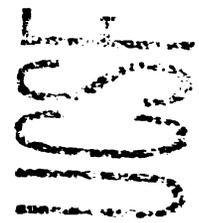

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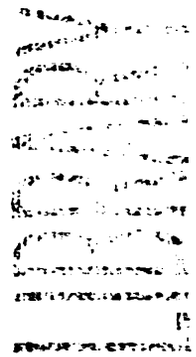

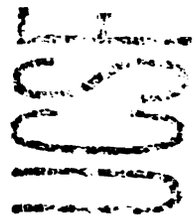

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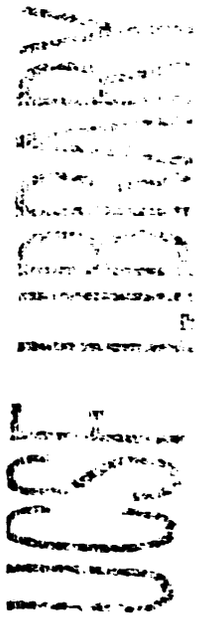

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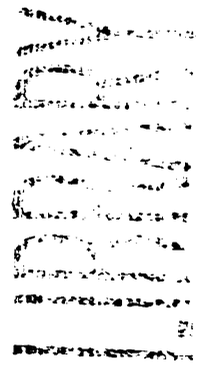

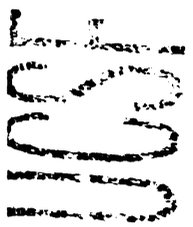

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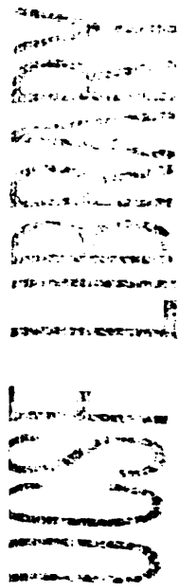
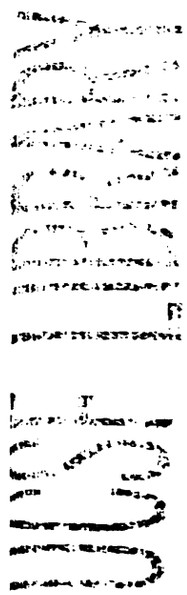


Chapter 2

Co-receptor Preference in Peripheral Lymphoid Populations



R5 HIV-1 Replicates More Efficiently in Primary CD4+ T Cell Cultures Than X4 HIV-1

Becky Schweighardt*, Ann-Marie Roy*, Duncan A. Meiklejohn, Edward J. Grace II, Walter J. Moretto, Jonas J. Heymann, and Douglas F. Nixon

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*Authors contributed equally to this work

Abstract

In this report, we present evidence that R5 HIV-1 replicates more efficiently in primary CD4+ T cells than X4 HIV-1. By comparing CD3/CD28 co-stimulated CD4+ T cell cultures infected by several X4 and R5 HIV-1 strains, we determined that R5-infected CD4+ T cell produce more virus over time than X4-infected CD4+ T cells. In the first comparison, we found that more cells were infected by the X4-tropic strain, LAI than the R5-tropic strain, JR-CSF, yet higher levels of viral production were detected in the R5-infected cultures. The differential viral production was partially due to the severe cytopathic effects of the X4 virus. We also compared cultures infected with the isogenic HIV-1 strains NL4-3 (X4) and 49.5 (R5). We found that fewer cells were infected by the R5 strain, yet similar levels of viral production were detected in both infected cultures. Cell death played less of a role in the differential viral production of these strains, as the cell viability remained comparable in both X4- and R5-infected cultures over time.

The final comparison involved the primary R5-tropic isolate KP1 and the primary dual-tropic isolate KP2. Although both strains infected similar numbers of cells and induced comparable levels of cytopathicity, viral production was considerably higher in the R5-infected culture. In summary, these data demonstrate that R5 HIV-1 has an increased

capacity to replicate in co-stimulated CD4+ T cells compared to X4 HIV-1.

Introduction

Human Immunodeficiency Virus type 1 (HIV-1) infects cells by binding to the CD4 receptor and one of several co-receptors expressed on the surface of target cells (2, 13, 15, 16, 18, 26). The chemokine receptors CCR5 and CXCR4 serve as the major co-receptors for HIV-1, although several other chemokine receptors have been linked to minor HIV-1 co-receptor usage (2, 8, 15, 18, 32, 53). Characteristically, non-syncytium-inducing (NSI) isolates utilize CCR5 as a co-receptor, and are referred to as R5 strains (2, 14-16). R5 strains often represent the dominant viral population detected during the early stages of clinical HIV-1 infection (9, 14, 41, 43, 47, 54). In contrast, syncytium-inducing (SI) isolates utilize CXCR4 as a co-receptor, and are referred to as X4 strains (18, 20, 24, 25, 28, 30, 40, 46, 49). X4 strains are typically detected in the later stages of infection, and are associated with rapid CD4+ T cell loss (11, 14, 24, 25, 47, 49, 50). Despite the link between X4 emergence and disease progression, approximately half of all individuals with AIDS continue to harbor predominantly R5 viruses, suggesting that CXCR4 co-receptor usage alone is not responsible for disease progression (9, 43, 47, 54).

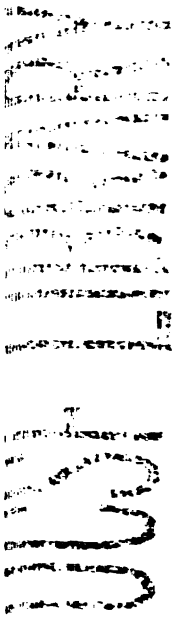
Several mechanisms have been proposed to explain the R5 dominance of early HIV-1 infection. There is evidence that R5 strains may be

transmitted at an increased frequency compared to X4 strains. For example, individuals that carry a 32 base pair deletion mutation (Δ32) in the CCR5 gene are highly resistant to HIV-1 infection (10, 33, 38, 45). Although these individuals are susceptible to X4 infection, very few cases have been reported, suggesting that X4 transmission occurs with lower frequency (3, 5, 23, 29, 37, 48, 51). Another mechanism leading to R5 dominance *in vivo* may involve preferential spread of R5 strains. It has been reported that dendritic cells preferentially transport R5 rather than X4 virions, which may lead to selective spread of R5 virus to lymph nodes (19, 41). Also, intestinal epithelial cells have been shown to selectively transport R5 virions to the lamina propria, which may lead to the preferential spread of R5 infection to activated CD4+ T cells (34). Recent evidence suggests that the immune response may also play a role in R5 selection *in vivo*. Harouse et al. report that both X4 and R5 SHIV replication can be detected in macaques early after co-infection; however, X4 replication is no longer detected after CD8+ T cell-mediated anti-viral immune responses are elicited (22). Furthermore, X4 replication re-emerges in co-infected animals following depletion of the anti-viral immune response by *in vivo* infusion with anti-CD8 antibodies. Collectively, these data suggest that X4-infected cells may be more susceptible to immune-mediated killing than R5-infected cells (22).

We propose that an additional mechanism may be involved in R5

dominance. In this report, we present evidence that R5 HIV-1 replicates more efficiently in CD3/CD28 co-stimulated CD4+ T cells than X4 HIV-1. We tested several viral strains in this system, including the molecular clones LAI (X4) and JR-CSF (R5), the isogenic viral pair NL4-3 (X4) and 49.5 (R5), and two primary isolates that were recovered from the same patient at different time points; KP1 (R5) and KP2 (X4/R5). We found that the R5 HIV-1 strain JR-CSF infected a smaller percentage of co-stimulated CD4+ T cells compared to the X4 HIV-1 strain LAI, yet produced more progeny virus over time than the X4-infected culture. It is likely that this result is partially due to the fact that the cell viability of X4-infected cultures decreased rapidly, whereas cell viability remained relatively high in R5-infected cultures over time. Analysis of cultures infected with the isogenic viral pair provided further confirmation that R5-infected CD4+ T cells produce more progeny virus than X4-infected CD4+ T cells. Despite the fact that fewer co-stimulated CD4+ T cells were infected by the R5-tropic 49.5 compared to its isogenic X4 counterpart NL4-3, similar amounts of viral production were detected in both infected cultures. Cell death seemed to play less of a role in the differential viral release between these two strains, as the viability of both infected cultures remained comparable over time. Interestingly, similar results were obtained with the primary R5 isolate KP1 and the primary dual-tropic isolate KP2. Although both KP1 and KP2 infected an equivalent number of co-stimulated CD4+ T cells and induced comparable amounts of cell

ral production was considerably higher in cultures infected by the
c KP1 strain. In summary, we report that R5 HIV-1 has an
d capacity to replicate in CD4+ T cells compared to X4 HIV-1, and
that this increased fitness may allow R5 viruses to out compete
es in the early stages of HIV-1 infection.



Materials and Methods

Antibodies. The following fluorescently labeled monoclonal antibodies were purchased from Becton Dickinson (San Jose, CA), and used for flow cytometric analysis: CD3-APC (clone SK7), CD69-FITC (clone FN50), CD38-PE (clone HIT2), CXCR4-APC (clone 12G5), CD4- PerCP (clone SK3), CCR5-APC (clone 2D7), IFNγ-PE (clone 25723.11). The monoclonal p24-FITC antibody (clone KC57) was purchased from Coulter Clone (Miami, Florida), and the annexin V-PE was purchased from Caltag Laboratories (Burlingame, CA). The following antibodies were used for CD3/CD28 stimulation: CD28 (LeuTM-28) clone L293 (Becton Dickinson) and CD3 clone SPV-T3b (Zymed, S. San Francisco, CA).

CD4+ T cell isolation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham BioSciences, Uppsala, Sweden) gradient centrifugation of leukopacks (Stanford Blood Bank, Stanford, CA) obtained by apheresis of healthy donors. CD4+ T cells were purified by negative selection using Miltenyi Microbeads (Miltenyi Biotec, Auburn, CA). Cell purity was determined by staining cells with fluorescently conjugated antibodies directed against CD4, CD3, CD8 and CD14. Cell populations were found to be >95% CD3+CD4+.

CD4+ T cell stimulation. CD4+ T cells were activated by PHA stimulation or by CD3/CD28 co-stimulation. For PHA stimulation, cells

were cultured at a density of 2x106 cells/ml with 1µg/ml PHA (Sigma, St. Louis, MO) for 24, 48, or 72hrs. Cells were then washed to remove PHA, and cultured for 48hrs in RPMI 1640 medium (MediaTech, Herndon, VA) supplemented with 15% fetal bovine serum (FBS) (Gemini, Woodland, CA) and 50 Units/ml IL-2 (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH). For CD3/CD28 co-stimulation, tissue culture plates were pre-coated with CD3 antibody. Briefly, wells were washed with 1x phosphate buffered saline (PBS) and then coated with a 50µg/ml stock solution of CD3 antibody. Excess liquid was removed and plates were incubated at 37°C until dry. Cells were then cultured on coated plates at a concentration of 2x106 cells/ml in the presence of 1µg/ml soluble CD28 antibody (Becton Dickinson) for 24, 48, or 72hrs. Cells were removed from the CD3 coated plates, washed to remove soluble CD28, and then cultured in RPMI 1640 medium supplemented with 15% FBS and 50 Units/ml IL-2.

Detection of cell surface protein expression by flow cytometry. To detect cell surface protein expression, 5x105 CD4+ T cells were incubated with appropriate concentrations of fluorescently conjugated monoclonal antibodies diluted in 1x PBS containing 1% bovine serum albumin (BSA; Sigma, St. Louis, MO). Cells were incubated with antibodies for 20 minutes at 4°C, washed twice, and then fixed in 1% paraformaldehyde. Samples were acquired on a FACS CaliburTM (Becton

Dickinson) instrument, and the resulting data was analyzed using CellQuestTM (Becton Dickinson) software.

Measurement of cellular proliferation and IFNγ expression. Stimulated CD4+ T cells were incubated with 2μM CFSE (5- and 6-carboxyfluorescein diacetate, succinimidyl ester) (Molecular Probes, Eugene, OR) for 10 minutes at 37°C, and then washed twice. Stained cells were incubated for 72 hours at 37°C. Brefeldin A (Sigma) was added at a final concentration of 10μg/ml for the last 6 hours of culturing. Cells were then washed, fixed in 4% PFA, permeabilized with 1% FACS Perm solution (Becton Dickinson), and stained with monoclonal antibodies against CD3, CD4, and IFNγ. After antibody staining, cells were washed twice, and then stored in 1% paraformaldehyde until acquisition on a FACS CaliburTM. Subsequent analysis was performed using FlowJo SoftwareTM (Tree Star, Inc).

Virus preparation. The HIV-1 strains LAI, JR-CSF, NL4-3, and 49.5 were prepared by introducing proviral constructs into 293T cells (ATCC, Manassas, VA) by CaPO4 transfection. The proviral plasmids were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pLAI.2 (39) from Dr. Keith Peden, courtesy of the MRC AIDS Directed Programme, pYK-JR-CSF (6, 21, 27) from Dr. Irvin SY Chen and Dr. Yoshio Koyanagi, pNL4-3 (1) from Dr. Malcolm

Martin, and p49.5 (52) from Dr. Bruce Chesebro. After changing the media approximately 16hrs after transfection, the virus-containing supernatants were harvested 72 hours post-transfection. Viral stocks were centrifuged at 2,000 rpm for 10 minutes to remove cell debris, and then passed through a 45μm filter. The infectious titer of each viral preparation was determined by TCID50 assay. Briefly, PHA stimulated PBMCs from multiple donors were pooled and infected with serially diluted virus in quadruplicate wells. Cell supernatants were collected five days post-infection and HIV p24 antigen was quantitated by p24 ELISA. Infections were scored positive for replication if p24 levels were greater than 50pg/ml. The TCID50 value represents the virus dilution at which 50% of wells scored positive for infection.

Co-receptor phenotyping assay. GHOST indicator cells were used to determine co-receptor usage of each viral strain. The GHOST-X4 and GHOST-Hi5 cell lines (35) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Vineet N. KewalRamani and Dr. Dan R. Littman. These cell lines were originally derived from human osteosarcoma (HOS) cells. The GHOST-X4 cell line was transduced with a retroviral vector that confers high level CXCR4 expression, and the GHOST-Hi5 cell line was transduced with a retroviral vector that confers high level CCR5 expression. The GHOST-Hi5 cells also express low levels of CXCR4 due to endogenous expression in the parental

HOS cells. Each cell line was also transduced with a CD4-expressing retroviral vector, and a construct that drives expression of GFP under the control of the HIV LTR promoter. GHOST-X4 and GHOST-Hi5 cells were seeded on 12 well plates at a density of 5x105 cells per well. Cells were cultured at 37°C overnight prior to infection with HIV-1 strains. Cells were infected with each viral strain at a multiplicity of infection (moi) of 0.01 in the presence of 20µg/ml polybrene to enhance infection efficiency. Each infection was performed in a total volume of 300µl at 37°C for 4hrs. After incubation, virus was removed and 1ml of fresh culture medium was added to each well. Cells were then incubated for an additional 48hrs prior to harvest. GFP expression was analyzed by FACS analysis. Samples were acquired on a FACS CaliburTM instrument, and the resulting data was analyzed using Cell QuestTM software.

HIV infection. CD4+ T cells were activated by CD3/CD28 co-stimulation for 72 hours prior to infection. Cells were then washed and incubated with virus at an moi of 0.01 (LAI, JR-CSF, NL4-3, and 49.5) or 0.001 (KP1 and KP2) for 4 hours at 37°C. After infection, cells were washed three times to remove any unbound virions, and then cultured in RPMI 1640 medium supplemented with 15% FBS and 50 Units/ml IL-2.

Quantification of viral replication. Viral replication was assessed by measuring the amount of soluble HIV p24 antigen in culture supernatants.

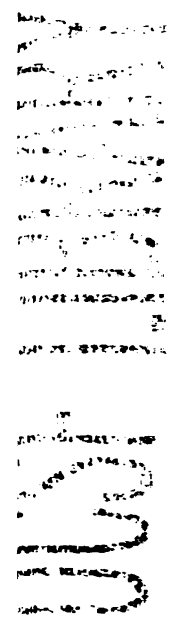
200µl aliquots of supernatant were removed from infected cell cultures at 3, 5, 7, and 10 days post-infection. Supernatants were stored at -80°C until completion of the experiment. Quantification of p24 was determined using an enzyme-linked immunosorbent assay (ELISA) (PerkinElmer Life Science, Inc., Boston, MA) according to the manufacturer protocol.

Intracellular p24 staining. The percentage of infected cells was determined by intracellular staining for the viral p24 antigen. 5x105 CD4+ T cells were removed from infected cultures at 3, 5, 7, and 10 days post-infection. Cells were washed, fixed in 4% paraformaldehyde, permeabilized with 1% FACS Perm solution (Becton Dickinson), and then incubated with a fluorescently conjugated monoclonal p24 antibody for 30 minutes at 4°C. Cells were then washed twice, and suspended in 1% paraformaldehyde. Samples were acquired by FACS CaliburTM, and the resulting data was analyzed using FlowJoTM software.

Results

Co-stimulation with plate-bound CD3 and soluble CD28 antibodies induces high levels of activation in CD4+ T cell cultures. In order to study X4 and R5 HIV replication in primary CD4+ T cells, we first optimized *in vitro* stimulation conditions for these cells. CD4+ T cells were isolated from the peripheral blood mononuclear cells (PBMC) of three donors by magnetic separation. The isolated cells were found to be >95% CD3+CD4+ (data not shown). *In vitro* stimulation conditions were optimized by treating cells with increasing concentrations of plate-bound CD3 antibody and increasing concentrations of soluble CD28 antibody for varying lengths of time (data not shown). Cellular activation was assessed by monitoring the expression levels of the activation markers CD38 and CD69 by flow cytometry at 0, 24, 48, and 72 hrs post-stimulation. The highest level of activation was achieved following treatment with 50µg/ml of plate-bound CD3 antibody and 1µg/ml of soluble CD28 antibody (Fig. 1A&B). CD38 and CD69 expression increased rapidly in these cultures, and by 48 hrs, an average of 29% of cells co-expressed both activation markers (Fig. 1A&B). Activation marker expression was two-fold lower in PHA-stimulated cultures, indicating that CD3/CD28 co-stimulation is a better method for activating purified CD4+ T cells *in vitro* (Fig. 1A&B). Cellular activation was also assessed by a

second, independent assay that measures cellular proliferation and interferon-γ (IFNγ) expression. To measure activation-induced proliferation, stimulated CD4+ T cells were stained with the green fluorescent dye CFSE (5- and 6- carboxyfluorescein diacetate, succinimidyl ester). CFSE is a cytoplasmic dye that is equally divided among daughter cells during cell division. Consequently, the fluorescence of each daughter cell is half as bright as the parental cell, allowing for the reduction in fluorescence to be used as a marker of cellular proliferation. After CFSE staining, cells were cultured for 72 hrs, and then fixed, permeabilized, and stained with fluorescently labeled antibodies directed against IFN-γ, CD3, and CD4. A large population of proliferating, IFN-γ expressing CD4+ T cells was detected in CD3/CD28 co-stimulated cultures (Fig. 2). This population of cells was not detected in PHA-stimulated cultures, confirming that CD3/CD28 co-stimulation induces higher levels of activation and proliferation in purified CD4+ T cell cultures compared to PHA treatment (Fig. 2).



The HIV-1 co-receptors, CXCR4 and CCR5, are expressed on CD3/CD28 co-stimulated CD4+ T cells. To assess the potential susceptibility of co-stimulated CD4+ T cells to HIV-1 infection, we examined co-receptor expression on these cells by flow cytometry. Cells from three independent donors were stimulated with either CD3/CD28 antibodies or PHA. At 0, 24, 48, and 72 hrs post-stimulation, cells were

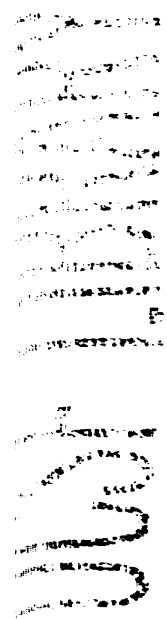
stained with fluorescently conjugated monoclonal antibodies directed against CD4, CXCR4, and CCR5. At each time point, nearly all CD4+ T cells from each donor expressed high levels of CXCR4, regardless of stimulation method (Fig. 3). In contrast, very low levels of the R5 co-receptor, CCR5, were detected in PHA and CD3/CD28 stimulated CD4+ T cell culture, and a high degree of variation was detected among donors (Fig. 4). CCR5 expression levels were found to increase over time in both PHA and CD3/CD28 co-stimulated cultures from each donor, with expression levels peaking at 72 hrs post-stimulation (Fig. 4).

The co-receptor usage of each HIV-1 strain was determined by GHOST cell assay.

Prior to assessing the susceptibility of co-stimulated CD4+ T cells to X4 and R5 strains of HIV-1, we first examined co-receptor usage of each strain using GHOST-X4 and GHOST-Hi5 indicator cells. These cell lines were originally derived from human osteosarcoma (HOS) cells, and express low levels of endogenous CXCR4. In addition, the GHOST-X4 cells were transduced with retroviral vectors carrying the human genes CD4 and CXCR4, and therefore express high levels of each receptor. The GHOST-Hi5 cells were transduced with vectors carrying the human genes CD4 and CCR5, and express high levels of each receptor, as well as low levels of endogenous CXCR4. Each of these cell lines was also stably transfected with a construct carrying the green fluorescent protein (GFP)

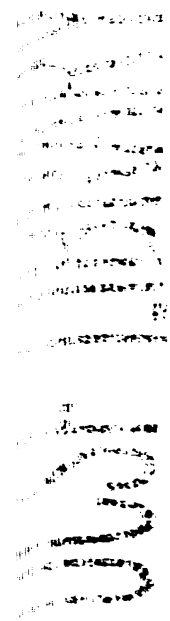
gene under the control of the HIV-1 LTR promoter. This permits HIV infection of these cells to be detected by flow cytometric analysis of GFP expression.

We infected the GHOST-X4 and GHOST-Hi5 cells with the following HIV-1 strains; LAI, JR-CSF, NL4-3, 49.5, KP1, and KP2. GFP expression was detected in GHOST-X4 cells infected with LAI, NL4-3, and KP2, confirming X4 co-receptor usage of these viral strains (Fig. 5). JR-CSF, 49.5, and KP1 did not induce GFP expression in these cells, indicating that these strains do not utilize the X4 co-receptor. High levels of GFP expression were detected in GHOST-Hi5 cells infected with JR-CSF, 49.5, KP1, and KP2, indicating R5 co-receptor usage of these strains (Fig. 5). The X4-tropic strain NL4-3 did not infect the GHOST-Hi5 cells, however, the X4-tropic strain LAI did infect a small population of these cells. This low-level infection was likely due to utilization of the endogenous CXCR4 expressed on the GHOST-Hi5 cells. The ability of KP2 to infect both GHOST-X4 and GHOST-Hi5 cells at an equivalent level indicates that this primary isolate has a dual-tropic phenotype.



R5-infected CD4+ T cells produce more progeny virus over time than X4-infected CD4+ T cells. CD4+ T cells were isolated from PBMC and then stimulated for 72 hrs with CD3/CD28 antibodies. Co-stimulated cultures were infected with several strains of X4 and R5 HIV-1.

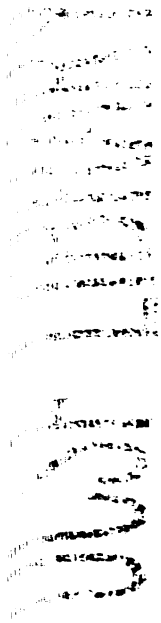
Strains included the HIV-1 molecular clones; LAI (X4) and JR-CSF (R5), an isogenic viral pair that differs only in the V1-V3 loop of gp120; NL4-3 (X4) and 49.5 (R5), and two primary viral isolates recovered from the same patient at different stages of clinical infection; KP1 (R5, early infection) and KP2 (X4/R5, late infection). Cells were infected with LAI, JRCSF, NL4-3, and 49.5 at an moi of 0.01 and with KP1 and KP2 at an moi of 0.001. At several time points post-infection, the percentage of apoptotic cells was determined by surface staining with fluorescently labeled annexin V, and the percentage of infected cells was determined by intracellular p24 staining with a fluorescently conjugated antibody directed against HIV-1 p24 antigen (Figs. 6 and 7). The FACS plots from the flow cytometric analysis of the Annexin V and intracellular p24 staining are shown in Fig. 6. Percentages of infected cells detected in each culture over time are summarized graphically in Fig. 7. In addition, the amount of viral production released by each infected culture was determined by measuring the amount of HIV-1 p24 antigen in the culture supernatants by p24 enzyme-linked immunosorbent assay (ELISA). Results of these assays are shown in Fig. 7, with error bars representing the standard deviation among triplicate infections. Also, the cell viability of each infected culture was monitored over time using the trypan blue exclusion assay. Results of these assays are depicted in Fig. 7.



Our data demonstrate that R5-infected co-stimulated CD4+ T cell

cultures produce more progeny virus than X4-infected CD4+ T cell cultures. In comparing cultures infected by the molecular clones LAI (X4) and JR-CSF (R5), we found that a much larger percentage of cells were infected by LAI compared to JR-CSF at the early time points post-infection (Figs. 6 & 7A). For example, 29% of CD4+ T cells were infected by LAI at day 3 post-infection, but only 8% were infected by JR-CSF (Figs. 6 & 7A). This was likely due to the high expression level of CXCR4 and low expression level of CCR5 on these cells (Figs. 3 & 4). At the later time points post-infection, however, the percentage of apoptotic cells increased in the LAI-infected cultures, and the number of infected cells decreased (Figs. 6 & 7A). By day 7 post-infection, more than half of the cells in the LAI-infected culture were dead, obscuring the FACS analysis due to the large amount of background auto-fluorescence caused by the dead and dying cells (Figs. 6 & 7A). Despite the small number of JR-CSF infected cells, high levels of viral production were detected in these cultures (Fig. 7A). The differential in viral production between the R5-tropic JR-CSF and the X4-tropic LAI was partially due to the fact that the cell viability in the LAI infected cultures decreased rapidly, whereas the cell viability remained relatively high in the JRCSF-infected cultures over time (Fig. 7A). Analysis of co-stimulated CD4+ T cell cultures infected with the isogenic pair NL4-3 (X4) and 49.5 (R5), provided further confirmation that R5-infected CD4+ T cells produce more progeny virus than X4-infected CD4+ T cells. A smaller percentage of co-stimulated

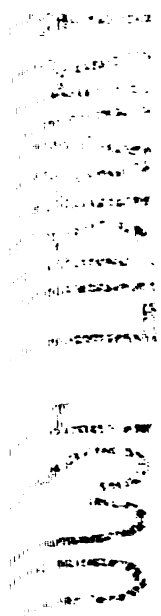
CD4+ T cells were infected by the R5-tropic 49.5 at the early time points post-infection compared to its isogenic X4 counterpart, NL4-3 (Figs. 6 & 7B). At day 5, 11.5% of CD4+ T cells were infected by the X4 strain, whereas only 4.45% were infected by the R5 strain (Figs. 6 & 7B). Despite the presence of fewer R5-infected cells, similar levels of viral production were detected in both the R5- and X4-infected cultures (Fig. 7B). Cell death seemed to play less of a role in the differential viral production between these two strains, as the viability of both infected cultures remained comparable over time (Fig. 7B). Similar results were obtained when comparing cultures infected with the primary R5 isolate KP1 and the primary dual-tropic isolate KP2. Although both KP1 and KP2 were found to infect a similar number of cells and induce comparable amounts of cell death, viral production was considerably higher in cultures infected by the R5-tropic KP1 strain compared to the dual-tropic KP2 strain (Figs. 6 & 7C). In summary, these data demonstrate that R5 HIV-1 has an increased capacity to replicate in co-stimulated CD4+ T cells compared to X4 HIV-1.



Discussion

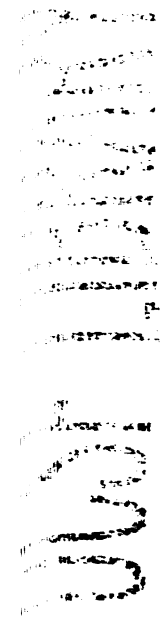
Our data provide evidence that R5 HIV-1 replicates more efficiently in primary CD4+ T cells than X4 HIV-1. Our first experiments focused on optimizing the *in vitro* stimulation conditions of primary CD4+ T cells. We

found that co-stimulation with plate bound anti-CD3 antibodies and soluble anti-CD28 antibodies induced high levels of activation and rendered cells permissible to X4 and R5 HIV-1 infection. This contradicts earlier reports that stimulation with CD3 and CD28 antibodies induced an R5-resistant state in CD4+ T cell cultures (7, 31, 42). Subsequent studies have reported that the R5 resistance only occurred when CD4+ T cells were stimulated with CD3 and CD28 antibodies immobilized on magnetic beads. This phenotype was thought to be mediated by down-regulation of the CCR5 receptor and increased expression levels of β-chemokines (4, 12). Following co-stimulation with our protocol, CD4+ T cells were found to express low levels of CCR5, but were still able to replicate R5 virus efficiently. This may, in part, be due to the high activation state of the CD4+ T cells following CD3/CD28 co-stimulation. In addition, co-stimulated CD4+ T cells were found to express high levels of CXCR4 and were determined to be highly susceptible to infection by X4 HIV-1.



We present evidence that R5-infected CD4+ T cells produce more progeny virus than X4- infected cells. Direct comparisons were made between co-stimulated CD4+ T cell cultures infected with X4 and R5 HIV-1 strains. We first compared cultures infected with two molecular clones of HIV-1, LAI (X4) and JR-CSF (R5). The R5-tropic JR-CSF infected far fewer cells than the X4-tropic LAI, yet produced greater amounts of progeny virus over time. The striking difference in the replication capacity

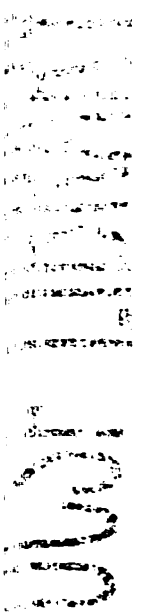
between these two strains can partly be explained by the variation in virus-induced cell death in these infected cultures. Cell viability decreased rapidly in LAI-infected cultures, yet remained relatively high in JR-CSF-infected cultures during the course of the experiment. These data demonstrate that X4 replication can be limited in CD4+ T cell cultures by extensive virus-induced cell death, whereas viral production remains high in cultures infected by less cytopathic viruses. We next compared cultures infected with the isogenic HIV-1 strains NL4-3 (X4) and 49.5 (R5). Because these viruses differ only in the V3 region of the envelope gene, differences in viral replication kinetics are likely mediated by co-receptor usage. We found that a smaller percentage of co-stimulated CD4+ T cells were infected by the R5-tropic 49.5 compared to its isogenic X4 counterpart NL4-3, yet similar levels of viral production were detected in both infected cultures. Virus-induced cell death played less of a role in the differential viral production of these two viruses, since the viability of both infected cultures remained comparable over time. The final comparison between the R5-tropic primary isolate KP1 and the dual-tropic primary isolate KP2 yielded similar results. Although KP1 and KP2 infected similar percentages of co-stimulated CD4+ T cells and induced comparable amounts of cell death, viral production was considerably higher in KP1-infected cultures. Taken together, these data provide evidence that R5-infected CD4+ T cells produce more virus over time than X4-infected CD4+ T cells.



Other groups have also observed a connection between R5 co-receptor usage and increased viral replication. In these studies, X4 and R5 isogenic strains were used to infect human lymphoid tissue cultures (17, 20, 44). These cultures contain various cell types in addition to CD4+ T cells, and require no exogenous stimulation to confer susceptibility to HIV-1 infection. Infection with R5 and X4 HIV-1 isogenic strains resulted in similar amounts of viral production in the lymphoid cultures, despite the presence of fewer R5-infected cells (17, 20, 44). The results of these studies support the conclusion that R5 HIV-1 has a higher replication capacity than X4 HIV-1.

We propose that the increased replication capacity of R5 strains may contribute to the R5 dominance of early HIV-1 infection. R5 viruses have the selective advantage of targeting the more activated CD4+ T cells that express higher levels of transcription factors, such as NFκB, which have been linked to increased HIV LTR promoter activity (36). This may lead to higher viral production levels and preferential spread of R5 viruses *in vivo*. In addition, X4 viral strains are often associated with increased cytopathicity, which may lead to a differential life span of X4- and R5-infected CD4+ T cells. As a result, R5-infected CD4+ T cells may live longer and release more virus over time than X4-infected CD4+ T cells.

It has been suggested that R5-infected CD4+ T cells may be less susceptible to immune mediated killing than X4-infected CD4+ T cells. Experiments performed in the macaque model have shown that both X4 and R5 SHIV replication can be detected early after co-infection, however R5 dominance develops within weeks of the initial infection (22). Interestingly, experimental depletion of CD8+ T cells in these animals results in re-emergence of X4 replication, suggesting that the CD8-mediated immune response is more effective at eliminating X4-infected cells (22). This may partially be due to compartmentalization of X4- and R5- infected cells. X4 viruses are more likely to infect circulating CD4+ T cells, which may be more accessible to CD8+ T cell surveillance than R5-infected activated CD4+ T cells and macrophages that are located deep within tissues. The idea that X4-infected cells are more susceptible to immune-mediated elimination suggests that the emergence of X4 strains in the later stages of disease may be the result of immune exhaustion.



In summary, we present evidence that R5 HIV-1 strains replicate more efficiently in CD3/CD28 co-stimulated CD4+ T cells than X4 HIV-1 strains. We found that non-cytopathic R5 HIV-1 has a greater capacity to replicate in CD4+ T cells than cytopathic X4 strains. In addition, we further analyzed the impact of co-receptor usage on viral production by comparing X4 and R5 viruses that share greater homology and induce similar cytopathic effects. These experiments have provided evidence

that R5-infected CD4+ T cells produce more virus over time than X4-infected CD4+ T cells. We suggest that this replication advantage may contribute to the preferential spread of R5 viruses during the early stages of HIV-1 infection.

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Figures

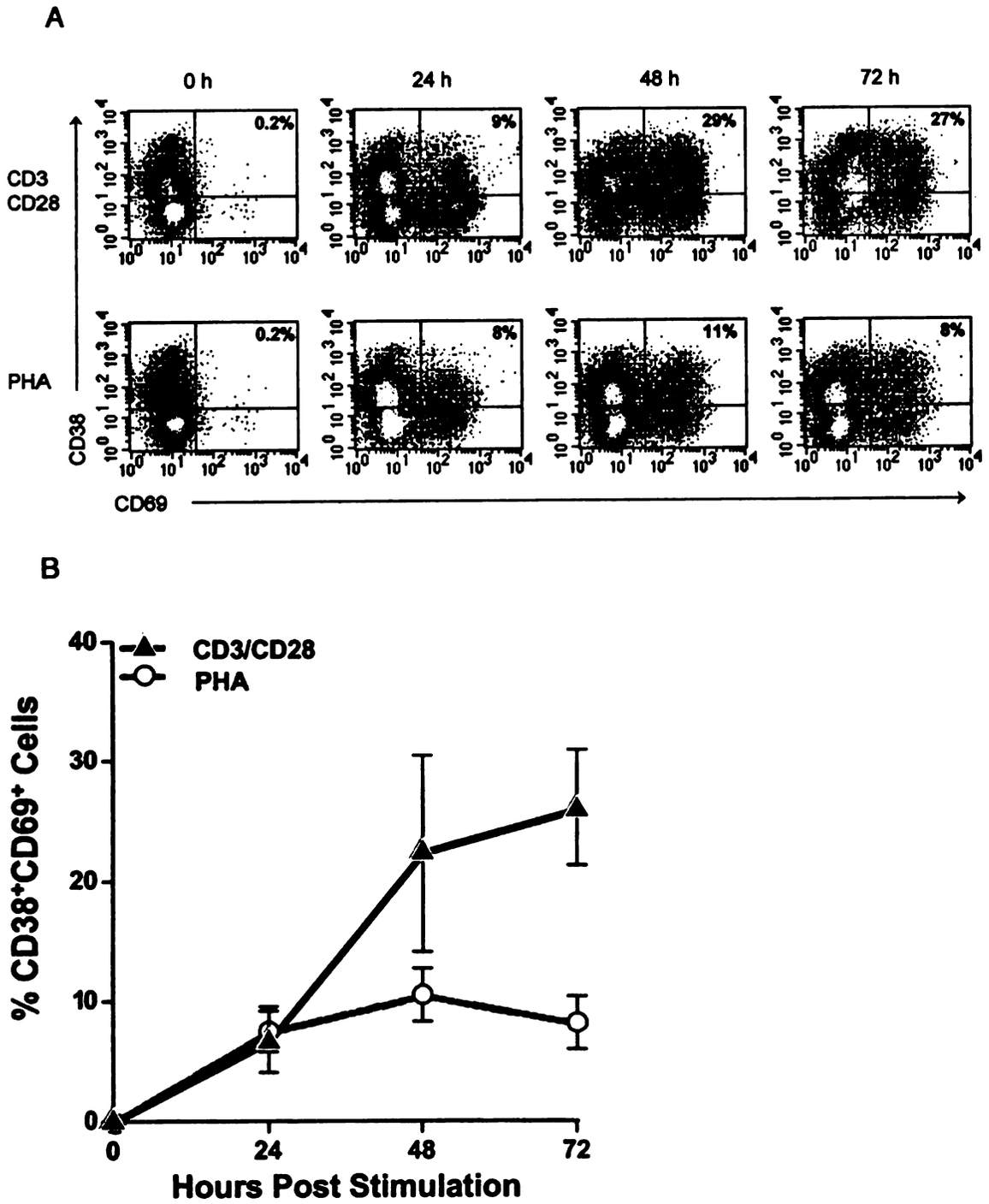


Figure 1. CD3/CD28 co-stimulation induces high levels of activation

marker expression on CD4+ T cells. CD4+ T cells were isolated from PBMC of 3 donors, and then stimulated with CD3/CD28 antibodies or PHA for 0, 24, 48, or 72hrs. At each time point, cells were stained with fluorescently conjugated monoclonal antibodies directed against CD4 and the activation markers CD38 and CD69. Samples were acquired on a FACS CaliburTM (Becton Dickinson), and the resulting data were analyzed using CellQuestTM (Becton Dickinson) software. (A) Representative flow cytometric results from one donor are shown in panel A. The number in the upper-right hand corner of each FACS plot represents the percentage of CD38+CD69+ co-expressing cells. (B) The percentage of CD38+CD69+ co-expressing cells detected in each donor was averaged and plotted as a line graph, with error bars representing the variation among donors.

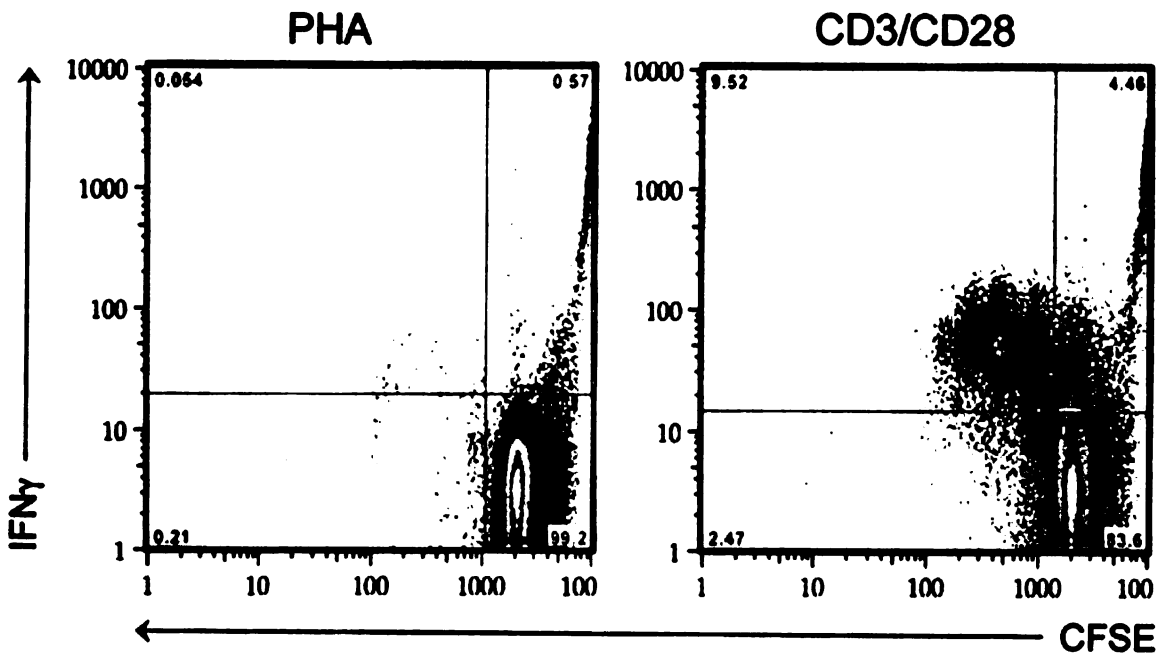


Fig. 2. CD3/CD28 co-stimulation induces high levels of cellular proliferation and IFNγ expression in CD4+ T cell cultures. Stimulated CD4+ T cells were stained with CFSE and then cultured for 72 hours. Cells were then fixed, permeabilized, and stained with fluorescently labeled monoclonal antibodies directed against CD3, CD4, and IFNγ. Samples were acquired on a FACS CaliburTM (Becton Dickinson), and the resulting data were analyzed using FlowJo SoftwareTM (Tree Star, Inc.). Numbers in the corner of each FACS plot represent the percentage of cells in that quadrant.

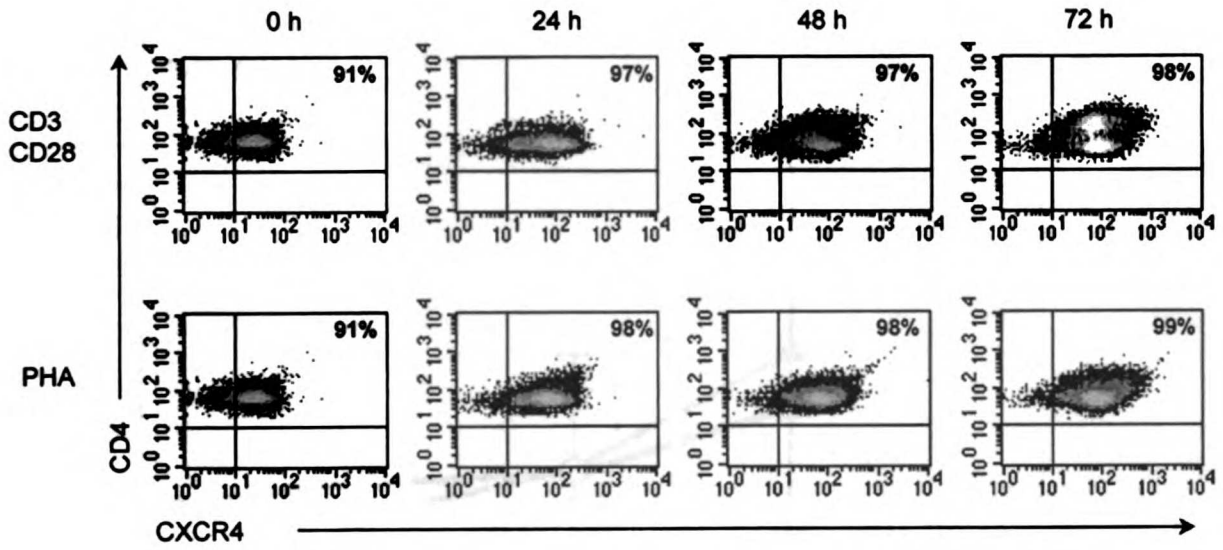


Figure 3. CD3/CD28 co-stimulated CD4+ T cells express high levels of CXCR4. CD4+ T cells were isolated from PBMC of 3 donors, and then stimulated with CD3/CD28 antibodies or PHA for 0, 24, 48, or 72hrs. At each time point, cells were stained with fluorescently conjugated monoclonal antibodies directed against CD4 and CXCR4. Samples were acquired on a FACS CaliburTM (Becton Dickinson), and the resulting data were analyzed using CellQuestTM (Becton Dickinson) software. Representative results from one donor are shown. The number in the upper-right hand corner of each FACS plot represents the percentage of cells that express both CD4 and CXCR4.

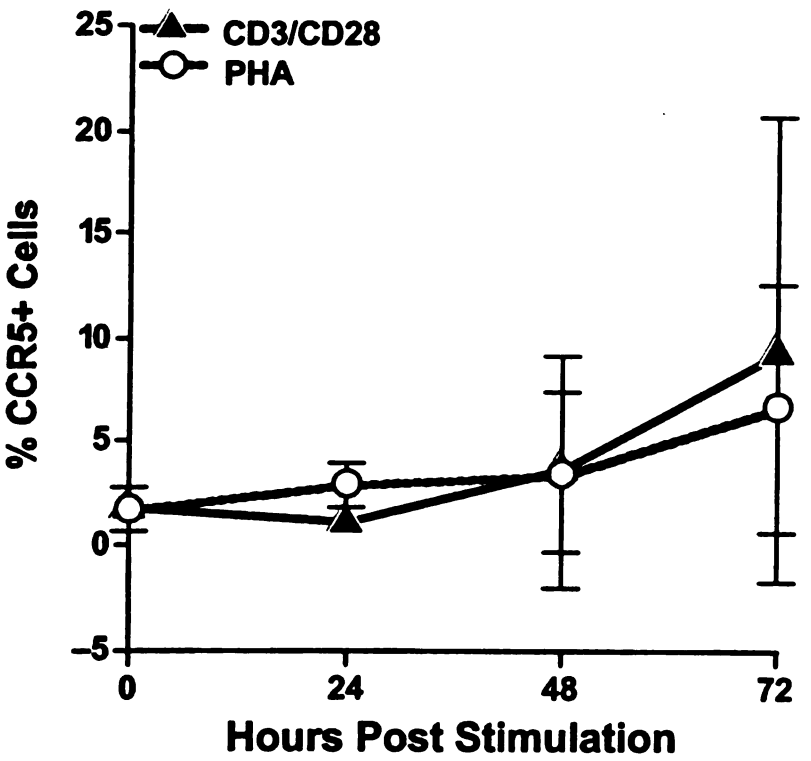


Figure 4. CD3/CD28 co-stimulated CD4+ T cells express low levels of CCR5. CD4+ T cells were isolated from PBMC of 3 donors, and then stimulated with CD3/CD28 antibodies or PHA for 0, 24, 48, or 72hrs. At each time point, cells were stained with fluorescently conjugated monoclonal antibodies directed against CD4 and CCR5. Samples were acquired on a FACS CaliburTM (Becton Dickinson), and the resulting data were analyzed using CellQuestTM (Becton Dickinson) software. The average CCR5 expression of all 3 donors was plotted as a line graph, with error bars representing the standard deviation among donors.

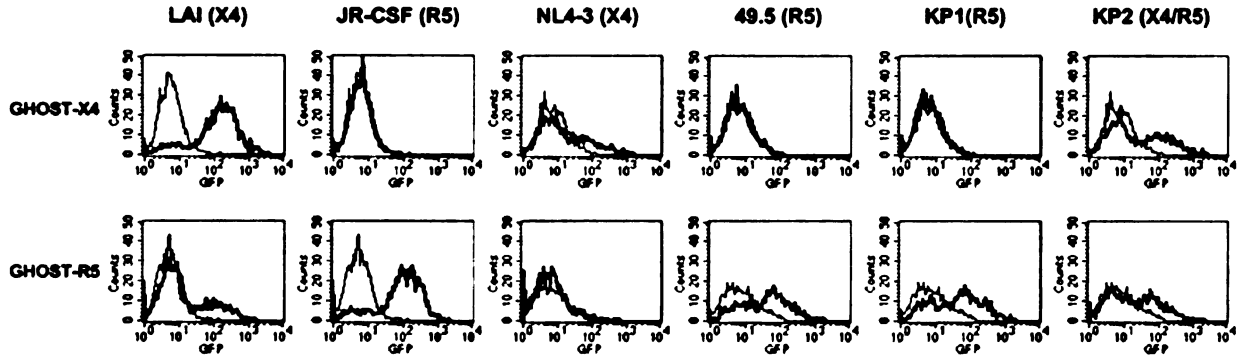


Figure 5. Co-receptor usage of each HIV-1 strain was determined by GHOST cell assay. The GHOST-X4 and GHOST-Hi5 cells were infected with virus for 48hrs prior to measurement of GFP expression by flow cytometry. Samples were acquired on a FACS CaliburTM (Becton Dickinson), and the resulting data were analyzed using CellQuestTM (Becton Dickinson) software. The top row of FACS plots represents GHOST-X4 infections, and the bottom row represents GHOST-Hi5 infections. The thin-lined peak in each plot represents the background fluorescence of uninfected cells, and the bold-lined peak represents the GFP fluorescence detected in cells infected with the indicated HIV-1 strain. The HIV-1 strains LAI and NL4-3 utilize the X4 co-receptor, the strains JR-CSF, 49.5, and KP1 utilize the R5 co-receptor, and the KP2 strain utilizes both the X4 and R5 co-receptors.

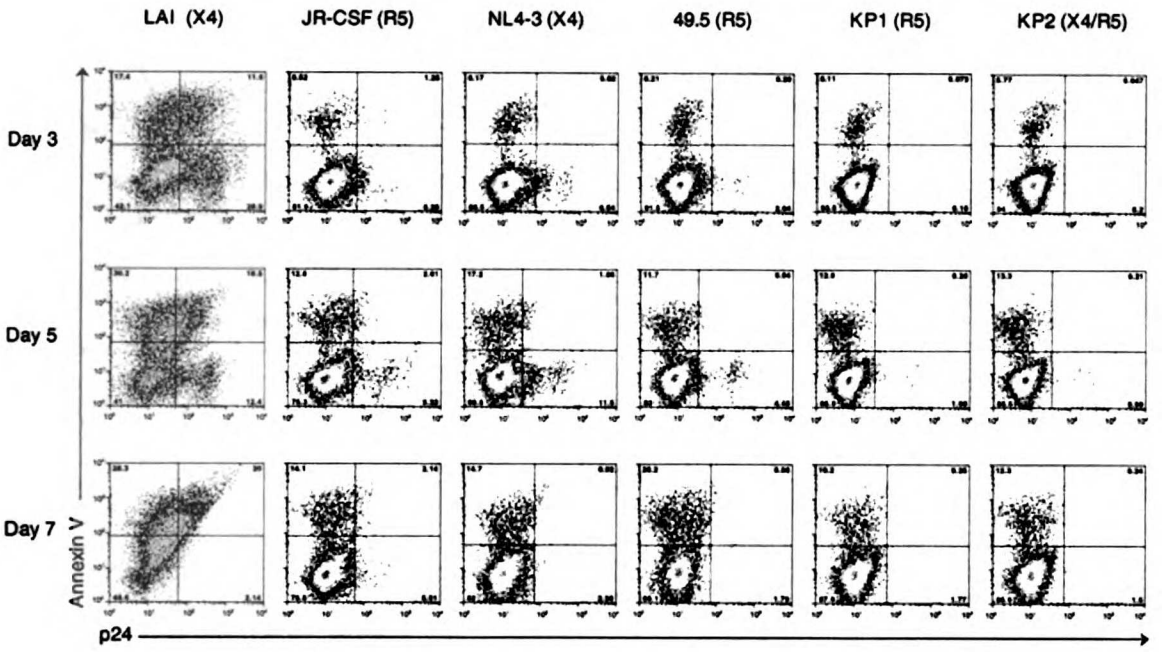


Figure 6. The percentage of co-stimulated CD4+ T cells infected by each viral strain was determined by intracellular p24 staining. CD3/CD28 co-stimulated CD4+ cells were infected with LAI, JRCSF, NL4-3, and 49.5 at an moi of 0.01, and infected with KP1 and KP2 at an moi of 0.001. Apoptotic cells were detected by surface staining with fluorescently conjugated Annexin-V, and infected cells were detected by intracellular staining for HIV-1 p24 antigen. Samples were acquired on a FACS CaliburTM (Becton Dickinson), and the resulting data were analyzed using FlowJo SoftwareTM (Tree Star, Inc.). Results from days 3, 5, and 7 days post-infection are shown. Numbers in the corner of each FACS plot represent the percentage of cells in that quadrant.

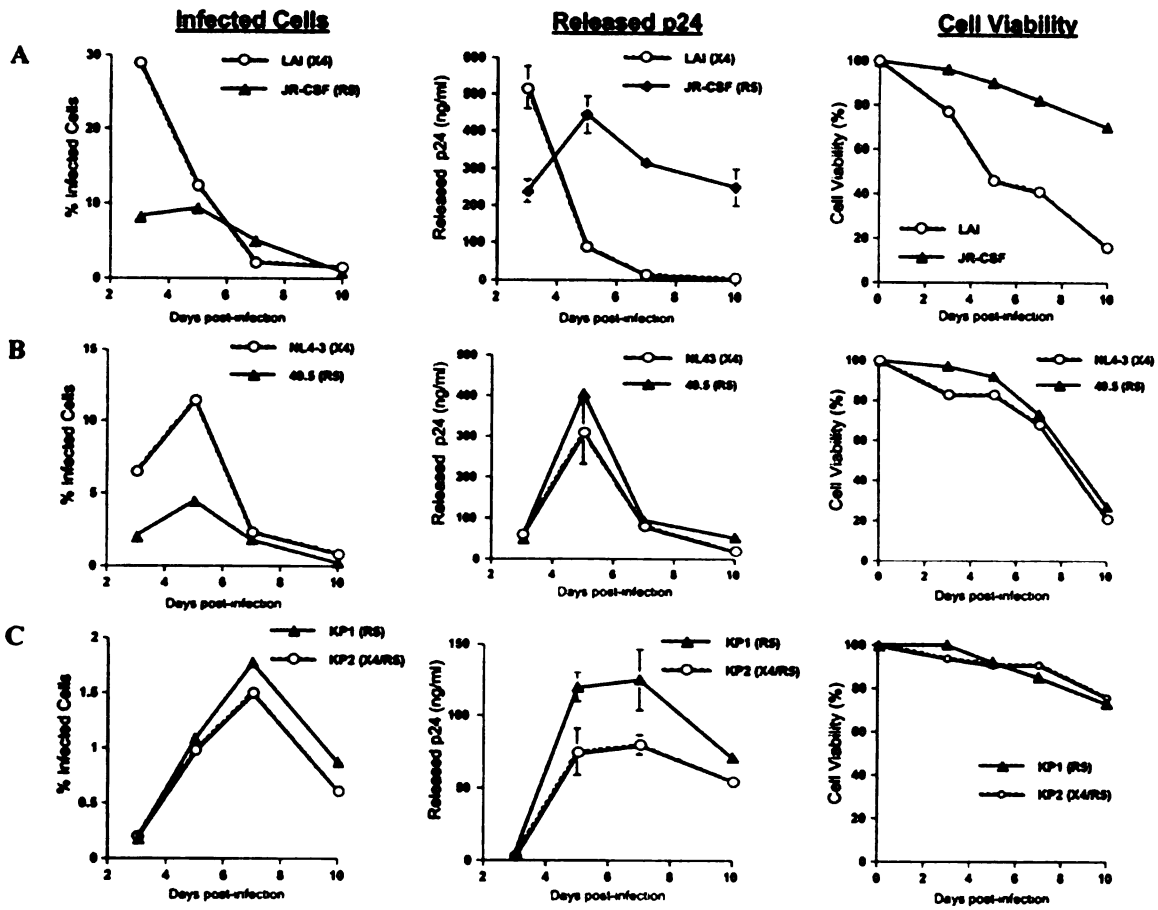
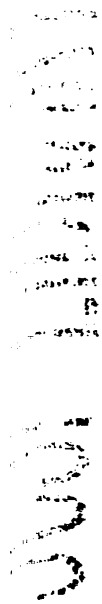


Figure 7. R5-infected CD4+ T cells produce more progeny virus over time than X4-infected CD4+ T cells. CD4+ T cells were isolated from PBMC and then stimulated for 72 hrs with CD3/CD28 antibodies prior to HIV-1 infection. At 3, 5, 7, and 10 days post-infection, the percentage of infected cells in each culture was determined by intracellular p24 staining and flow cytometry, the amount of virus released from each culture was measured by p24 ELISA, and the cell viability of each culture was determined by trypan blue exclusion assay. **(A)** Comparison of the percentage of infected cells, viral release, and cell viability in co-

stimulated CD4+ T cell cultures infected with the HIV-1 molecular clones LAI (X4) and JR-CSF (R5). **(B)** Comparison of the percentage of infected cells, viral release, and cell viability in co-stimulated CD4+ T cell cultures infected with the isogenic HIV-1 strains NL4-3 (X4) and 49.5 (R5). **(C)** Comparison of the percentage of infected cells, viral release, and cell viability in co-stimulated CD4+ T cell cultures infected with the primary HIV-1 isolates KP1 (R5) and KP2 (X4/R5).



Chapter 3

Co-receptor Preference in Secondary Lymphoid Tissue

Enhanced Replication of R5 HIV-1 over X4 HIV-1 in CD4+CCR5+CXCR4+ T Cells

Ann-Marie Roy, Becky Schweighardt, Lauren A. Eckstein, Mark A. Goldsmith, and Joseph M. McCune

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Abstract

To enter human cells, human immunodeficiency virus type 1 (HIV-1) usually uses CD4 and one of two co-receptors, CCR5 and CXCR4. Interestingly, even though CCR5 is expressed on far fewer T cells than is CXCR4, many patients in both early and late-stage HIV disease maintain high levels of CCR5-tropic (R5) viruses. We hypothesized that such high R5 viral loads may be sustained because, relative to CXCR4-tropic (X4) HIV-1 infection, R5 HIV-1 infection of permissive CD4+ CCR5+ CXCR4+ T cells results in the production of significantly more infectious virus particles per target cell. To investigate this possibility, we compared the levels of virus production per target cell following isogenic R5 and X4 HIV-1 infection of two *in vitro* primary human lymphocyte culture systems: T-cell receptor stimulated, blood-derived CD4+ T cells and tonsil histoculture (which requires no exogenous stimulation for *ex vivo* infection). We provide evidence that R5 HIV-1 does indeed compensate for a small target cell population by producing, on average, 5-10 times more infectious virus per CCR5+ target cell than X4 HIV-1. This replicative advantage may contribute to the predominance of R5 HIV-1 *in vivo*.

Introduction

The disease course induced by the human immunodeficiency virus type 1 (HIV-1) typically passes through three distinct phases. The first phase, lasting for a period of weeks to several months and associated with flu-like symptoms and CD4+ T cell depletion, is often dominated by "R5" HIV-1 variants that utilize the chemokine co-receptor CCR5 in addition to CD4 for entry into target cells (12, 15, 25). Following the generation of a virus-specific immune response, levels of virus replication recede, CD4 counts stabilize, and a period (variably lasting 4 or more years) of relative clinical quiescence ensues (32). Finally, in later stages of disease, levels of viral replication rebound, associated with quantitative and qualitative changes in the CD4+ and CD8+ T cell compartments, immunodeficiency, and mortality due to opportunistic infections (44). In approximately 50% of infected individuals, this last phase is marked by the emergence of "X4" HIV-1 variants that utilize the chemokine co-receptor CXCR4 in addition to CD4 for target cell entry (11, 14, 18). Since X4 variants have an increased propensity to induce cytopathicity *in vitro* and a larger target pool size *in vivo*, their emergence might be responsible for accelerated disease progression (7, 21, 31, 36, 39). Yet, 40-50% of those infected with B clade virus progress to AIDS with R5 variants alone (10, 40, 47).

Several hypotheses have been advanced to explain the intrinsic

pathogenicity of R5 variants of HIV-1. For instance, some studies suggest that R5 HIV-1 is selectively transported across mucosal epithelial barriers (30). Studies in primate models, however, have shown that although both R5 HIV-1 and X4 HIV-1 can be efficiently transmitted through vaginal infection, X4 variants eventually disappear and R5 HIV-1 strains disseminate throughout the body (24). This observation is more consistent with the possibility that R5 HIV-1 may possess an advantage in replication and/or spread after target cell infection, perhaps a function of the differential representation of CCR5+ macrophages and dendritic cells at these interfaces (22, 30, 35). Evidence that longer-lived cells such as macrophages are capable of producing greater amounts of virus than their CD4+ T cell counterparts has been provided by several recent studies (17, 26, 27). In one such study utilizing human lymphoid histoculture (HLH), donor tissues were infected with an isogenic pair of HIV-1 strains differing only in co-receptor preference. Despite the fact that 5-10 times more CD3+ cells were infected with X4 HIV-1 than R5 HIV-1, the X4 and R5 HIV-1 infected cultures released approximately equivalent amounts of HIV-1 Gag protein (detected as p24) over time (23). Since macrophages are abundant in HLH, the high output of R5 HIV-1 was attributed to production by infected macrophages that are not susceptible to X4 HIV-1. This explanation was supported by later work in the same system (17), examining isolates of HIV-1 that were defective in Vpr. Vpr is an HIV-1 protein required for replication in non-dividing cells

such as macrophages; Vpr-deficient R5 HIV-1 strains showed a 50% reduction in p24 production while the absence of Vpr had no significant impact on X4 HIV-1 infection, demonstrating that macrophage infection contributes a significant amount of the virus generated after R5 HIV-1 infection of lymphoid tissue. However, this study did not rule out the possibility that the surprisingly high viral output of R5 HIV-1 is partially due to an advantage in CCR5+CXCR4+ T cells.

Initially, we tested this hypothesis by isolating CD4+ T cells from peripheral blood of healthy donors and rendering them susceptible to HIV-1 infection via stimulation with antibodies against CD3 and CD28. This study demonstrated that such cultures produced more virus over the course of a 10 day *in vitro* infection (as measured by p24 production in the supernatant) when infected by R5 as compared to X4 HIV-1, a difference in virus production that did not appear to be the result of enhanced X4 HIV-1 cytopathicity *in vitro* (41). We have extended these observations in the current study, using *ex vivo* lymphoid histoculture and a quantitative approach that assesses not simply p24 production but also the production of infectious virions. We directly addressed the possibility that CD4+CCR5+CXCR4+ T cells may produce a larger number of virions per infected cell after R5 HIV-1 infection than after X4 HIV-1 infection. We show here that infection with R5 HIV-1 does in fact result in the generation of more progeny per infected CD4+ T cell than infection with

Materials and Methods

Antibodies

For analysis and sorting of cells by flow cytometry as well as for stimulation of cells in tissue culture, commercially-available monoclonal antibodies were used (diluted in PBS with 1% FBS), including: CD3 PerCP at 1:10 (BD Immunocytometry), CD4 APC at 1:10 (BD PharMingen), CD8 PE at 1:10 for analysis, 1:20 for cell sorting (BD Immunocytometry), CCR5 FITC at 1:5 or 1:7.5 (BD PharMingen), CXCR4 APC at 1:10 (BD PharMingen), CD25 PE at 1:10 (BD PharMingen), CD69 APC at 1:10 (BD PharMingen), p24 PE at 1:100 (Beckman Coulter), anti-FITC rabbit IgG, Alexa 488 conjugate at 2.5 μg/10^6 cells (Molecular Probes), goat anti-rabbit IgG, Alexa 488 conjugate at 5 μg/10^6 cells (Molecular Probes), CD14 PE at 1:20 (BD Immunocytometry), CD19 PE at 1:20 (BD Immunocytometry), CD3 pure at 50 μg/ml (Zymed), and CD28 pure at 100 μg/ml (BD Biosciences).

Virus Preparation

Virus stocks of the molecular clones NL4-3 (gift from Malcolm Martin via the NIH AIDS Research and Reference Reagent Program), 49-5, and 81A (gifts from Bruce Chesebro) were derived from plasmid transfection of 293T cells. 49-5 and 81A are viruses that are isogenic to NL4-3 except for determinants in the V1, V2, and V3 regions which specify co-receptor

preference (1, 9, 42). Specifically, 81A substitutes the V1-V3 loops of BaL into the NL4-3 backbone, while 49-5 incorporates only the V3 region of BaL into NL4-3. Transfection was performed using the calcium phosphate method. Virus-containing supernatants were harvested at 72 and 120 hours post transfection, sterile filtered, and titered to determine a "tissue culture infectious dose-50" ($TCID_{50}$). Briefly, peripheral blood mononuclear cells (PBMC) from human blood buffy coats (Stanford Blood Bank) were separated by ficoll separation (see below) and stimulated with phytohemaglutinin (PHA). The cells were then brought to a concentration of 2×10^6 cells/ml in RPMI 1640 medium (Mediatech-Cellgro) supplemented with 10% heat inactivated (HI) FBS (Gemini Bioproducts), 2 µg/ml PHA (Sigma), 1% penicillin/streptomycin (Mediatech-Cellgro), and 1% L-glutamine (Mediatech-Cellgro). After 24 hours, the cells were rinsed in fresh media without PHA and cultured in RPMI 1640 with 10% HI FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 5 units/ml human recombinant IL-2 (Boehringer Mannheim) until use in the $TCID_{50}$ assay. To perform the $TCID_{50}$ assay itself, serial half-log dilutions of virus supernatants were prepared in IL-2-containing RPMI, and 25 µl of each dilution was added to quadruplicate wells of PHA-stimulated PBMCs (3 donor pool) at $1x10^5$ cells/well in a U-bottom 96 well culture plate (Corning). After incubation for 2 hours at 37°C, 200 µl of IL-2 containing culture media were added to each well and the plates were incubated at

37°C for 5 days. The $TCID_{50}$ is the reciprocal of the dilution at which 50% of the wells contained detectable (>30 pg/ml) p24 (capsid) protein by ELISA (NEN Perkin-Elmer)

Cell Culture

293T cells for virus preparation were cultured in DMEM (Mediatech-Cellgro) supplemented with 10% HI FBS and penicillin/streptomycin, passaging every 3-4 days. Human tonsil tissue (from the National Disease Research Institutes, Cooperative Health Tissue Network, Kaiser San Rafael, Kaiser South San Francisco, and Kaiser San Francisco) was obtained with approval from the University of California, San Francisco Committee on Human Research and was processed into human lymphoid aggregate culture (HLAC), as previously described (16). Briefly, fresh tonsil tissue from routine tonsillectomies was fully dispersed to create a single cell suspension in RPMI media (supplements detailed below). The cells were then counted and plated at a concentration of $2x10^6$ cells per well in a 96 well U-bottom culture plate. The cells settled naturally to form high density aggregates at the base of each well. HLAC cultures were maintained in RPMI medium supplemented with 10% FBS, 1X non-essential amino acids (Mediatech-Cellgro), 100 mM sodium pyruvate (Mediatech-Cellgro), 10 µg/ml gentamicin (Gibco Invitrogen), 100 ng/ml ampicillin (Sigma), and 250 µg/ml amphotericin B (Mediatech-Cellgro). PHA-stimulated PBMC cultures were prepared as described above and

maintained in RPMI medium supplemented with 10% FBS, penicillin/streptomycin, and 5 units/ml IL-2 (Boehringer Mannheim). CD4+ T cells isolated from PBMC were maintained following stimulation in RPMI supplemented with 10% FBS and penicillin/streptomycin.

Virus Infections

All HIV-1 infections were carried out at low multiplicity of infection (m.o.i. = 0.001) in a designated BSL-3 facility. Target cell cultures were incubated overnight (12 to 16 hours) with virus-containing media, which was then replaced with fresh media. Infections were monitored via FACS analysis of intracellular p24 each day after day 4 and infected cultures were manipulated as described for individual experiments.

Assessment of Infection Kinetics in R5 vs. X4 HIV-1

HLAC cultures were infected with NL4-3 or 81A at an m.o.i. of 0.001 on day 0 and incubated at 37°C, 5% CO_2. On day 4, samples were removed and stained for surface expression of CD3, CD4, and CD8 as well as for intracellular expression of p24. Samples were fixed in 1% paraformaldehyde (Sigma)/PBS (Mediatech-Cellgro) and permeabilized in 0.1% Triton X-100 (Sigma)/PBS to permit intracellular staining. Supernatants from the same wells were harvested and assessed for p24 content by p24 ELISA analysis. All samples were assessed in triplicate. This procedure was repeated until day 8-9, depending on culture viability.

Infection kinetic experiments in PBMC-derived CD4+ T cells were performed using the same methods.

Flow Cytometric Sorting of CD4+ T Cell Subsets From HLAC and PBMC

On day 5-7 of HIV-1 infection in HLAC culture, 360 x 10^6 infected cells per infection condition were harvested and rinsed in PBS/1% FBS (FACS buffer). Cultures were then re-suspended in FACS buffer containing CCR5 FITC (1:7.5), CD8 PE, CD14 PE, and CD19 PE (each 1:20) antibodies and incubated at °C for 30 minutes in the dark. The cells were rinsed in a large volume of FACS buffer with 2 mM EDTA (KD Medical), re-suspended in FACS buffer with EDTA containing anti-PE microbeads at 1:5 (Miltenyi Biotec), and incubated at 4°C for 30 minutes in the dark. Cells were then rinsed in FACS buffer, re-suspended in 4 ml FACS buffer with 2mM EDTA, and passed through a MACS magnetic bead sorter (Miltenyi Biotec) to remove PE-labeled cells. The negative fraction, consisting primarily of CD4+ T cells, was retained and the positive fraction was discarded. The enriched CD4+ T cells were re-suspended in FACS buffer containing 2.5 µg/10^6 cells rabbit anti-FITC IgG-Alexa 488 and incubated at 4°C for 30 minutes (in the dark). After rinsing, the cells were re-suspended in FACS buffer containing 5 µg/10^6 cells goat anti-rabbit IgG-Alexa 488 for an

additional 30 minutes under the same conditions. These final two staining steps resulted in significant amplification of anti-CCR5 fluorescence (data not shown). The cells were then rinsed for a final time, re-suspended in 4 ml FACS buffer, passed through a 5 ml polystyrene tube with cell strainer cap (Falcon), and sorted into FITC/Alexa 488+ CCR5+ and FITC/Alexa 488- CCR5- populations on a FACS Vantage (Becton Dickinson), gating on the live lymphocyte population by forward and side scatter, and gating out any remaining PE positive events to electronically eliminate residual cells positive for CD8, CD14, or CD19. The cells were sorted into 15 ml Falcon tubes (Fisher) containing 1 ml FBS, and the number of FITC/Alexa 488 positive and negative events recorded for future calculations. Sorted cells were then rinsed in preparation for further manipulations to determine the average viral output per infected cell (see below). PBMC-derived CD4+ T cells were subjected to the same procedure, with the elimination of steps needed for removal of CD8, CD14, and CD19 positive cell types, which were performed prior to infection (see below).

PBMC Derived CD4+ T Cell Isolation and Stimulation

Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte/buffy coats (Stanford Blood Bank) by ficoll (Sigma) separation. Briefly, 30 ml of buffy coat were diluted 1:5 in sterile PBS and underlayed with 14 ml ficoll per 30 ml diluted cells in 50 ml Falcon tubes (Fisher). The tubes were spun at 1400 rpm in a Beckman GS-6R centrifuge for 30

minutes at room temperature with centrifuge brake disengaged. The upper layer of liquid was removed and the PBMC layer set aside. When needed, 10 ml ACK lysis buffer (Quality Biological) was added to remove erythrocytes, incubating at room temperature for 5 minutes. The cells were rinsed three times in large volumes of PBS and counted on a hemocytometer. CD4+ T cells were negatively selected using a CD4+ T cell isolation kit (Miltenyi Biotec), as per the manufacturer's instructions, in combination with LS Columns for Midi MACS (Miltenyi Biotec) and the Midi MACS Separation Unit (Miltenyi Biotec). This method typically yielded CD4+ T cells with greater than 95% purity. These cells were then stimulated with 50 µg/ml plate-bound purified anti-CD3 (Zymed) and 100 µg/ml soluble purified anti-CD28 (BD Biosciences) antibodies for 72 hours at 37°C (6, 8, 37, 41), at which time they were rinsed and plated in 96 well U bottom plates at 5×10^5 cells per ml. These cells were then infected in the same manner as HLAC cultures and manipulated as required for individual experiments.

Determination of Average Viral Output per Infected Cell

Following isolation of CCR5+ and CCR5- CD4+ T cells, one third of the total was assessed for the presence of intracellular p24 by p24 ELISA assay. The remaining cells were cultured at 37°C for 36 hours after which the supernatant was harvested and assessed for the presence of p24 by ELISA and for infectious virus by $TCID_{50}$ assay. These values were then

used, in combination with the percent of CD4+CCR5+ and CD4+CCR5- cells infected at the time of sorting (as determined by FACS analysis of intracellular p24) and the absolute number of cells in each sorted population (corrected for the fraction of cells used to assess viral production in each case) to determine the average output of viral protein/infectious virus per infected cell, according to the following equation:

Average virus production per cell =

ng intracellular p24 or p24 produced or $TCID_{50}$s produced/number of infected cells in each subset

The number of infected cells in each subset = [absolute number of sorted cells in each subset (CD4+CCR5+ or CD4+CCR5-)] x (% of infected cells in each subset, as determined by FACS analysis for intracellular p24) x (fraction of total sorted cells used to assess each measure of viral production, i.e., 0.33 for intracellular p24 and 0.67 for supernatant p24 and $TCID_{50}$)

Results

High Levels of R5 HIV-1 p24 Production in Human Lymphoid Aggregate Cultures

Previous experiments revealed equivalent levels of R5 and X4 HIV-1 production after infection of intact blocks of tonsillar tissue in human lymphoid histoculture (HLH) (20, 23). We further examined these observations in the context of an alternative culture system, the human lymphoid aggregate culture (HLAC) (16). In the preparation of such cultures, a fixed number ($2x10^6$) of dispersed tonsillar cells was plated in each well of 96 well U bottom plates and allowed to settle into a high density aggregate. Thereafter, and in contradistinction to other *in vitro* systems, HIV-1 replication proceeded in the absence of exogenous stimulation. This *in vitro* culture system has two advantages over HLH: first, the number of cells placed into each well is known; secondly, all wells are equivalent. A potential disadvantage of HLAC is that some cells (e.g., CD4+ myeloid cells) that are contained in HLH are only poorly represented after transfer of dispersed tonsillar cells into the 96-well plate (data not shown). Such selective loss of macrophage populations was advantageous in the case of these experiments, permitting focused attention on HIV-1 interactions with CD4+ T cells.

HLAC cultures were infected in triplicate with equivalent inputs (0.001 m.o.i.) of either X4 (NL4-3) or R5 (81A) HIV-1. These viruses are identical except for determinants in the V1-V3 loops that confer differential tropism for CXCR4 or CCR5, respectively. On each day after infection (day 4 through day 8-9), culture supernatants were harvested and the amount of p24 (HIV-1 capsid protein) produced was measured by p24 ELISA. Concomitantly, cells from the infected cultures were permeabilized and stained with antibodies against HIV-1 p24 to determine the percentage of infected cells. As shown by example of two representative donors (n=19), HIV-1 infection of HLAC resulted in a time-dependent increase in supernatant p24 (Fig. 1). In some cases, represented by Donor 1 (Fig. 1A, left panel), more p24 was produced after R5 HIV-1 than after X4 HIV-1 infection; in other cases, represented by Donor 2 (Fig 1B, left panel), the opposite was observed. In all cases, however, the percentage of p24+ cells after R5 HIV-1 infection was much smaller, on the range of 1 to 15% at day 8-9 than that generated after X4 HIV-1 challenge, 30 to 40% at the same time point (Figs. 1A and B, middle panels). When supernatant p24 levels were normalized for the percent of cells infected, the average amount of virus (p24) produced after R5 HIV-1 infection was 5-10 fold higher than that observed after X4 HIV-1 infection (Figs. 1A and B, right panels).

This apparent disparity in virus production might be due to a virus-

intrinsic property, i.e., a given target cell might produce more p24 after infection with R5 than X4 HIV-1. Alternatively, it might be due to a cell intrinsic property, i.e., R5 and X4 viruses infect different target cells, and those cells infected with R5 HIV-1 are more efficient in replicating and/or releasing HIV-1. In either case, it is also possible that the ratio of replication competent/defective virus released into the supernatant is non-identical after R5 or X4 infection.

The Apparent Replicative Advantage of R5 HIV-1 Is Virus Intrinsic

To discriminate between the above possibilities, the amount of infectious virus made per cell after R5 or X4 infection was calculated more precisely. In HLAC cultures, 80-100% of CD3+CD4+ T cells express CXCR4 while 5-15% express CCR5; notably, nearly all CD3+CD4+CCR5+ T cells are also CXCR4+ and therefore permissive for entry by R5 and X4 HIV-1(Fig. 2A). To determine whether such CD4+CCR5+CXCR4+ T cells might produce more virus after R5 than after X4 HIV-1 infection, HLAC cultures were infected with equivalent amounts (0.001 m.o.i.) of NL4-3 (X4) or the isogenic isolate 49-5 (R5). At an early time point post-infection (day 5-6), before noticeable levels of cell death in infected cultures (as assessed by CD4/CD8 ratio in infected as compared to mock-infected cultures), a small portion of each was analyzed by intracellular p24 staining to determine the percentage of infected (p24+) cells that were CD4+CCR5+

and CD4+CCR5- (Fig. 2B). The remaining cells were enriched for CD4+ T cells (by bead depletion of cells expressing CD8, CD14, or CD19) and then sort-purified into subpopulations that were negative for these lineage markers and were either CD4+CCR5+ or CD4+CCR5- (Fig. 2C). The sorted populations were divided into two portions. One third was lysed immediately to quantitate p24 content (via p24 ELISA). The other two-thirds were cultured *in vitro* for an additional 1.5 days so that viral replication could continue. At the end of this time, supernatants were harvested and assessed for p24 production (by ELISA) and for infectious virus release (by $TCID_{50}$ assay).

The average amount of virus made per cell was calculated by relating the above three values [intracellular p24 at the time of sort purification and the amount of p24 and of $TCID_{50}$ made during the 1.5 day culture period) to the absolute number of infected cells (determined by multiplying the percentage of p24+ cells (CD4+CCR5+ or CD4+CCR5-) times the absolute number of sort-purified cells in each subset]. As shown in Fig. 3, significant variability was observed between donors in the absolute amount of p24 made per infected cell. However, HLAC cultures prepared from multiple independent donors (n=9) revealed two consistent qualitative patterns. First, after X4 HIV-1 infection, the average amount of p24 and $TCID_{50}$s produced per CD4+CCR5+ cell was greater (by 1 to 100 fold) than the average amount made per CD4+CCR5- cell. Secondly,

and more strikingly, the average amount of p24 and $TCID_{50}$s made per CD4+CCR5+ cell was 5-10 fold higher after R5 HIV-1 infection than after X4 HIV-1 infection.

The Replicative Advantage of R5 HIV-1 Is Observed In Activated CD4+ T Cells

Although HLAC cultures are compromised primarily of T cells, myeloid cells permissive for HIV-1 infection may also be present. To exclude measurement of viral production from such cells, we extended the above analysis to *in vitro* culture conditions designed to preferentially stimulate and expand T cells in a purified culture. CD4+ T cells were purified from PBMC and incubated with plate-bound monoclonal antibodies against CD3 and soluble antibodies against CD28. As a function of time thereafter, CD4+ T cells in these cultures showed evidence of stimulation, expressing CXCR4 (98%), CCR5 (10%), and a number of activation markers (e.g., 29% were positive for CD38+ and CD69+) (41). A comparable level of activation was seen in HLAC, i.e., 20% of CD4+T cells in HLAC were CD69+ (data not shown). After 72 hours of stimulation, the cultures were thoroughly rinsed and then infected with either X4 HIV-1 (NL4-3) or an R5 isolate (49-5) isogenic in the V3 loop, at an m.o.i. of 0.001. The average amount of virus made per infected cell was then calculated using the approach used previously for HLAC. CD4+ T cells stimulated with anti-CD3 and anti-CD28 were harvested 6-7 days after infection with X4 (NL4-

3) or R5 (49-5) HIV-1, stained with antibodies against CCR5, and sorted into CD4+CCR5+ and CD4+CCR5- populations. The sorted populations were assessed for the average amount of intracellular p24 at the time of sort-purification and for the amount of p24 and $TCID_{50}$s produced during 1.5 days of further culture *in vitro*. As shown in Fig. 4A-C for two independent donors, CD4+CCR5+CXCR4+ T cells infected with R5 HIV-1 produced an average of 5-fold more p24 and 5-fold more $TCID_{50}$s per infected cell than CD4+CCR5+CXCR4+ T cells infected with X4 HIV-1.

DISCUSSION

We have recently reported (41) that non-cytopathic R5 HIV-1 strains are able to replicate more efficiently in co-stimulated CD4+ T cell cultures than cytopathic X4 HIV-1 strains over the course of a 10 day infection and previous studies described similar observations in HLAC (23). Here, we have examined this issue more closely, analyzing and comparing the relative replicative capacity of X4 and R5 HIV-1 within human CD4+CCR5+CXCR4+ T cells that should be permissive for infection by each type of virus. Two different culture conditions were used: un-stimulated human lymphoid aggregate culture and human peripheral blood derived CD4+ T cells stimulated with antibodies against CD3 and CD28. Sort-purified CD4+CCR5+CXCR4+ T cells from each of these sources were found to make, on average, 5-10 fold more infectious virus per infected

cell after challenge with R5 HIV-1 than after challenge with X4 HIV-1. This difference was reflected as higher levels per infected cell of intracellular p24 as well as higher levels of p24 and infectious virus particles released into the supernatant. It is interesting to note (e.g., as in Fig. 3) that, in most cases, CD4+CCR5+CXCR4+ T cells also generated higher levels of virus than did CD4+CCR5-CXCR4+ T cells after challenge with X4 HIV-1. Possibly, CD4+CCR5+CXCR4+ T cells are more permissive for viral replication and/or are more long-lived after infection than are CD4+CCR5-CXCR4+ T cells. In either case, CCR5+ T cells are able to sustain R5 HIV-1 replication to a greater degree than X4 HIV-1 replication.

As shown by experiments using purified CD4+ T cells, the replicative advantage of R5 HIV-1 was not dependent on interaction with other cell lineages (e.g., myeloid cells). Given similar results using T cells from multiple donors, it also appears to be a generalized finding. The high average output of R5 HIV-1 per infected cell could be reflective of a cell population that is relatively homogenous with respect to viral production. Alternatively, the CD4+CCR5+CXCR4+ T cell population may be comprised of discrete subpopulations (e.g., with varying degrees of activation, in different stages of the cell cycle, etc), some of which are more permissive for viral production than others. Although our current assay system cannot discriminate between these possibilities on the single cell level, it is clear that R5 HIV-1 challenge does result in up to 100 fold higher levels

of viral production per cell when averaged across the entire CD4+CCR5+CXCR4+ population. This difference in relative production may contribute to the predominance of R5 isolates of HIV-1 *in vivo*.

Given the finding that R5 output is higher on a single cell basis, what might be the mechanism behind this difference? It may be that R5, but not X4, HIV-1 is able to infect a subpopulation of T cells that is able to generate a large amount of virus per unit time (45). Alternatively, the frequency of superinfected cells may be higher in the context of R5 as opposed to X4 HIV-1 infection, allowing for a larger number of virions to be produced per infected target cell. Another possibility is that R5 HIV-1 is less cytopathic, allowing cumulatively increased virus production over the lifespan of individual cells. Experiments to discriminate between these possibilities await the development of an assay that can follow single infected cells over time. It seems likely, however, that the last possibility (differential cytopathicity between X4 and R5 HIV-1 viruses) is not playing a major role under the conditions focused on in this study. During the early stages (days 5-7) of infection that we have studied, there appears to be no significant difference in cell viability in the cell cultures infected by the isogenic R5 and X4 HIV-1 isolates (data not shown).

Another possibility is that R5 HIV-1 signaling through CCR5 generates an intracellular environment more suitable for productive viral infection than

does X4 HIV-1 signaling through CXCR4. Although these two pathways share at least one common downstream signaling intermediate (13), they are likely to be divergent (19, 29, 33, 38, 43). It should be noted that the consequences of co-receptor signaling in HIV-1 infection are a subject of some debate. While several studies have suggested that co-receptor signaling can enhance infection by HIV-1 (4, 28, 34, 46), others indicate that co-receptor signaling has no impact on HIV-1 infection (2, 3, 5). The HLAC system may be used as a means of investigating the impact of co-receptor signaling in un-stimulated primary human cells, generating a comparison based on viral co-receptor preference. In this manner, we hope to better understand the mechanisms at play behind the differences in viral output observed in this study.

The replicative advantages we have reported of R5 HIV-1 over X4 HIV-1 may have important implications in HIV-1 pathogenesis. In combination with other factors (e.g., the presence of susceptible cells at sites of transmission, high viral output by infected macrophages, selective uptake by antigen presenting cells such as follicular dendritic cells, and increased immunogenicity of X4 HIV-1) (17, 22-24, 26, 27, 30, 35), the replicative advantage of R5 HIV-1 reported here could underlie the patterns of infection seen *in vivo*. If so, the question still remains: why is there a switch to the use of CXCR4 in some patients? Some studies have shown that X4 HIV-1 may be capable of viral production equivalent to R5 HIV-1

in a highly activated immune system (34), such as is present in late stages of HIV-1 infection *in vivo,* and other factors may tip the balance as well. Since such X4 switches are only observed in 50% of those infected with B clade virus (10, 40, 41, 47), they are not a necessary precondition for the development of AIDS. Further studies examining the replicative advantage of R5 HIV-1 may shed light both on the mechanisms of disease progression and on methods to prevent transmission or to slow progression after infection.

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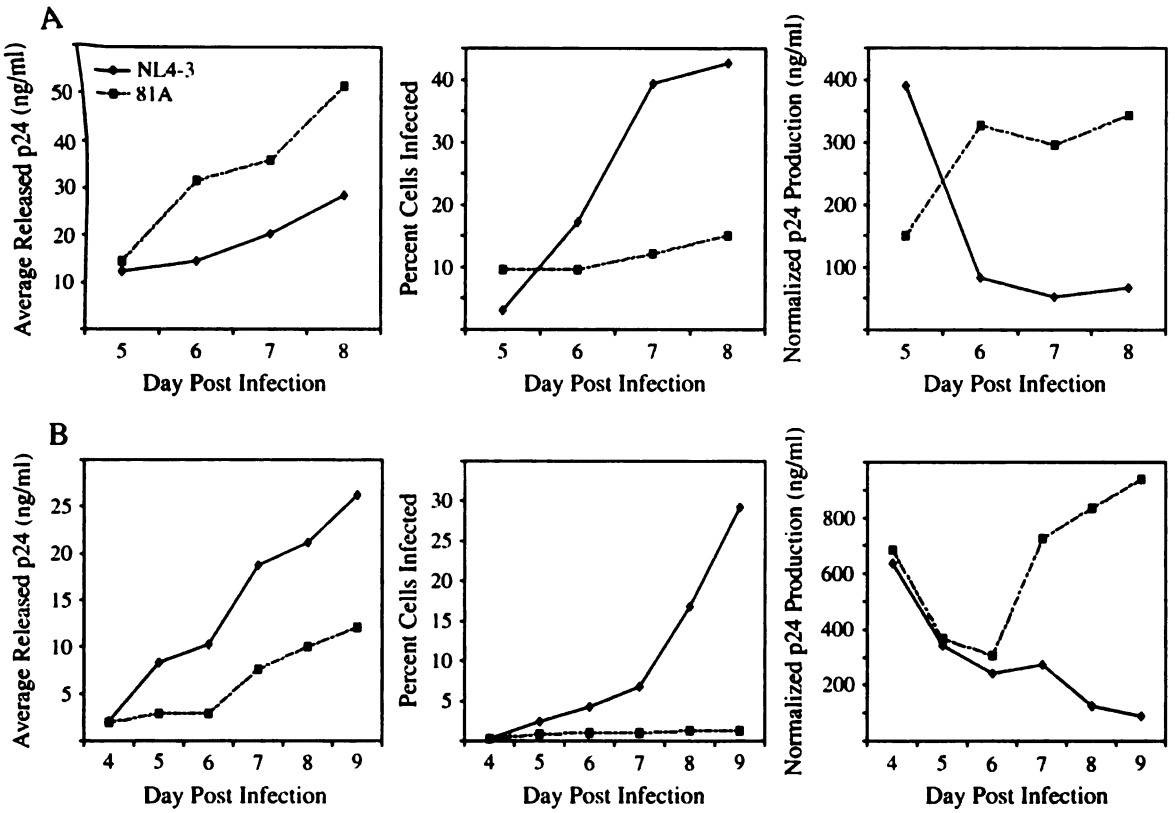

Figure 1. Assessment of HIV-1 Replication Kinetics in HLAC.

HLAC cultures were infected at an m.o.i. of 0.001 with either X4 HIV-1 (NL4-3) or R5 HIV-1 (81A). Data for two independent tonsil donors are shown in A (Donor 1) and B (Donor 2). Supernatants of the infected cultures were collected between days 4 - 9 and assessed for the amount of released p24 (ng/ml) by p24 ELISA (left panels). In parallel, the percent of infected CD3+CD4+ cells was measured by flow cytometry (center panels). Values shown represent the average of triplicate wells. The supernatant p24 levels were then divided by the corresponding fraction of infected cells in order to yield the normalized supernatant p24 data shown in C, expressed as "Normalized p24 production (ng/ml)"

(right panels). These data are representative of experiments using 19 different donors.

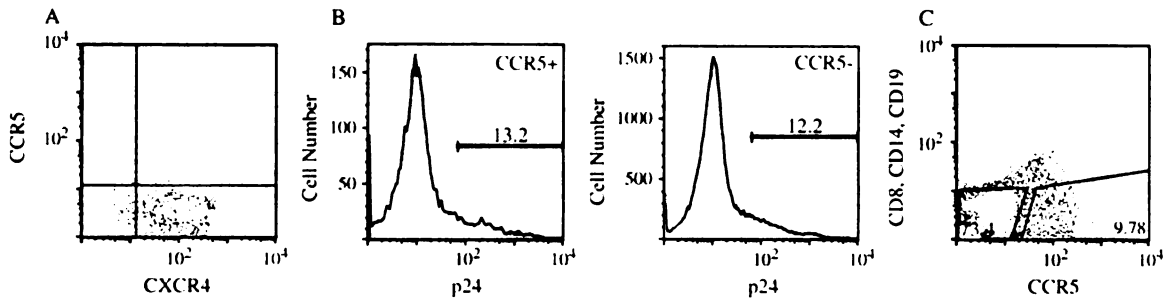

Figure 2. Characterization and Purification of CD4+ T Cell Populations from HLAC.

A. Most CD3+CD4+ T cells that express CCR5 also express CXCR4. Cells from HLAC were first gated on the lymphocyte population by forward and side scatter, and then on CD3+CD4+ T cells by extracellular staining for CD3 and CD4. Shown are representative results for CCR5 and CXCR4 staining on the CD3+CD4+ T cell population. B. Representative example of intracellular p24 staining in CD3+CD4+CCR5+ and CD3+CD4+CCR5- populations. Cells from HLAC cultures were assessed for expression of viral p24 at the conclusion of HIV-1 infection. In this example, the cultures were infected with NL4-3 at an m.o.i. of 0.001. On day 5 after infection, cultures were harvested and assessed for expression of CD3, CD4, CCR5, and p24. Background CCR5 staining was assessed by comparison to a sample stained for CD3, CD4 and p24 only. The percent of p24+ cells in each population was then determined by comparison to a mock-infected control. C. Purification of CD3+CD4+CCR5+ and CD3+CD4+CCR5- cells from HLAC. Cells from HLAC cultures were first depleted of CD8+, CD14+, and CD19+ populations by magnetic bead

depletion and then sort-purified into CCR5+ and CCR5- populations, gating first on lymphocytes and then events that were negative for the lineage markers CD8, 14, and 19. Shown is a representative sample of final gating of CCR5- and CCR5+ populations. These fractions were collected and analyzed for virus production (e.g., as in Fig. 3).

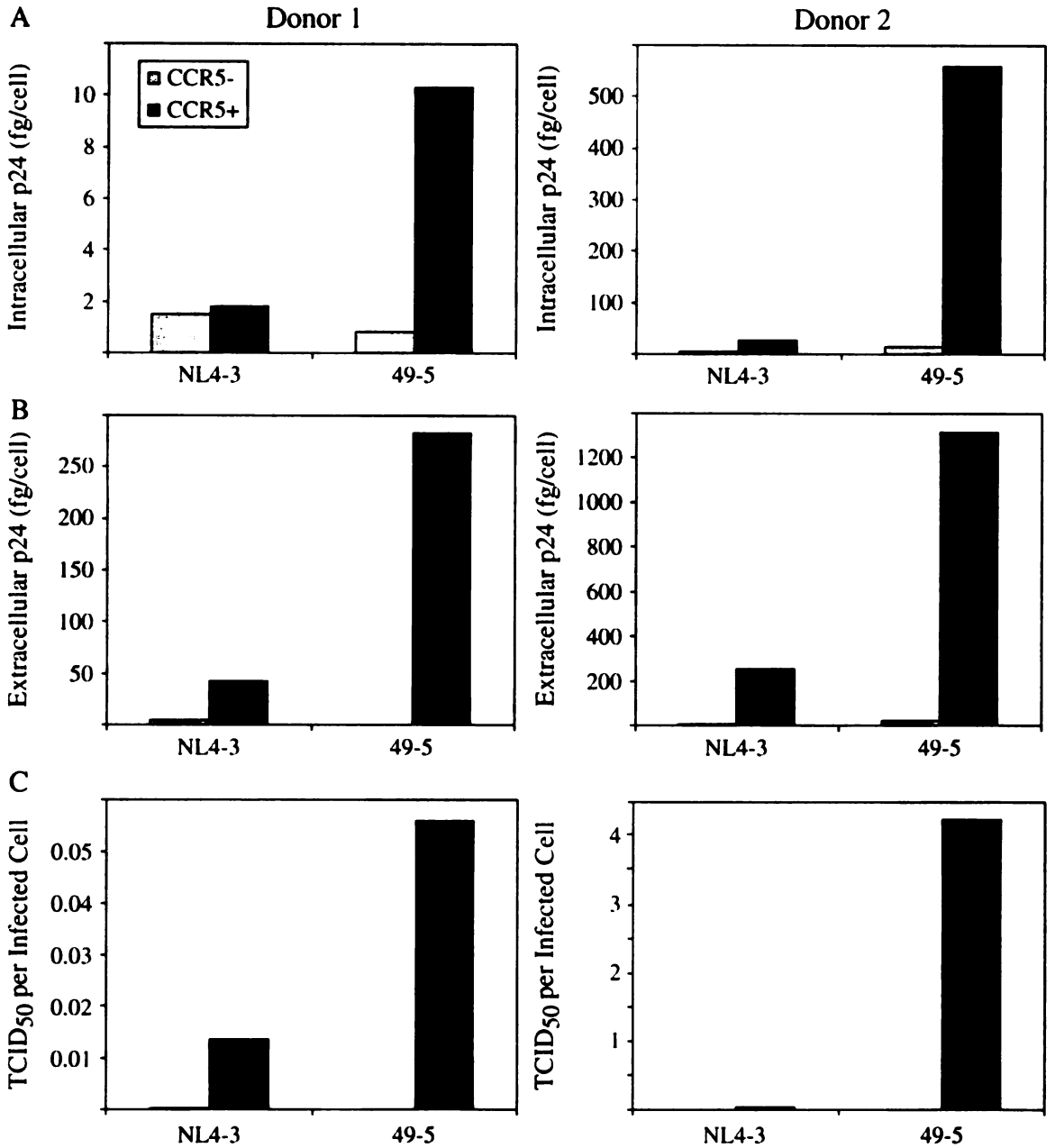

Figure 3. HLAC Infected with R5 HIV-1 (49-5) Yields a Higher Viral Output per Infected Cell than X4 HIV-1 (NL4-3) Infected Cultures. HLAC cultures were infected with NL4-3 or 49-5 at an m.o.i. of 0.001 for 5 to 6 days. Cultures were harvested and CD3+CD4+CCR5+ and CD3+CD4+CCR5- cells were purified as described in Fig. 2. In parallel,

unsorted HLAC cultures were analyzed to determine the percentage of CCR5+ (FITC) and CCR5- infected cells (p24+). The sorted cells were then separated into two groups. One third of the cells were analyzed immediately by p24 ELISA to determine their internal p24 concentration. The remaining cells were placed back into culture for 1.5 days to allow viral replication to continue. At the conclusion of this time period, supernatants from each well were harvested and measured for p24 production (by ELISA) and for infectious virus (by $TCID_{50}$ assay). As detailed in Materials and Methods, these data were used to determine the average intracellular p24 concentration per infected cell (A), the average extracellular p24 concentration per infected cell (B) and the average $TCID_{50}$ per infected cell (C). These data are representative of differences seen in 9 different donors.

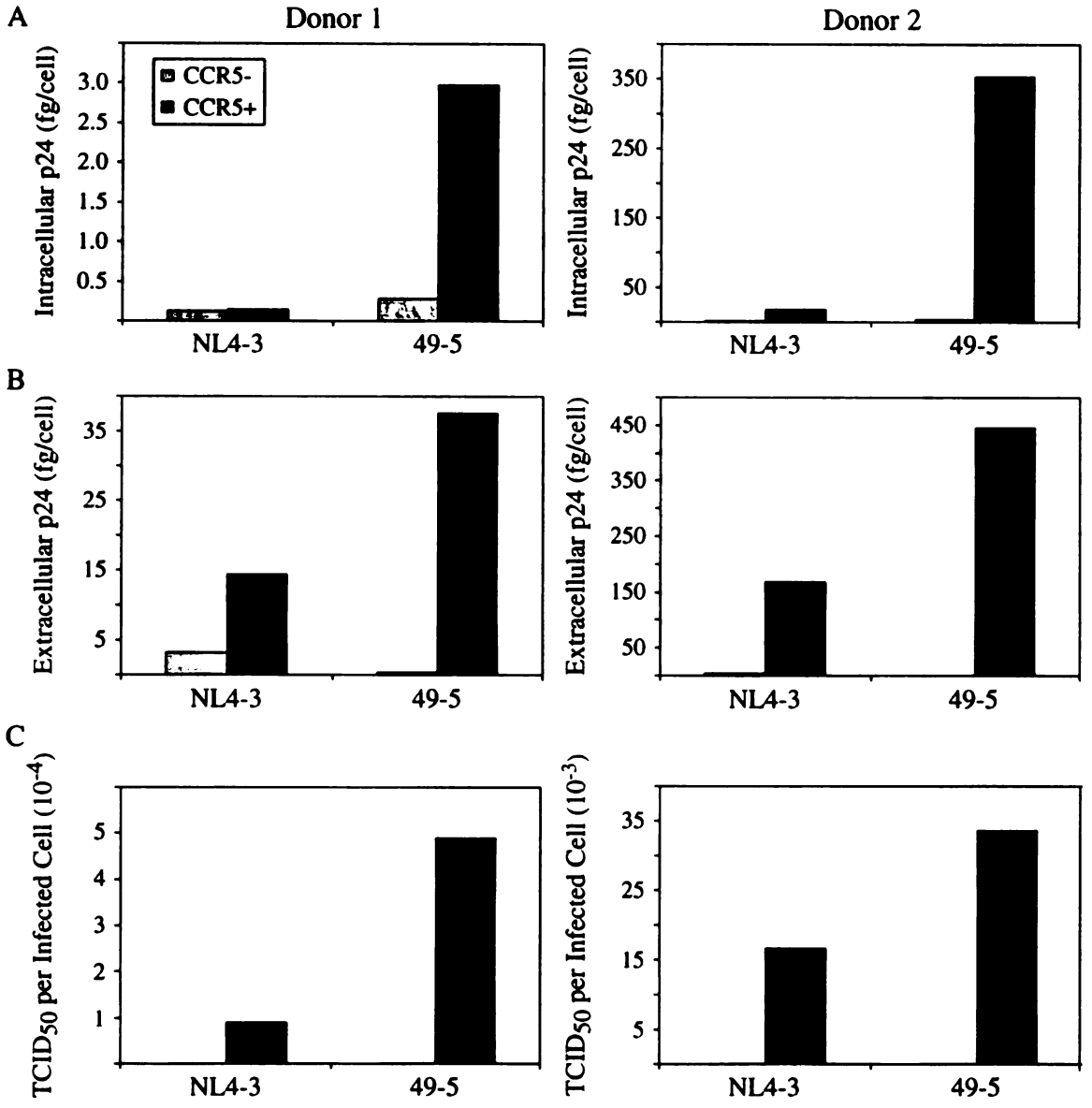

Figure 4. PBMC-Derived CD4+ T Cells Infected with R5 HIV-1 (49-5) Yield a Higher Viral Output per Infected Cell than X4 HIV-1 (NL4-3) Infected Cultures.

CD4+ T cells were purified from human PBMC, stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies (as described in Materials and Methods), and then infected (at an m.o.i. of 0.001) with

either NL4-3 or 49-5 for 6-7 days. Infected cells were harvested and analyzed as described in Figs. 2 and 3 (except that the removal of CD8, CD14, and CD19+ cells via magnetic bead separation was not required, as the CD4+ T cells had already been purified). These data were used to determine the average intracellular p24 concentration per infected cell (A), the average extracellular p24 concentration per infected cell (B) and the average $TCID_{50}$ per infected cell (C). The data shown are from two independent PBMC donors.

Chapter 4

Discussion: Conclusions, Implications, and Future Directions

In this study, we have asked whether a difference in viral output per infected CD4+ T cell could be partially responsible for R5 HIV-1 predominance over X4 HIV-1 in most stages of HIV disease. Utilizing human lymphoid histoculture and TCR-stimulated peripheral blood derived CD4+ T cells, we observed that R5 HIV-1 is capable of producing, on average, 10-100 fold more virions per infected cell than X4 HIV-1. This advantage seems to be partially independent of cell types other than CD4+ T cells and is maintained across donors and experimental systems (37, 39).

In past studies, it has been noted that macrophage infection by R5 HIV-1 could give a distinct advantage over T cell restricted X4 HIV-1, with estimates of ten fold greater viral output by long lived macrophages over T cell targets (12). The studies of this thesis indicates that variation in viral production in T cells may also be important to R5 HIV-1's advantage. Even in systems in which macrophages and monocytes are severely depleted if not completely absent, R5 HIV-1 is capable of producing much greater levels of virus per infected cell than X4 HIV-1. A combination of enhanced production of virus in CD4+ T cells and access to long-lived macrophage targets may contribute significantly to R5 HIV-1's ability to persist in vivo despite a much smaller target cell population than X4 HIV-1.

Because of the large numbers of cells required by the experiments carried out herein to estimate viral output per cell, it was not possible to use tissue blocks as in past studies (17, 19, 33). Such tissue culture models have the advantage of preservation of tissue architecture and native cell-cell interactions, which may very well have an impact on the viral output of individual target cells in lymphoid tissues. It was also only possible to generate an average viral output per cell. The sorted populations may be relatively homogeneous with respect to viral output, producing approximately equal amounts of virus in each individual cell. Alternatively, they may consist of several subpopulations, with the majority of the viral burden produced by the minority of infected cells. Preliminary data indicate that in some cases, the first scenario is correct. In a comparison of the intracellular p24 (as measured by FACS staining of cells infected with two non-isogenic HIV-1 strains), the mean fluoresence intensity of p24 staining in R5 HIV-1 infected cells was twice that of X4 infected cells. However, these experiments were performed with a non-isogenic pair of viruses and many other factors may have come into play. Other past studies have indicated that most of the viral burden is produced by the minority of infected cells. Support for this comes from the idea that many cellular factors seem to contribute to viral output, including but not limited to activation state, experience with antigen (i.e., memory vs. naïve populations), and ease of interaction with other infected cells, allowing for direct cell-cell transmission of virus.

Single Cell Assay Development

This question would be extremely difficult to answer utilizing the experimental design in these studies; as such a large number of cells is needed to calculate viral production. To study subsets of infected cells efficiently and compare them to each other, it would be necessary to develop an assay capable of assessing viral output on a single cell level. We have initiated attempts to create such an assay using two separate strategies. The first utilizes an indicator cell line to measure the amount of virus produced per target cell. Many indicator cell lines have been developed to study HIV-1 replication, and most function using the same basic principle. An easily quantitated protein is inserted into the cell line linked to the HIV-1 LTR. In this way, when the cell line is infected by HIV-1, viral replication and protein expression leads to activation of the LTR and transcription of the indicator protein, which can then be quantitated and used as a proxy for the level of HIV-1 protein production itself. Several different proteins have been used for this purpose. Green fluorescent protein (GFP), for example, can be detected by FACS or immunofluorescence. GFP expression is only a semi-quantitative reflection of HIV-1 replication at best, but can be isolated to individual infected indicator cells. Luciferase, another fluorescent protein, can also be detected via fluorescence, but only after lysis of the indicator cells. GFP or luciferase expressing lines are often used in combination with

differential co-receptor expression to assess tropism of primary HIV-1 isolates. A third indicator protein sometimes used is β-galactosidase (β-gal). β-gal is an enzyme capable of cleaving the substrate X-gal into a blue product visible within intact cells.

The indicator cell line chosen in this case is called JC53BL, and was developed to assess the efficacy of the HIV-1 fusion inhibitor T-20 in the biotechnology industry (45). JC53BL expresses the two indicator proteins luciferase and β-galactosidase under the control of the HIV-1 LTR. Previously, the cells were directly infected with free HIV particles in the presence or absence of T-20 in order to determine the impact of T-20 on HIV-2 entry and protein production. For the purpose of our experiments, JC53BL cells were used to capture and quantify infectious virus released from other infected cells. In short, primary T-cells from peripheral blood or lymphoid tissue were infected as in our previous studies, then overlayed on top of a JC53BL monolayer. Theoretically, any virus released from these producer cells could infect the JC53BL indicator cell line and trigger expression of luciferase and β-gal. The amount of virus produced per primary cell could then be assessed by either intensity of X-gal staining on each indicator cell line or the size of individual stained JC53BL colonies. Either parameter should give a quantitative assessment of the amount of virus being released by individual primary T-cells (Figure 1). Unfortunately, we were never able to detect cell-released virus

effectively, despite efficient infection of JC53BL cells by free virus of the same strains used to infect primary T-cells in our experiments (data not shown). Even PHA stimulated PBMC cultures, which produce extremely high levels of HIV-1, were unable to transfer virus to the indicator cell monolayer.

The second strategy used to develop a single cell assay is based on a previously developed method of assessing antibody or antigen release from individual cells called ELISPOT (8, 40). In this assay, an antibody against the antigen of interest is used to coat the surface of a filter lined enzyme linked immunosorbant assay (ELISA) plate. The cells to be tested are then added on top of the coated plate and incubated to allow the release of antigen, often in the presence of exogenous stimulation. The cells are then removed and the antigen detected by adding a second antigen specific, biotin labeled antibody followed by an appropriate substrate such as streptavidin. Spots indicating the previous location of cells releasing the antigen of interest can then be detected either by eye using a dissection microscope or by the use of specialized equipment and visualization software. In short, antibodies against the viral p24 protein and/or gp120 are used to coat an ELISA plate, then overlayed with a known number of infected cells. These cells are then allowed to settle and adhere. At this point, the cells can either be lysed immediately to determine the amount of viral protein contained within each or incubated

further to allow virions to be released and captured by the antibody coat. Following this capture step, non-specifically bound cells are rinsed away, and a biotin labeled antibody to a second p24 epitope added. The spots can then be developed using a streptavidin substrate and quantitated using specialized software (Fig. 2).

For the purpose of our studies, it is important to detect not only the number of spots generated by HIV-1 infected cell cultures, but also the size, intensity, and/or saturation of these spots. Theoretically, any or all of these parameters could enable the relative quantitation of the amount of virus released per infected cell. Infected cells indeed generated a specific signal proportional to the number of infected cells added to the plate. Unfortunately, it was not possible to detect significant differences in spot size, intensity or saturation using either peripheral blood or lymphoid tissue. In order to determine whether the assay is sensitive enough to detect even large differences in viral output, a TNFα inducible HIV-1 producer cell line (ACH2) was stimulated and infected cells sorted into low, medium, and highly infected populations based on GFP expression, which should be proportional to viral production in ACH2 cells (11). The data from these studies are as yet inconclusive, but may in the future provide a means of determining viral protein production at the single cell level. These studies should allow us to work with smaller numbers of cells, as in tissue blocks or HIV+ patient samples, as well as

helping us to understand the cellular factors which contribute to viral output in T cells. It may be that R5 HIV-1 is simply able to access a subpopulation of T cells that allows for large amounts of virus to be made, a population not accessible to X4 HIV-1 (43). However, it is also possible that the intracellular environment of target cells could be differentially altered by R5 HIV-1 or X4 HIV-1 infection.

For instance, it has been shown that R5 HIV-1 and X4 HIV-1 have a differential impact of on target cell viability. As previously mentioned, the switch from R5 to X4 HIV-1 *in vivo* is typically accompanied by a rapid loss of CD4+ T cell numbers in the periphery (7, 38). This is most likely due to a phenomenon called bystander killing, in which uninfected T cells in the vicinity of HIV-1 infected cells are killed without being directly infected. Bystander killing has been shown to be far more common in X4 HIV-1 infection, perhaps due to differences in gp120 released from infected cells, or simply due to the expression of CXCR4 on a higher proportion of susceptible bystanders (14, 22, 24, 42). However, in the experiments performed in this study, preliminary evidence suggests that there is no significant difference in cell death between R5 HIV-1 and X4 HIV-1 infected cultures, at least at the early time points observed. After this point, a rapid decrease in viability of X4 HIV-1 cultures has been seen (data not shown). This indicates that while viability may be a significant

factor in R5 HIV-1 predominance over time, it is not the only mechanism at play.

We must also consider the possibility that the difference in viral output may be fated before HIV-1 ever enters the target cell interior. It is possible that the lower percentage of cells infected by R5 HIV-1 each receive a higher level of input virus than their X4 HIV-1 counterparts. In other words, the incidence of super-infection may be higher in R5 HIV-1 than in X4 HIV-1 infection. This could lead to a larger number of virions produced per R5 HIV-1 infected cell, even without any advantage for replication after the virus has entered its target; there would simply be a higher number of input viral genomes to contribute to virus production. Why would there be a difference in the level of super-infection? Several lines of evidence support this theory. First, it has been shown that while CD4 and CCR5 are found in lipid rafts on the surface of lymphocytes, CXCR4 is not (10, 21, 27). This could lead to an increased chance for an R5 HIV-1 virion to find its receptor and co-receptor in close proximity to each other and in large numbers, increasing the overall avidity of the interaction and the odds of successful docking and fusion (32). Second, we and others have observed that as CCR5 levels increase on a given cell, CXCR4 levels decrease slightly (2). This could lead to an overrepresentation of CCR5 on activated T cells, giving R5 HIV-1 a greater chance of coming in contact with its co-receptor frequently. One

must keep in mind, however, that the possibility of differential super-infection depends very heavily on a high concentration of virus in the immediate proximity of the target cell. After the initial virus has entered the cell, CD4 is rapidly down regulated by Nef, drastically lowering the chance of subsequent super-infection (29). It may be that the concentration of virus is in fact high enough, due to the close proximity of cells to each other in lymphoid tissues, allowing for cell to cell transmission of large amounts of virus simultaneously (26, 30), and due to the presence of follicular dendritic cells, which have been shown to take up and present virus in high concentration to T cell targets (13, 16, 18, 20, 23, 30).

One final possibility involves the fact that inherently CCR5 and CXCR4 are G protein linked chemokine receptors. Upon contact with their natural ligands, they initiate a cascade of signaling intermediates within the cell, leading to chemotaxis toward the source of the ligand, as well as cellular activation. CCR5 and CXCR4 belong to two different classes of chemokine receptors, and initiate different downstream signaling pathways upon stimulation and different intracellular consequences (6, 41, 44, 47). Unfortunately, these cascades are not completely characterized, and have not been directly compared in detail. However, it has been shown that both their natural ligands and HIV-1 gp120 are capable of initiating signaling cascades (9, 15, 28, 34, 36, 42).

Therefore, it may be that upon signaling through CCR5, R5 HIV-1 generates an intracellular environment more suitable to productive viral infection than X4 HIV-1 through CXCR4. It should be noted that the consequences of co-receptor signaling in HIV-1 infection are controversial. While several studies have shown that co-receptor signaling can enhance infection by both R5 and X4 HIV-1 (4, 25, 35, 46), there are an equal number of studies that indicate the opposite, that co-receptor signaling has no impact on HIV-1 infection (1, 3, 5). Unfortunately, because laboratory strains of HIV-1 are not capable of infecting un-stimulated peripheral blood cells, to the best of our knowledge, all investigations of HIV-1 co-receptor signaling have been performed either in cell lines or in peripheral blood stimulated with PHA or cytokines. In either of these cases, the manipulation of the target cells could have a highly significant impact on the results of the experiments. It has been previously shown that intense TCR stimulation has a positive impact on HIV-1 infection, especially on X4 HIV-1 (35, 43), so there is reason to believe that any form of stimulation could impact the need for signaling in productive infection, and that the impact could be different for R5 and X4 HIV-1. With this in mind, the HLAC system could be a useful means of investigating the impact of co-receptor signaling in un-stimulated primary human cells, and to determine whether signaling through CCR5 gives a replicative advantage to R5 HIV-1. Although this system will not permit direct ablation of signaling and comparison to an

intact system, signaling pathways can be isolated through the use of pseudotyped HIV-1 and other methods. Through this and other lines of experimentation previously mentioned, it may be possible to better understand the mechanisms at play behind the differences in viral output observed in our study, and perhaps this understanding can be used to gain a further therapeutic advantage in the clinical setting.

The replicative advantages enjoyed by R5 HIV-1 over X4 HIV-1 that are seen in this study may have implications in HIV-1 pathogenesis. In combination with possible advantages reported by other groups, including the presence of susceptible cells at transmission sites, high viral output by infected macrophages, selective uptake by antigen presenting cells such as follicular dendritic cells, and others, the replicative advantage of R5 HIV-1 could explain the patterns of infection seen *in vivo*. So, if R5 HIV-1 presents so many advantages, why switch later on to predominantly use CXCR4? Studies have shown that X4 HIV-1 may present different immunodominant epitopes to the immune system (31), and may be capable of viral production equivalent to R5 HIV-1 in a highly activated immune system (35), such as is present in late stages of HIV-1 infection *in vivo*. These and other factors may well tip the balance. However, as it has been observed that only 50% of infected individuals make the switch from CCR5 use to CXCR4 use at all, this balance is obviously far more complicated than we know. Hopefully, the better we

understand the battle HIV-1 fights for survival in the human body, the better we will be able to fight back.

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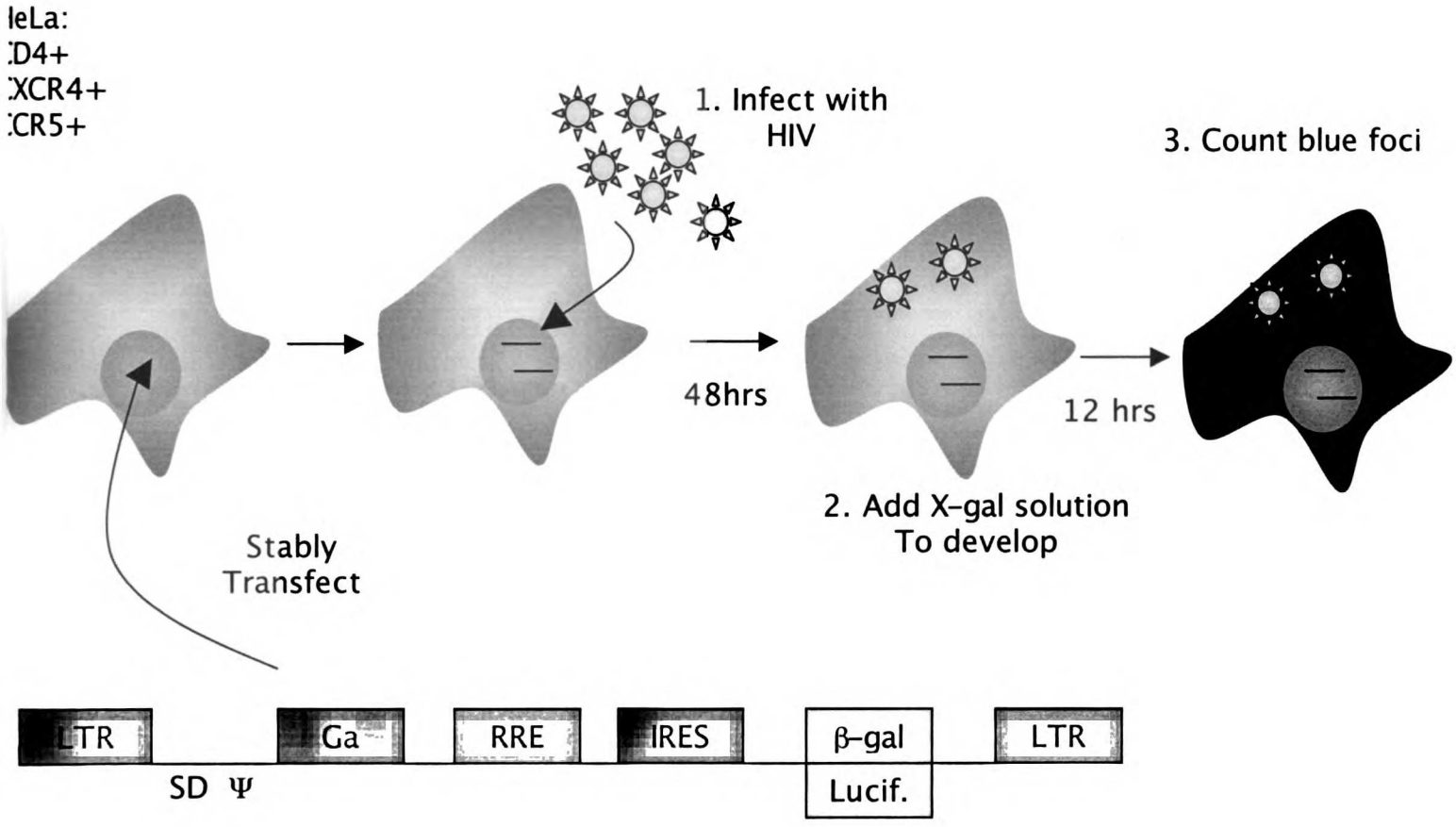

Figure 1. JC53BL Indicator Cell Assay Design.

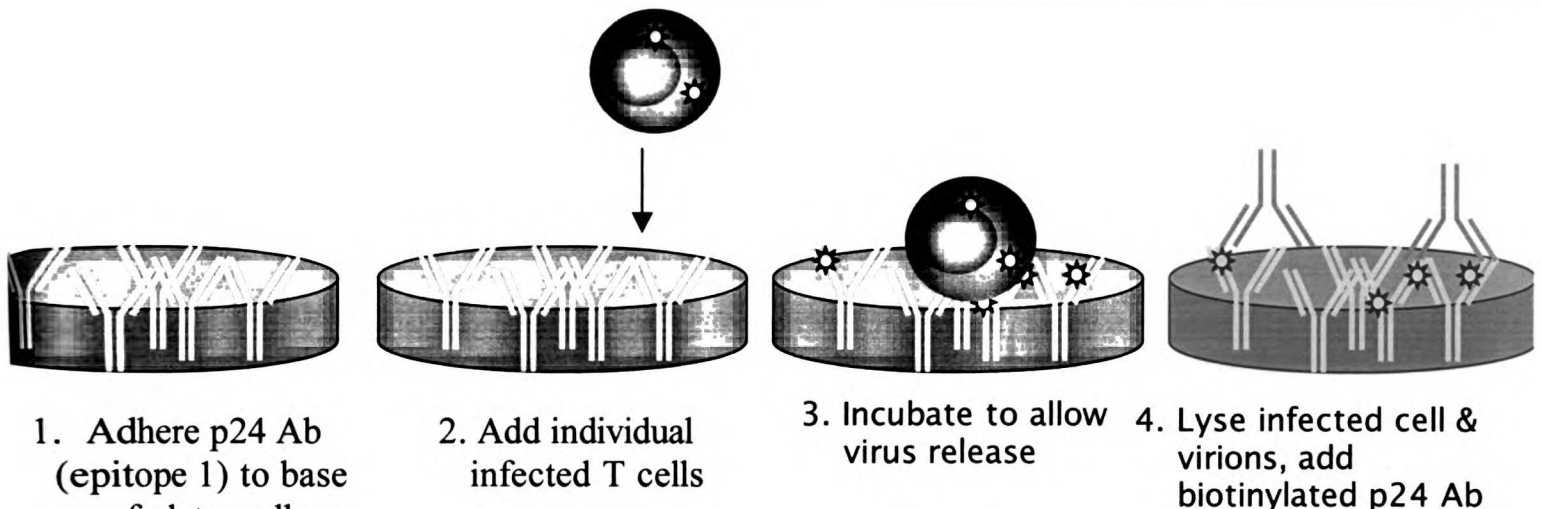

Figure 2. Virospot Assay Design.

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R5 Human Immunodeficiency Virus Type 1 (HIV-1) Replicates More Efficiently in Primary CD4$^+$ T-Cell Cultures Than X4 HIV-1

Becky Schweighardt,*† Ann-Marie Roy,† Duncan A. Meiklejohn, Edward J. Grace II, Walter J. Moretto, Jonas J. Heymann, and Douglas F. Nixon

Gladstone Institute of Virology and Immunology, University of California, San Francisco, California

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In this report, we present evidence that R5 human immunodeficiency virus type 1 (HIV-1) replicates more efficiently in primary CD4$^+$ T cells than X4 HIV-1. By comparing CD3/CD28-costimulated CD4$^+$ T-cell cultures infected by several X4 and R5 HIV-1 strains, we determined that R5-infected CD4$^+$ T cells produce more virus over time than X4-infected CD4$^+$ T cells. In the first comparison, we found that more cells were infected by the X4-tropic strain LAI than by the R5-tropic strain JR-CSF and yet that higher levels of viral production were detected in the R5-infected cultures. The differential viral production was partially due to the severe cytopathic effects of the X4 virus. We also compared cultures infected with the isogenic HIV-1 strains NL4-3 (X4) and 49.5 (R5). We found that fewer cells were infected by the R5 strain, and yet similar levels of viral production were detected in both infected cultures. Cell death played less of a role in the differential viral production of these strains, as the cell viability remained comparable in both X4- and R5-infected cultures over time. The final comparison involved the primary R5-tropic isolate KP1 and the primary dual-tropic isolate KP2. Although both strains infected similar numbers of cells and induced comparable levels of cytopathicity, viral production was considerably higher in the R5-infected culture. In summary, these data demonstrate that R5 HIV-1 has an increased capacity to replicate in costimulated CD4$^+$ T cells compared to X4 HIV-1.

Human immunodeficiency virus type 1 (HIV-1) infects cells by binding to the CD4 receptor and to one of several coreceptors expressed on the surface of target cells (2, 13, 15, 16, 18, 26). The chemokine receptors CCR5 and CXCR4 serve as the major coreceptors for HIV-1, although several other chemokine receptors have been linked to minor HIV-1 coreceptor usage (2, 8, 15, 18, 32, 52). Characteristically, non-syncytium-inducing (NSI) isolates utilize CCR5 as a coreceptor and are referred to as R5 strains (2, 14–16). R5 strains often represent the dominant viral population detected during the early stages of clinical HIV-1 infection (9, 14, 41, 43, 46, 53). In contrast, syncytium-inducing (SI) isolates utilize CXCR4 as a coreceptor and are referred to as X4 strains (18, 20, 24, 25, 28, 30, 40, 45, 48). X4 strains are typically detected in the later stages of infection and are associated with rapid CD4$^+$ T-cell loss (11, 14, 24, 25, 46, 48, 49). Despite the link between X4 emergence and disease progression, approximately half of all individuals with AIDS continue to harbor predominantly R5 viruses, suggesting that CXCR4 coreceptor usage alone is not responsible for disease progression (9, 43, 46, 53).

Several mechanisms have been proposed to explain the R5 dominance of early HIV-1 infection. There is evidence that R5 strains may be transmitted at an increased frequency compared to X4 strains. For example, individuals that carry a 32-bp deletion mutation (Δ32) in the CCR5 gene are highly resistant to HIV-1 infection (10, 33, 38, 44). Although these individuals are susceptible to X4 infection, very few cases have been reported, suggesting that X4 transmission occurs with lower frequency (3, 5, 23, 29, 37, 47, 50). Another mechanism leading to R5 dominance in vivo may involve preferential spread of R5 strains. It has been reported that dendritic cells preferentially transport R5 rather than X4 virions, which may lead to selective spread of R5 virus to lymph nodes (19, 41). Also, intestinal epithelial cells have been shown to selectively transport R5 virions to the lamina propria, which may lead to the preferential spread of R5 infection to activated CD4$^+$ T cells (34). Recent evidence suggests that the immune response may also play a role in R5 selection in vivo. Harouse et al. report that both X4 and R5 SHIV replication can be detected in macaques early after coinfection; however, X4 replication is no longer detected after CD8$^+$ T-cell-mediated antiviral immune responses are elicited (22). Furthermore, X4 replication reemerges in coinfected animals following depletion of the antiviral immune response by in vivo infusion with anti-CD8 antibodies. Collectively, these data suggest that X4-infected cells may be more susceptible to immune-mediated killing than R5-infected cells (22).

We propose that an additional mechanism may be involved in R5 dominance. In this report, we present evidence that R5 HIV-1 replicates more efficiently in CD3/CD28-costimulated CD4$^+$ T cells than X4 HIV-1. We tested several viral strains in this system, including the molecular clones LAI (X4) and JR-CSF (R5), the isogenic viral pair NL4-3 (X4) and 49.5 (R5), and two primary isolates that were recovered from the same patient at different time points, KP1 (R5) and KP2 (X4/R5). We found that the R5 HIV-1 JR-CSF strain infected a smaller percentage of costimulated CD4$^+$ T cells than the X4 HIV-1 strain LAI and yet produced more progeny virus over time than the X4-infected culture. It is likely that this result is partially due to the fact that the cell viability of X4-infected

* Corresponding author. Mailing address: Gladstone Institute of Virology and Immunology, P.O. Box 419100, San Francisco, CA 94141-9100. Phone: (415) 695-3826. Fax: (415) 826-8449. E-mail: bschweighardt@gladstone.ucsf.edu.

† B.S. and A.-M.R. contributed equally to this manuscript.

cultures decreased rapidly, whereas cell viability remained relatively high in R5-infected cultures over time. Analysis of cultures infected with the isogenic viral pair provided further confirmation that R5-infected CD4+ T cells produce more progeny virus than X4-infected CD4+ T cells. Despite the fact that fewer costimulated CD4+ T cells were infected by the R5-tropic 49.5 strain than by its isogenic X4 counterpart NL4-3, similar amounts of viral production were detected in both infected cultures. Cell death seemed to play less of a role in the differential levels of viral release between these two strains, as the viability characteristics of both infected cultures remained comparable over time. Interestingly, similar results were obtained with the primary R5 isolate KP1 and the primary dual-tropic isolate KP2. Although both KP1 and KP2 infected equivalent numbers of costimulated CD4+ T cells and induced comparable amounts of cell death, viral production was considerably higher in cultures infected by the R5-tropic KP1 strain. In summary, we report that R5 HIV-1 has an increased capacity to replicate in CD4+ T cells compared to X4 HIV-1 and propose that this increased fitness may allow R5 viruses to out-compete X4 viruses in the early stages of HIV-1 infection.

MATERIALS AND METHODS

Antibodies. The following fluorescently labeled monoclonal antibodies were purchased from Becton Dickinson (San Jose, Calif.) and used for flow cytometric analysis: CD3-APC (clone SK7), CD69-FITC (clone FN50), CD38-PE (clone HIT2), CXCR4-APC (clone 12G5), CD4-PerCP (clone SK3), CCR5-APC (clone 2D7), and IFN-γ-PE (clone 25723.11). Monoclonal p24-FITC antibody (clone KC57) was purchased from Coulter Clone (Miami, Fla.), and annexin V-PE was purchased from Caltag Laboratories (Burlingame, Calif.). The following antibodies were used for CD3/CD28 stimulation: CD28 (Leu-28) clone L293 (Becton Dickinson) and CD3 clone SPV-T3b (Zymed, South San Francisco, Calif.).

CD4+ T-cell isolation. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham BioSciences, Uppsala, Sweden) gradient centrifugation of leukopacks (Stanford Blood Bank, Stanford, Calif.) obtained by apheresis of healthy donors. CD4+ T cells were purified by negative selection using Microbeads (Miltenyi Biotec, Auburn, Calif.). Cell purity was determined by staining cells with fluorescently conjugated antibodies directed against CD4, CD3, CD8, and CD14. Cell populations were found to be >95% $CD3^+$ $CD4^+$.

CD4+ T-cell stimulation. CD4+ T cells were activated by phytohemagglutinin (PHA) stimulation or by CD3/CD28 costimulation. For PHA stimulation, cells were cultured at a density of 2×10^6 cells/ml with 1 μg of PHA (Sigma, St. Louis, Mo.)/ml for 24, 48, or 72 h. Cells were then washed to remove PHA and cultured for 48 h in RPMI 1640 medium (MediaTech, Herndon, Va.) supplemented with 15% fetal bovine serum (FBS) (Gemini, Woodland, Calif.) and 50 U of interleukin-2 (IL-2) (AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH])/ml. For CD3/CD28 costimulation, tissue culture plates were precoated with CD3 antibody. Briefly, wells were washed with 1× phosphate-buffered saline (PBS) and then coated with a 50 μg/ml stock solution of CD3 antibody. Excess liquid was removed, and plates were incubated at 37°C until dry. Cells were then cultured on coated plates at a concentration of 2×10^6 cells/ml in the presence of 1 μg of soluble CD28 antibody (Becton Dickinson)/ml for 24, 48, or 72 h. Cells were removed from the CD3 coated plates, washed to remove soluble CD28, and then cultured in RPMI 1640 medium supplemented with 15% FBS and 50 U of IL-2/ml.

Detection of cell surface protein expression by flow cytometry. To detect cell surface protein expression, 5×10^5 $CD4^+$ T cells were incubated with appropriate concentrations of fluorescently conjugated monoclonal antibodies diluted in 1× phosphate-buffered saline containing 1% bovine serum albumin (Sigma). Cells were incubated with antibodies for 20 min at 4°C, washed twice, and then fixed in 1% paraformaldehyde. Samples were acquired on a FACSCalibur instrument (Becton Dickinson), and the resulting data were analyzed using CellQuest software (Becton Dickinson).

Measurement of cellular proliferation and IFN-γ expression. Stimulated $CD4^+$ T cells were incubated with 2 μM CFSE (5- and 6-carboxyfluorescein diacetate, succinimidyl ester) (Molecular Probes, Eugene, Oreg.) for 10 min at 37°C and then washed twice. Stained cells were incubated for 72 h at 37°C. Brefeldin A (Sigma) was added at a final concentration of 10 μg/ml for the last 6 h of culturing. Cells were then washed, fixed in 4% PFA, permeabilized with 1% fluorescence activated cell sorter (FACS) Perm solution (Becton Dickinson), and stained with monoclonal antibodies against CD3, CD4, and gamma interferon (IFN-γ). After antibody staining, cells were washed twice and then stored in 1% paraformaldehyde until acquisition on a FACSCalibur instrument. Subsequent analysis was performed using FlowJo software (Tree Star, Inc).

Virus preparation. The HIV-1 strains LAI, JR-CSF, NL4-3, and 49.5 were prepared by introducing proviral constructs into 293T cells (American Type Culture Collection, Manassas, Va.) by $CaPO_4$ transfection. The following proviral plasmids were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pLAI.2 (39) from Keith Peden, courtesy of the Medical Research Council AIDS Directed Programme; pYK-JR-CSF (6, 21, 27) from Irvin S. Y. Chen and Yoshio Koyanagi; pNL4-3 (1) from Malcom Martin; and p49.5 (51) from Bruce Chesebro. After the media were changed approximately 16 h after transfection, the virus-containing supernatants were harvested 72 h posttransfection. Viral stocks were centrifuged at $1,000 \times g$ for 10 min to remove cell debris and then passed through a 45-μm-pore-size filter. The infectious titer of each viral preparation was determined by 50% tissue culture infective dose assay. Briefly, PHA-stimulated PBMCs from multiple donors were pooled and infected with serially diluted virus in quadruplicate wells. Cell supernatants were collected 5 days postinfection, and HIV p24 antigen was quantitated by p24 enzyme-linked immunosorbent assay (ELISA). Infections were scored positive for replication when p24 levels were higher than 50 pg/ml. The 50% tissue culture infective dose value represents the virus dilution at which 50% of wells scored positive for infection.

Coreceptor phenotyping assay. GHOST indicator cells were used to determine coreceptor usage of each viral strain. The GHOST-X4 and GHOST-Hi5 cell lines (35) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Vineet N. Kewalramani and Dan R. Littman. These cell lines were originally derived from human osteosarcoma (HOS) cells. The GHOST-X4 cell line was transduced with a retroviral vector that confers high-level CXCR4 expression, and the GHOST-Hi5 cell line was transduced with a retroviral vector that confers high-level CCR5 expression. The GHOST-Hi5 cells also express low levels of CXCR4 due to endogenous expression in the parental HOS cells. Each cell line was also transduced with a CD4-expressing retroviral vector and a construct that drives expression of green fluorescent protein (GFP) under the control of the HIV long-terminal-repeat (LTR) promoter.

GHOST-X4 and GHOST-Hi5 cells were seeded on 12-well plates at a density of 5×10^5 cells per well. Cells were cultured at 37°C overnight prior to infection with HIV-1 strains. Cells were infected with each viral strain at a multiplicity of infection (MOI) of 0.01 in the presence of 20 μg of Polybrene/ml to enhance infection efficiency. Each infection was performed in a total volume of 300 μl at 37°C for 4 h. After incubation, virus was removed and 1 ml of fresh culture medium was added to each well. Cells were then incubated for an additional 48 h prior to harvest. GFP expression was analyzed by FACS analysis. Samples were acquired on a FACSCalibur instrument, and the resulting data were analyzed using Cell Quest software.

HIV infection. CD4+ T cells were activated by CD3/CD28 costimulation for 72 h prior to infection. Cells were then washed and incubated with virus at an MOI of 0.01 (for strains LAI, JR-CSF, NL4-3, and 49.5) or 0.001 (for strains KP1 and KP2) for 4 h at 37°C. After infection, cells were washed three times to remove any unbound virions and then cultured in RPMI 1640 medium supplemented with 15% FBS and 50 U of IL-2/ml.

Quantification of viral replication. Viral replication was assessed by measuring the amount of soluble HIV p24 antigen in culture supernatants. Aliquots (200 μl) of supernatant were removed from infected cell cultures at 3, 5, 7, and 10 days postinfection. Supernatants were stored at −80°C until completion of the experiment. Quantification of p24 was determined using an ELISA (PerkinElmer Life Science, Inc., Boston, Mass.) according to the manufacturer's protocol.

Intracellular p24 staining. The percentage of infected cells was determined by intracellular staining for the viral p24 antigen. $CD4^+$ T cells (5×10^5) were removed from infected cultures at 3, 5, 7, and 10 days postinfection. Cells were washed, fixed in 4% paraformaldehyde, permeabilized with 1% FACS Perm solution (Becton Dickinson), and then incubated with a fluorescently conjugated monoclonal p24 antibody for 30 min at 4°C. Cells were then washed twice and suspended in 1% paraformaldehyde. Samples were acquired by use of a FACSCalibur instrument, and the resulting data were analyzed using FlowJo software.

RESULTS

Costimulation with plate-bound CD3 and soluble CD28 antibodies induces high levels of activation in $CD4^+$ T-cell cultures. To study X4 and R5 HIV-1 replication in primary $CD4^+$ T cells, we first optimized in vitro stimulation conditions for these cells. $CD4^+$ T cells were isolated from the PBMC of three donors by magnetic separation. The isolated cells were found to be >95% $CD3^+$ $CD4^+$ (data not shown). In vitro stimulation conditions were optimized by treating cells with increasing concentrations of plate-bound CD3 antibody and increasing concentrations of soluble CD28 antibody for various lengths of time (data not shown). Cellular activation was assessed by monitoring the expression levels of the activation markers CD38 and CD69 by flow cytometry at 0, 24, 48, and 72 h poststimulation. The highest level of activation was achieved following treatment with 50 μg of plate-bound CD3 antibody/ml and 1 μg of soluble CD28 antibody/ml (Fig. 1). CD38 and CD69 expression increased rapidly in these cultures, and by 48 h, an average of 29% of cells coexpressed both activation markers (Fig. 1). Activation marker expression was twofold lower in PHA-stimulated cultures, indicating that CD3/CD28 costimulation is a better method for activating purified $CD4^+$ T cells in vitro (Fig. 1).

Cellular activation was also assessed by a second, independent assay that measures cellular proliferation and IFN-γ expression. To measure activation-induced proliferation, stimulated $CD4^+$ T cells were stained with the green fluorescent dye CFSE. CFSE is a cytoplasmic dye that is equally divided among daughter cells during cell division. Consequently, the fluorescence of each daughter cell is half as bright as that of the parental cell, allowing for the reduction in fluorescence to be used as a marker of cellular proliferation. After CFSE staining, cells were cultured for 72 h and then fixed, permeabilized, and stained with fluorescently labeled antibodies directed against IFN-γ, CD3, and CD4. A large population of proliferating, IFN-γ-expressing $CD4^+$ T cells was detected in CD3/CD28-costimulated cultures (Fig. 2). This population of cells was not detected in PHA-stimulated cultures, confirming that CD3/CD28 costimulation induces higher levels of activation and proliferation in purified $CD4^+$ T-cell cultures than PHA treatment (Fig. 2).

CXCR4 and CCR5, the HIV-1 coreceptors, are expressed on CD3/CD28-costimulated $CD4^+$ T cells. To assess the potential susceptibility of costimulated $CD4^+$ T cells to HIV-1 infection, we examined coreceptor expression on these cells by flow cytometry. Cells from three independent donors were stimulated with either CD3/CD28 antibodies or PHA. At 0, 24, 48, and 72 h poststimulation, cells were stained with fluorescently conjugated monoclonal antibodies directed against CD4, CXCR4, and CCR5. At each time point, nearly all $CD4^+$ T cells from each donor expressed high levels of CXCR4, regardless of the stimulation method (Fig. 3). In contrast, very low levels of CCR5, the R5 coreceptor, were detected in PHA- and CD3/CD28-stimulated $CD4^+$ T-cell culture, and a high degree of variation among donors was detected (Fig. 4). CCR5 expression levels in both PHA- and CD3/CD28-costimulated cultures from each donor were found to increase over time, with expression levels peaking at 72 h poststimulation (Fig. 4).

The coreceptor usage of each HIV-1 strain was determined by GHOST cell assay. Prior to assessing the susceptibility of costimulated $CD4^+$ T cells to X4 and R5 strains of HIV-1, we first used GHOST-X4 and GHOST-Hi5 indicator cells to examine coreceptor usage of each strain. These cell lines were originally derived from HOS cells and express low levels of endogenous CXCR4. In addition, the GHOST-X4 cells were transduced with retroviral vectors carrying the human genes CD4 and CXCR4 and therefore express high levels of each receptor. The GHOST-Hi5 cells were transduced with vectors carrying the human genes CD4 and CCR5 and express high levels of each receptor as well as low levels of endogenous CXCR4. Each of these cell lines was also stably transfected with a construct carrying the GFP gene under the control of the HIV-1 LTR promoter. This permits HIV infection of these cells to be detected by flow cytometric analysis of GFP expression.

We infected the GHOST-X4 and GHOST-Hi5 cells with the following HIV-1 strains: LAI, JR-CSF, NL4-3, 49.5, KP1, and KP2. GFP expression was detected in GHOST-X4 cells infected with LAI, NL4-3, and KP2, confirming X4 coreceptor usage of these viral strains (Fig. 5). JR-CSF, 49.5, and KP1 did not induce GFP expression in these cells, indicating that these strains do not utilize the X4 coreceptor. High levels of GFP expression were detected in GHOST-Hi5 cells infected with strains JR-CSF, 49.5, KP1, and KP2, indicating R5 coreceptor usage of these strains (Fig. 5). The X4-tropic strain NL4-3 did not infect the GHOST-Hi5 cells; however, the X4-tropic strain LAI did infect a small population of these cells. This low-level infection was likely due to utilization of the endogenous CXCR4 expressed on the GHOST-Hi5 cells. The ability of KP2 to infect both GHOST-X4 and GHOST-Hi5 cells at equivalent levels indicates that this primary isolate has a dual-tropic phenotype.

R5-infected $CD4^+$ T cells produce more progeny virus over time than X4-infected $CD4^+$ T cells. $CD4^+$ T cells were isolated from PBMC and then stimulated for 72 h with CD3/CD28 antibodies. Costimulated cultures were infected with several strains of X4 and R5 HIV-1. Strains included the HIV-1 molecular clones; LAI (X4) and JR-CSF (R5), an isogenic viral pair, which differ only in the V1-V3 loop of gp120; NL4-3 (X4) and 49.5 (R5), two primary viral isolates recovered from the same patient at different stages of clinical infection; and KP1 (R5, early infection) and KP2 (X4/R5, late infection). Cells were infected with strains LAI, JR-CSF, NL4-3, and 49.5 at an MOI of 0.01 and with strains KP1 and KP2 at an MOI of 0.001. At several time points postinfection, the percentage of apoptotic cells was determined by surface staining with fluorescently labeled annexin V, and the percentage of infected cells was determined by intracellular p24 staining with a fluorescently conjugated antibody directed against HIV-1 p24 antigen (Fig. 6 and 7). The FACS plots from the flow cytometric analysis of the annexin V and intracellular p24 staining are shown in Fig. 6. Percentages of infected cells detected in each culture over time are summarized graphically in Fig. 7. In addition, the amount of viral production released by each infected culture was determined by p24 ELISA by measuring the amount of HIV-1 p24 antigen in the culture supernatants. Results of these assays are shown in Fig. 7, with error bars representing the standard deviations among triplicate infec-

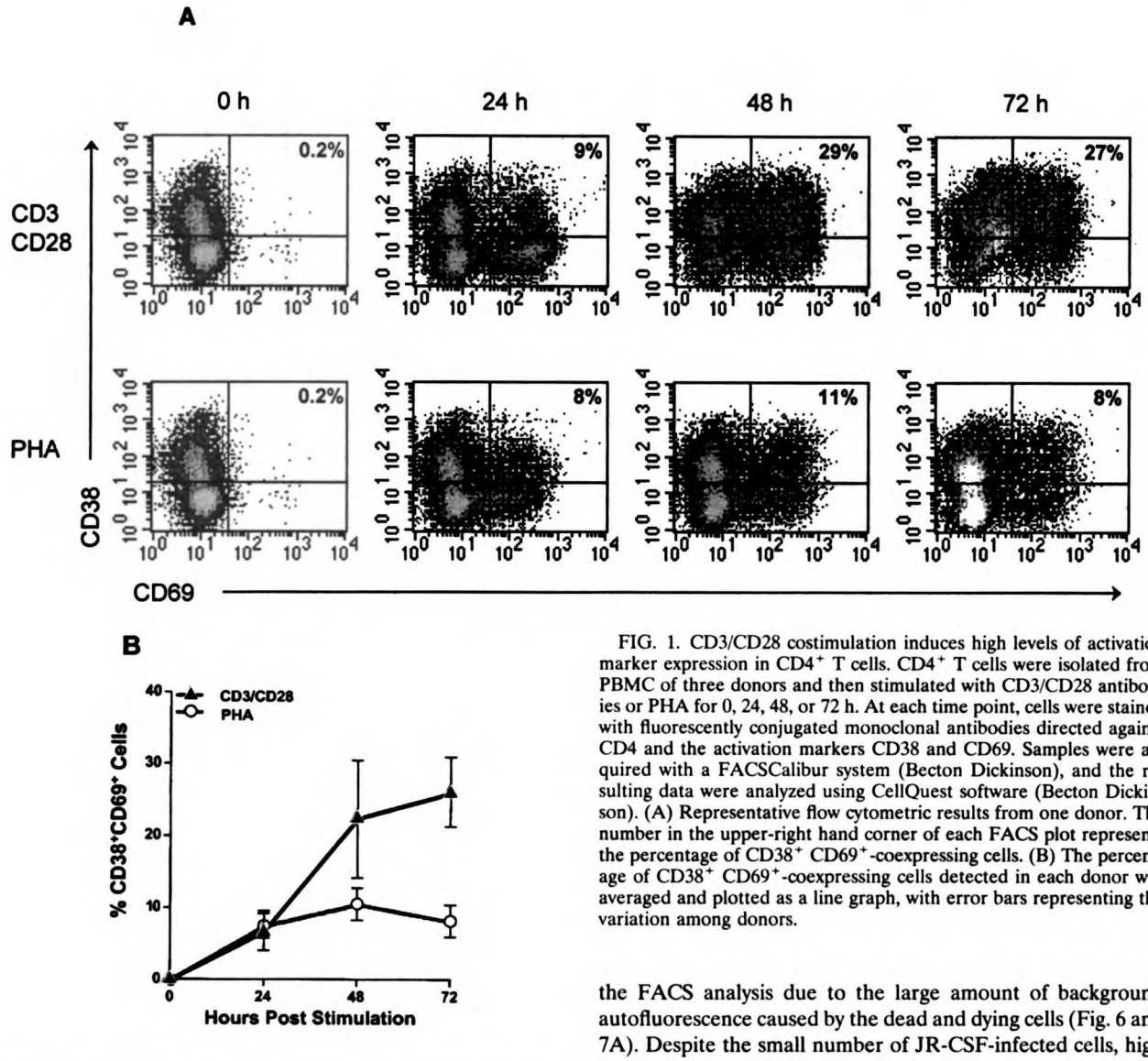

FIG. 1. CD3/CD28 costimulation induces high levels of activation marker expression in CD4+ T cells. CD4+ T cells were isolated from PBMC of three donors and then stimulated with CD3/CD28 antibodies or PHA for 0, 24, 48, or 72 h. At each time point, cells were stained with fluorescently conjugated monoclonal antibodies directed against CD4 and the activation markers CD38 and CD69. Samples were acquired with a FACSCalibur system (Becton Dickinson), and the resulting data were analyzed using CellQuest software (Becton Dickinson). (A) Representative flow cytometric results from one donor. The number in the upper-right hand corner of each FACS plot represents the percentage of $CD38^+$ $CD69^+$-coexpressing cells. (B) The percentage of $CD38^+$ $CD69^+$-coexpressing cells detected in each donor was averaged and plotted as a line graph, with error bars representing the variation among donors.

tions. Also, the cell viability of each infected culture over time was monitored using the trypan blue exclusion assay. Results of these assays are depicted in Fig. 7.

Our data demonstrate that R5-infected costimulated CD4+ T-cell cultures produce more progeny virus than X4-infected CD4+ T-cell cultures. In comparing cultures infected by the molecular clones LAI (X4) and JR-CSF (R5), we found that a much larger percentage of cells was infected by LAI than by JR-CSF at the early time points postinfection (Fig. 6 and 7A). For example, 29% of CD4+ T cells were infected by clone LAI at day 3 postinfection, but only 8% were infected by clone JR-CSF (Fig. 6 and 7A). This was likely due to the high expression level of CXCR4 and low expression level of CCR5 on these cells (Fig. 3 and 4). At the later time points postinfection, however, the percentage of apoptotic cells increased in the LAI-infected cultures, and the number of infected cells decreased (Fig. 6 and 7A). By day 7 postinfection, more than half of the cells in the LAI-infected culture were dead, obscuring the FACS analysis due to the large amount of background autofluorescence caused by the dead and dying cells (Fig. 6 and 7A). Despite the small number of JR-CSF-infected cells, high levels of viral production were detected in these cultures (Fig. 7A). The differential in viral production levels between the R5-tropic JR-CSF and the X4-tropic LAI clones was partially due to the fact that the cell viability in the LAI-infected cultures decreased rapidly whereas the cell viability remained relatively high in the JR-CSF-infected cultures over time (Fig. 7A). Analysis of costimulated CD4+ T-cell cultures infected with the isogenic pair NL4-3 (X4) and 49.5 (R5) provided further confirmation that R5-infected CD4+ T cells produce more progeny virus than X4-infected CD4+ T cells. A smaller percentage of costimulated CD4+ T cells was infected by the R5-tropic 49.5 at the early time points postinfection compared to the results seen with its isogenic X4 counterpart, NL4-3 (Fig. 6 and 7B). At day 5, 11.5% of CD4+ T cells were infected by the X4 strain whereas only 4.45% were infected by the R5 strain (Fig. 6 and 7B). Despite the presence of fewer R5-infected cells, similar levels of viral production were detected in the R5- and X4-infected cultures (Fig. 7B). Cell death seemed to play less of a role in the differential viral production

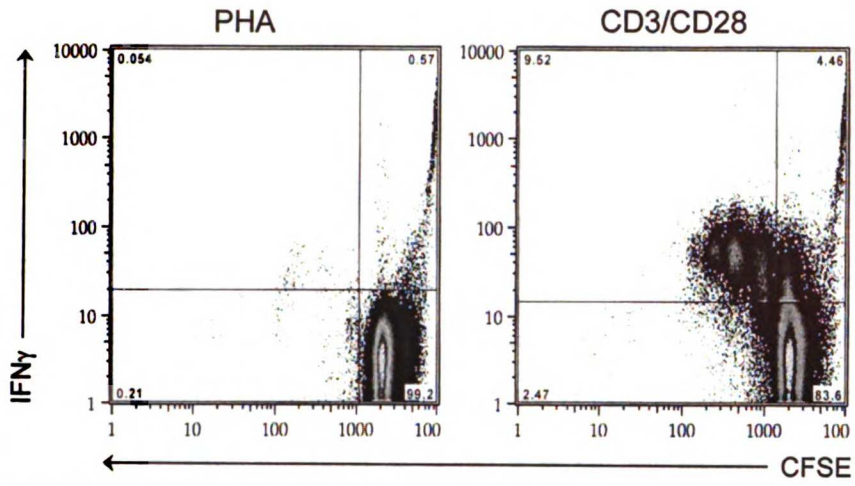

FIG. 2. CD3/CD28 costimulation induces high levels of cellular proliferation and IFN-γ expression in CD4+ T-cell cultures. Stimulated CD4+ T cells were stained with CFSE and then cultured for 72 h. Cells were then fixed, permeabilized, and stained with fluorescently labeled monoclonal antibodies directed against CD3, CD4, and IFN-γ. Samples were acquired on a FACSCalibur instrument (Becton Dickinson), and the resulting data were analyzed using FlowJo software (Tree Star, Inc.). The number in the each corner of each FACS plot represents the percentage of cells in that quadrant.

levels between these two strains, as the viability characteristics of both infected cultures remained comparable over time (Fig. 7B). Similar results were obtained in comparisons of cultures infected with the primary R5 isolate KP1 and the primary dual-tropic isolate KP2. Although KP1 and KP2 were found to infect similar numbers of cells and induce comparable amounts of cell death, viral production was considerably higher in cultures infected by the R5-tropic KP1 strain compared to the results seen with the dual-tropic KP2 strain (Fig. 6 and 7C). In summary, these data demonstrate that R5 HIV-1 has an increased capacity to replicate in costimulated CD4+ T cells compared to X4 HIV-1.

DISCUSSION

Our data provide evidence that R5 HIV-1 replicates more efficiently in primary CD4+ T cells than X4 HIV-1. Our first experiments focused on optimizing the in vitro stimulation conditions of primary CD4+ T cells. We found that costimulation with plate-bound anti-CD3 antibodies and soluble anti-

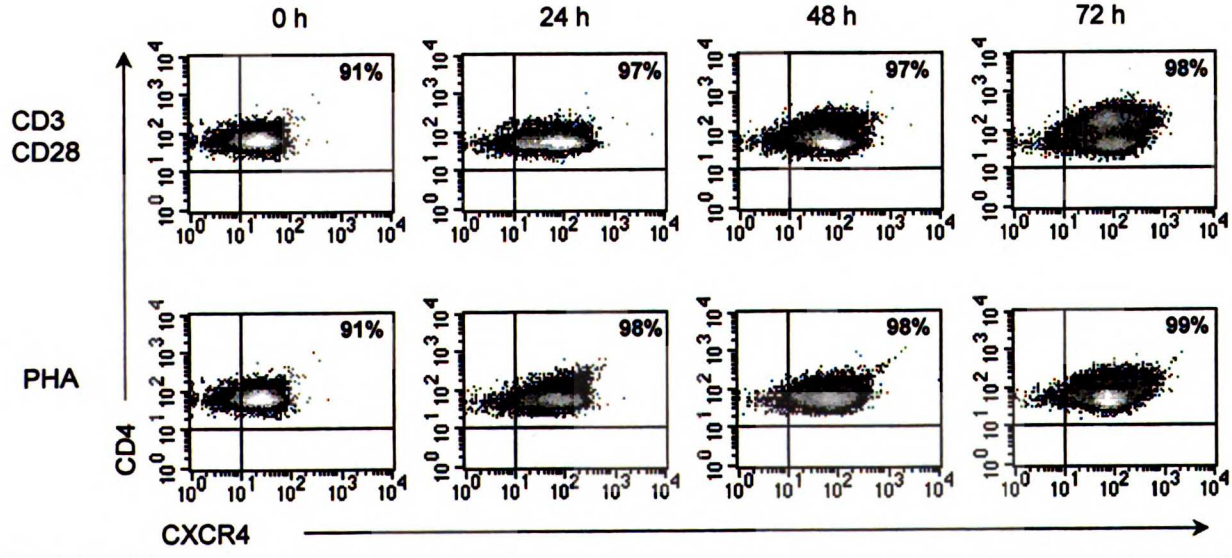

FIG. 3. CD3/CD28-costimulated CD4+ T cells express high levels of CXCR4. CD4+ T cells were isolated from PBMC of three donors and then stimulated with CD3/CD28 antibodies or PHA for 0, 24, 48, or 72 h. At each time point, cells were stained with fluorescently conjugated monoclonal antibodies directed against CD4 and CXCR4. Samples were acquired on a FACSCalibur instrument (Becton Dickinson), and the resulting data were analyzed using CellQuest software (Becton Dickinson). Representative results from one donor are shown. The number in the upper-right hand corner of each FACS plot represents the percentage of cells that express both CD4 and CXCR4.

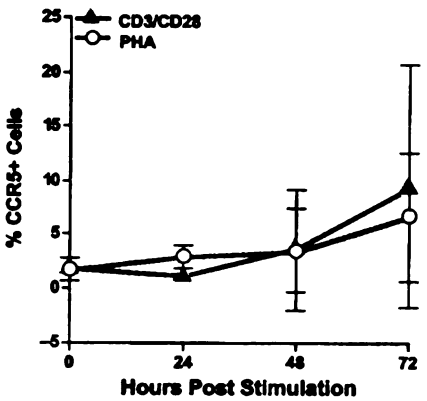

FIG. 4. CD3/CD28-costimulated CD4+ T cells express low levels of CCR5. CD4+ T cells were isolated from PBMC of 3 donors and then stimulated with CD3/CD28 antibodies or PHA for 0, 24, 48, or 72 h. At each time point, cells were stained with fluorescently conjugated monoclonal antibodies directed against CD4 and CCR5. Samples were acquired on a FACSCalibur instrument (Becton Dickinson), and the resulting data were analyzed using CellQuest software (Becton Dickinson). The average of the CCR5 expression levels of all three donors was plotted as a line graph, with error bars representing the standard deviations among donors.

CD28 antibodies induced high levels of activation and rendered cells permissible to X4 and R5 HIV-1 infection. This contradicts earlier reports that stimulation with CD3 and CD28 antibodies induced an R5-resistant state in CD4+ T-cell cultures (7, 31, 42). Reports from subsequent studies have indicated that the R5 resistance only occurred when CD4+ T cells were stimulated with CD3 and CD28 antibodies immobilized on magnetic beads. This phenotype was thought to be mediated by down-regulation of the CCR5 receptor and increased expression levels of β-chemokines (4, 12). Following costimulation with our protocol, CD4+ T cells were found to express low levels of CCR5 but were still able to replicate R5 virus efficiently. This may be due in part to the high activation state of the CD4+ T cells following CD3/CD28 costimulation. In addition, costimulated CD4+ T cells were found to express high levels of CXCR4 and to be highly susceptible to infection by X4 HIV-1.

We present evidence that R5-infected CD4+ T cells produce more progeny virus than X4-infected cells. Direct comparisons were made between costimulated CD4+ T-cell cultures infected with X4 and R5 HIV-1 strains. We first compared cultures infected with two molecular clones of HIV-1, LAI (X4) and JR-CSF (R5). The R5-tropic JR-CSF clone infected far fewer cells than the X4-tropic LAI clone and yet produced greater amounts of progeny virus over time. The striking difference in the replication capacity characteristics of these two strains can partly be explained by the variation in virus-induced cell death in these infected cultures. Cell viability decreased rapidly in LAI-infected cultures and yet remained relatively high in JR-CSF-infected cultures during the course of the experiment. These data demonstrate that X4 replication can be limited in CD4+ T-cell cultures by extensive virus-induced cell death, whereas viral production remains high in cultures infected by less-cytopathic viruses. We next compared cultures infected with the isogenic HIV-1 strains NL4-3 (X4) and 49.5 (R5). Because these viruses differ only in the V3 region of the envelope gene, differences in viral replication kinetics are likely mediated by coreceptor usage. We found that a smaller percentage of costimulated CD4+ T cells was infected by the R5-tropic 49.5 strain than by its isogenic X4 counterpart strain NL4-3, and yet similar levels of viral production were detected in both infected cultures. Virus-induced cell death played less of a role in the differential viral production of these two viruses, since the viability characteristics of the two infected cultures remained comparable over time. The final comparison between the R5-tropic primary isolate KP1 and the dual-tropic primary isolate KP2 yielded similar results. Although KP1 and KP2 infected similar percentages of costimulated CD4+ T cells and induced comparable amounts of cell death, viral production was considerably higher in KP1-infected cultures. Taken together, these data provide evidence that R5-infected CD4+

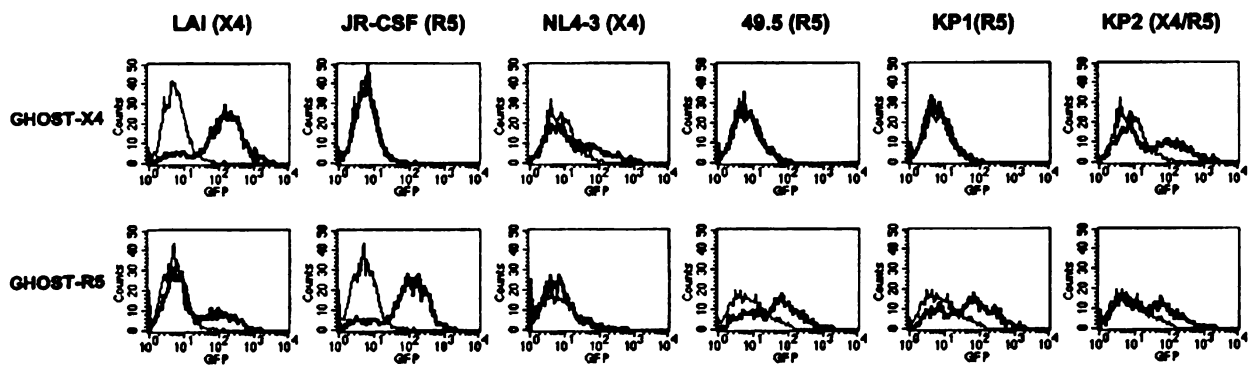

FIG. 5. Coreceptor usage of each HIV-1 strain was determined by GHOST cell assays. The GHOST-X4 and GHOST-Hi5 cells were infected with virus for 48 h prior to measurement of GFP expression by flow cytometry. Samples were acquired on a FACSCalibur instrument (Becton Dickinson), and the resulting data were analyzed using CellQuest software (Becton Dickinson). The top row of FACS plots represents GHOST-X4 infections, and the bottom row represents GHOST-Hi5 infections. The thin-lined peak in each plot represents the background fluorescence of uninfected cells, and the bold-lined peak represents the GFP fluorescence detected in cells infected with the indicated HIV-1 strain. The HIV-1 strains LAI and NL4-3 utilize the X4 coreceptor, the strains JR-CSF, 49.5, and KP1 utilize the R5 coreceptor, and the KP2 strain utilizes both the X4 and R5 coreceptors.

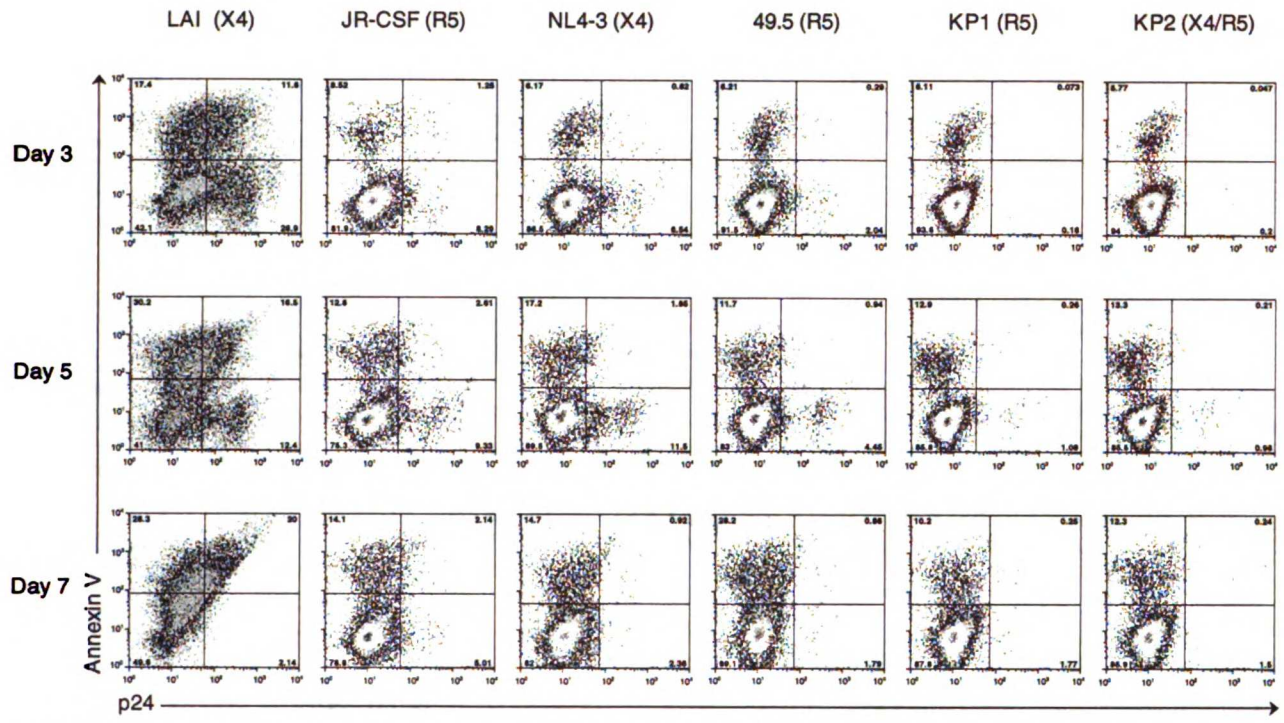

FIG. 6. The percentage of costimulated CD4$^+$ T cells infected by each viral strain was determined by intracellular p24 staining. CD3/CD28-costimulated CD4$^+$ cells were infected with LAI, JR-CSF, NL4-3, and 49.5 at an MOI of 0.01 and with KP1 and KP2 at an MOI of 0.001. Apoptotic cells were detected by surface staining with fluorescently conjugated annexin V, and infected cells were detected by intracellular staining for HIV-1 p24 antigen. Samples were acquired on a FACSCalibur instrument (Becton Dickinson), and the resulting data were analyzed using FlowJo software (Tree Star, Inc.). Results from days 3, 5, and 7 postinfection are shown. The number in each corner of each FACS plot represents the percentage of cells in that quadrant.

T cells produce more virus over time than X4-infected CD4$^+$ T cells.

Other groups have also observed a connection between R5 coreceptor usage and increased viral replication. In these studies, X4 and R5 isogenic strains were used to infect human lymphoid tissue cultures (17, 20; A. M. Roy, D. A. Eckstein, and M. A. Goldsmith, Abstr. 2002 Keystone Symposia: HIV Pathogenesis, abstr. 329, 2002). These cultures contain various cell types in addition to CD4$^+$ T cells and require no exogenous stimulation to confer susceptibility to HIV-1 infection. Infection with R5 and X4 HIV-1 isogenic strains resulted in similar amounts of viral production in the lymphoid cultures despite the presence of fewer R5-infected cells (17, 20; Roy et al., 2002 Keystone Symposia). The results of these studies support the conclusion that R5 HIV-1 has a higher replication capacity than X4 HIV-1.

We propose that the increased replication capacity of R5 strains may contribute to the R5 dominance of early HIV-1 infection. R5 viruses have the selective advantage of targeting the more activated CD4$^+$ T cells that express higher levels of transcription factors, such as NFκB, which have been linked to increased HIV LTR promoter activity (36). This may lead to higher viral production levels and preferential spread of R5 viruses in vivo. In addition, X4 viral strains are often associated with increased cytopathicity, which may lead to differential life spans of X4- and R5-infected CD4$^+$ T cells. As a result, R5-infected CD4$^+$ T cells may live longer and release more virus over time than X4-infected CD4$^+$ T cells.

It has been suggested that R5-infected CD4$^+$ T cells may be less susceptible to immune-mediated killing than X4-infected CD4$^+$ T cells. Experiments performed in the macaque model have shown that both X4 and R5 SHIV replication can be detected early after coinfection; however, R5 dominance develops within weeks of the initial infection (22). Interestingly, experimental depletion of CD8$^+$ T cells in these animals results in reemergence of X4 replication, suggesting that the CD8-mediated immune response is more effective at eliminating X4-infected cells (22). This may partially be due to compartmentalization of X4- and R5-infected cells. X4 viruses are more likely to infect circulating CD4$^+$ T cells, which may be more accessible to CD8$^+$ T-cell surveillance than R5-infected activated CD4$^+$ T cells and macrophages that are located deep within tissues. The idea that X4-infected cells are more susceptible to immune-mediated elimination suggests that the emergence of X4 strains in the later stages of disease may be the result of immune exhaustion.

In summary, we present evidence that R5 HIV-1 strains replicate more efficiently in CD3/CD28-costimulated CD4$^+$ T cells than X4 HIV-1 strains. We found that noncytopathic R5 HIV-1 has a greater capacity to replicate in CD4$^+$ T cells than cytopathic X4 strains. In addition, we further analyzed the impact of coreceptor usage on viral production by comparing

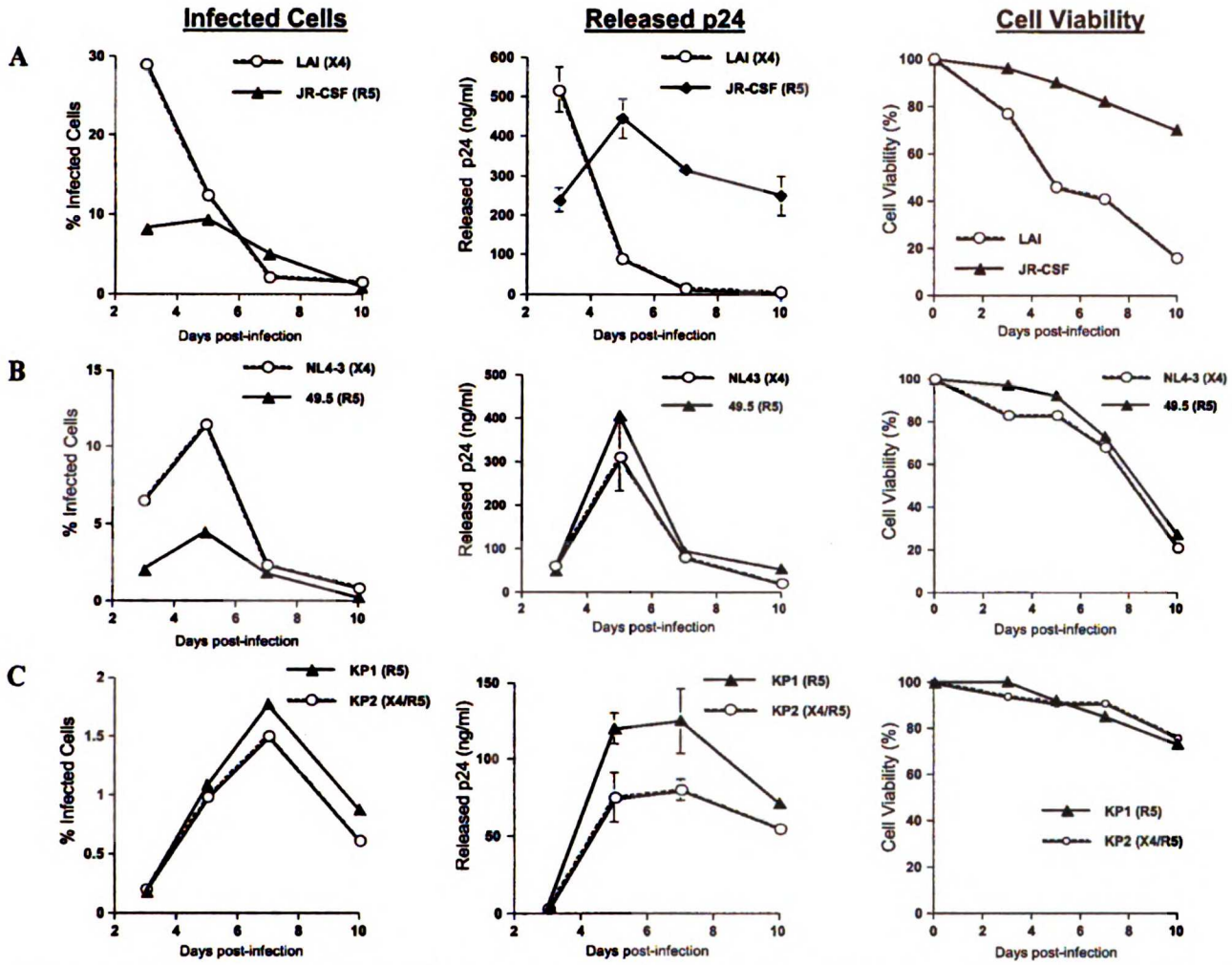

FIG. 7. R5-infected CD4+ T cells produce more progeny virus over time than X4-infected CD4+ T cells. CD4+ T cells were isolated from PBMC and then stimulated for 72 h with CD3/CD28 antibodies prior to HIV-1 infection. At 3, 5, 7, and 10 days postinfection, the percentage of infected cells in each culture was determined by intracellular p24 staining and flow cytometry, the amount of virus released from each culture was measured by p24 ELISA, and the cell viability of each culture was determined by trypan blue exclusion assay. (A) Comparison of percentages of infected cells, viral release characteristics, and percentages of cell viability in costimulated CD4+ T-cell cultures infected with the HIV-1 molecular clones LAI (X4) and JR-CSF (R5). (B) Comparison of percentages of infected cells, viral release characteristics, and percentages of cell viability in costimulated CD4+ T-cell cultures infected with the isogenic HIV-1 strains NL4-3 (X4) and 49.5 (R5). (C) Comparison of percentages of infected cells, viral release characteristics, and percentages of cell viability in costimulated CD4+ T-cell cultures infected with the primary HIV-1 isolates KP1 (R5) and KP2 (X4/R5).

X4 and R5 viruses that share greater homology and induce similar cytopathic effects. These experiments have provided evidence that R5-infected CD4+ T cells produce more virus over time than X4-infected CD4+ T cells. We suggest that this replication advantage may contribute to the preferential spread of R5 viruses during the early stages of HIV-1 infection.

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Enhanced Replication of R5 HIV-1 Over X4 HIV-1 in CD4$^+$CCR5$^+$CXCR4$^+$ T Cells

Ann-Marie Roy, BS, Becky Schweighardt, PhD,* Lauren A. Eckstein, MD, PhD,* Mark A. Goldsmith, MD, PhD,* and Joseph M. McCune, MD, PhD*†*

Summary: To enter human cells, HIV-1 usually uses CD4 and 1 of 2 coreceptors: CCR5 and CXCR4. Interestingly, even though CCR5 is expressed on far fewer T cells than is CXCR4, many patients in early- and late-stage HIV disease maintain high levels of CCR5-tropic (R5) viruses. We hypothesized that such high R5 viral loads may be sustained because, relative to CXCR4-tropic (X4) HIV-1 infection, R5 HIV-1 infection of permissive CD4$^+$CCR5$^+$CXCR4$^+$ T cells results in the production of significantly more infectious virus particles per target cell. To investigate this possibility, we compared the levels of virus production per target cell after isogenic R5 and X4 HIV-1 infection of 2 in vitro primary human lymphocyte culture systems: T-cell receptor–stimulated blood-derived CD4$^+$ T cells and tonsil histoculture (which requires no exogenous stimulation for ex vivo infection). We provide evidence that R5 HIV-1 does indeed compensate for a small target cell population by producing, on average, 5 to 10 times more infectious virus per CCR5$^+$ target cell than X4 HIV-1. This replicative advantage may contribute to the predominance of R5 HIV-1 in vivo.

Key Words: HIV, coreceptor, pathogenesis, viral output per target cell

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The disease course induced by HIV-1 typically passes through 3 distinct phases. The first phase, lasting for a period of weeks to several months and associated with flu-like symptoms and CD4$^+$ T-cell depletion, is often dominated by CCR5-tropic (R5) HIV-1 variants that use the chemokine coreceptor CCR5 in addition to CD4 for entry into target cells.[1–3] After the generation of a virus-specific immune response, levels of virus replication recede, CD4 counts stabilize, and a period (variably lasting 4 or more years) of relative clinical quiescence ensues.[4] Finally, in later stages of disease, levels of viral replication rebound, associated with quantitative and qualitative changes in the CD4$^+$ and CD8$^+$ T-cell compartments, immunodeficiency, and mortality attributable to opportunistic infections.[5] In approximately 50% of infected individuals, this last phase is marked by the emergence of CXCR4-tropic (X4) HIV-1 variants that use the chemokine coreceptor CXCR4 in addition to CD4 for target cell entry.[6–8] Because X4 variants have an increased propensity to induce cytopathicity in vitro and a larger target pool size in vivo, their emergence might be responsible for accelerated disease progression.[9–13] Yet, 40% to 50% of those infected with B clade virus progress to AIDS with R5 variants alone.[14–16]

Several hypotheses have been advanced to explain the intrinsic pathogenicity of R5 variants of HIV-1. For instance, some studies suggest that R5 HIV-1 is selectively transported across mucosal epithelial barriers.[17] Studies in primate models, however, have shown that although R5 HIV-1 and X4 HIV-1 can be efficiently transmitted through vaginal infection, X4 variants eventually disappear and R5 HIV-1 strains disseminate throughout the body.[18] This observation is more consistent with the possibility that R5 HIV-1 may possess an advantage in replication and/or spread after target cell infection, perhaps a function of the differential representation of CCR5$^+$ macrophages and dendritic cells at these interfaces.[17,19,20] Evidence that longer lived cells such as macrophages are capable of producing greater amounts of virus than their CD4$^+$ T-cell counterparts has been provided by several recent studies.[21–23] In 1 such study using human lymphoid histoculture (HLH), donor tissues were infected with an isogenic pair of HIV-1 strains differing only in coreceptor preference. Despite the fact that 5 to 10 times more CD3$^+$ cells were infected with X4 HIV-1 than with R5 HIV-1, the X4 and R5 HIV-1–infected cultures released approximately equivalent amounts of HIV-1 Gag protein (detected as p24) over time.[24] Because macrophages are abundant in HLH, the high output of R5 HIV-1 was attributed to production by infected macrophages that are not susceptible to X4 HIV-1. This explanation was supported by later work in the same system21 examining isolates of HIV-1 that were defective in Vpr. Vpr is an HIV-1 protein required for replication in nondividing cells such as macrophages; Vpr-deficient R5 HIV-1 strains showed a 50% reduction in p24 production, whereas the absence of Vpr had no significant impact on X4 HIV-1 infection, demonstrating that macrophage infection contributes a significant amount of the virus generated after

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From the *Gladstone Institute of Virology and Immunology, University of California, San Francisco, CA; and †Department of Medicine, San Francisco General Hospital, University of California, San Francisco, CA.

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Reprints: Joseph M. McCune, Gladstone Institute of Virology and Immunology, 1650 Owens Street, San Francisco, CA 94158 (e-mail: mmccune@gladstone.ucsf.edu).

R5 HIV-1 infection of lymphoid tissue. This study did not rule out the possibility that the surprisingly high viral output of R5 HIV-1 is partially attributable to an advantage in $CCR5^+CXCR4^+$ T cells, however.

Initially, we tested this hypothesis by isolating $CD4^+$ T cells from peripheral blood of healthy donors and rendering them susceptible to HIV-1 infection via stimulation with antibodies against CD3 and CD28. This study demonstrated that such cultures produced more virus over the course of a 10-day in vitro infection (as measured by p24 production in the supernatant) when infected by R5 as compared with X4 HIV-1, a difference in virus production that did not seem to be the result of enhanced X4 HIV-1 cytopathicity in vitro.[25] We have extended these observations in the current study using ex vivo lymphoid histoculture and a quantitative approach that assesses not simply p24 production but the production of infectious virions. We directly addressed the possibility that $CD4+CCR5^+CXCR4^+$ T cells may produce a larger number of virions per infected cell after R5 HIV-1 infection than after X4 HIV-1 infection. We show here that infection with R5 HIV-1 does, in fact, result in the generation of more progeny per infected $CD4^+$ T cell than infection with X4 HIV-1. Such a difference in "burst size" might contribute to the prevalence of R5 HIV-1 in the setting of infections in vivo.

MATERIALS AND METHODS

Antibodies

For analysis and sorting of cells by flow cytometry as well as for stimulation of cells in tissue culture, commercially available monoclonal antibodies were used (diluted in phosphate-buffered saline [PBS] with 1% fetal bovine serum [FBS]), including CD3 Peridinin-chlorophyll protein complex (PerCP) at 1:10 (BD Immunocytometry), CD4 allophycocyanin (APC) at 1:10 (BD PharMingen), CD8 phycoerythrin (PE) at 1:10 for analysis and 1:20 for cell sorting (BD Immunocytometry), CCR5 fluorescein isothiocyanate (FITC) at 1:5 or 1:7.5 (BD PharMingen), CXCR4 APC at 1:10 (BD PharMingen), CD25 PE at 1:10 (BD PharMingen), CD69 APC at 1:10 (BD PharMingen), p24 PE at 1:100 (Beckman Coulter), anti-FITC rabbit IgG, Alexa 488 conjugate at 2.5 $\mu g/10^6$ cells (Molecular Probes), goat anti-rabbit IgG, Alexa 488 conjugate at 5 $\mu g/10^6$ cells (Molecular Probes), CD14 PE at 1:20 (BD Immunocytometry), CD19 PE at 1:20 (BD Immunocytometry), CD3 pure at 50 $\mu g/mL$ (Zymed), and CD28 pure at 100 $\mu g/mL$ (BD Biosciences).

Virus Preparation

Virus stocks of the molecular clones NL4-3 (a gift from Malcolm Martin via the National Institutes of Health [NIH] AIDS Research and Reference Reagent Program) and 49-5 and 81A (gifts from Bruce Chesebro) were derived from plasmid transfection of 293T cells. The 49-5 and 81A are viruses that are isogenic to NL4-3 except for determinants in the V1, V2, and V3 regions that specify coreceptor preference.[26–28] Specifically, 81A substitutes the V1 to V3 loops of BaL into the NL4-3 backbone, whereas 49-5 incorporates only the V3 region of BaL into NL4-3. Transfection was performed using the calcium phosphate method. Virus-containing supernatants were harvested at 72 and 120 hours after transfection, sterile filtered, and titered to determine a tissue culture infectious dose-50 ($TCID_{50}$). Briefly, peripheral blood mononuclear cells (PBMCs) from human blood buffy coats (Stanford Blood Bank) were separated by Ficoll separation (see below) and stimulated with phytohemagglutinin (PHA). The cells were then brought to a concentration of 2×10^6 cells/mL in RPMI 1640 medium (Mediatech-Cellgro) supplemented with 10% heat inactivated (HI) FBS (Gemini Bioproducts), 2 $\mu g/mL$ PHA (Sigma), 1% penicillin/streptomycin (Mediatech-Cellgro), and 1% L-glutamine (Mediatech-Cellgro). After 24 hours, the cells were rinsed in fresh media without PHA and cultured in RPMI 1640 with 10% HI FBS, 1% penicillin/streptomycin, 1% L-glutamine, and 5 U/mL of human recombinant interleukin (IL)-2 (Boehringer Mannheim) until use in the $TCID_{50}$ assay. To perform the $TCID_{50}$ assay itself, serial half-log dilutions of virus supernatants were prepared in IL-2–containing RPMI, and 25 μL of each dilution was added to quadruplicate wells of PHA-stimulated PBMCs (3-donor pool) at 1×10^5 cells per well in a U-bottom 96-well culture plate (Corning). After incubation for 2 hours at 37°C, 200 μL of IL-2–containing culture media was added to each well and the plates were incubated at 37°C for 5 days. The $TCID_{50}$ is the reciprocal of the dilution at which 50% of the wells contained detectable (>30 pg/mL) p24 (capsid) protein by enzyme-linked immunosorbent assay (ELISA; NEN Perkin-Elmer).

Cell Culture

The 293T cells for virus preparation were cultured in Dulbecco modified Eagle medium (DMEM; Mediatech-Cellgro) supplemented with 10% HI FBS and penicillin/streptomycin, passaging every 3 to 4 days. Human tonsil tissue (from the National Disease Research Institutes, Cooperative Health Tissue Network, Kaiser San Rafael, Kaiser South San Francisco, and Kaiser San Francisco) was obtained with approval from the University of California, San Francisco Committee on Human Research and was processed into human lymphoid aggregate cultures (HLACs). Briefly, fresh tonsil tissue from routine tonsillectomies was fully dispersed to create a single cell suspension in RPMI media (supplements detailed below). The cells were then counted and plated at a concentration of 2×10^6 cells per well in a 96-well U-bottom culture plate. The cells settled naturally to form high-density aggregates at the base of each well. HLACs were maintained in RPMI medium supplemented with 10% FBS, 1× nonessential amino acids (Mediatech-Cellgro), 100 mM of sodium pyruvate (Mediatech-Cellgro), 10 $\mu g/mL$ of gentamicin (Gibco Invitrogen), 100 ng/mL of ampicillin (Sigma), and 250 $\mu g/mL$ of amphotericin B (Mediatech-Cellgro). PHA-stimulated PBMC cultures were prepared as described previously and maintained in RPMI medium supplemented with 10% FBS, penicillin/streptomycin, and 5 U/mL of IL-2 (Boehringer Mannheim). $CD4^+$ T cells isolated from PBMCs were maintained after stimulation in RPMI supplemented with 10% FBS and penicillin/streptomycin.

Virus Infections

All HIV-1 infections were carried out at low multiplicity of infection (moi = 0.001) in a designated BSL-3 facility.

Target cell cultures were incubated overnight (12–16 hours) with virus-containing media, which was then replaced with fresh media. Infections were monitored via fluorescence-activated cell sorter (FACS) analysis of intracellular p24 each day after day 4, and infected cultures were manipulated as described for individual experiments.

Assessment of Infection Kinetics in R5 Versus X4 HIV-1

HLACs were infected with NL4-3 or 81A at an moi of 0.001 on day 0 and incubated at 37°C in 5% CO_2. On day 4, samples were removed and stained for surface expression of CD3, CD4, and CD8 as well as for intracellular expression of p24. Samples were fixed in 1% paraformaldehyde (Sigma)/PBS (Mediatech-Cellgro) and permeabilized in 0.1% Triton X-100 (Sigma)/PBS to permit intracellular staining. Supernatants from the same wells were harvested and assessed for p24 content by p24 ELISA analysis. All samples were assessed in triplicate. This procedure was repeated until day 8 or 9, depending on culture viability. Infection kinetic experiments in PBMC-derived $CD4^+$ T cells were performed using the same methods.

Flow Cytometric Sorting of $CD4^+$ T-Cell Subsets From Human Lymphoid Aggregate Cultures and Peripheral Blood Mononuclear Cells

On days 5 through 7 of HIV-1 infection in HLACs, 360×10^6 infected cells per infection condition were harvested and rinsed in PBS/1% FBS (FACS buffer). Cultures were then resuspended in FACS buffer containing CCR5 FITC (1:7.5), CD8 PE, CD14 PE, and CD19 PE (each 1:20) antibodies and incubated at 4°C for 30 minutes in the dark. The cells were rinsed in a large volume of FACS buffer with 2 mM of ethylenediamine tetra-acidic acid (EDTA; KD Medical), resuspended in FACS buffer with EDTA containing anti-PE microbeads at 1:5 (Miltenyi Biotec), and incubated at 4°C for 30 minutes in the dark. Cells were then rinsed in FACS buffer, resuspended in 4 mL of FACS buffer with 2 mM of EDTA, and passed through a MACS magnetic bead sorter (Miltenyi Biotec) to remove PE-labeled cells. The negative fraction, consisting primarily of $CD4^+$ T cells, was retained, and the positive fraction was discarded. The enriched $CD4^+$ T cells were resuspended in FACS buffer containing 2.5 $\mu g/10^6$ cells of rabbit anti-FITC IgG-Alexa 488 and incubated at 4°C for 30 minutes (in the dark). After rinsing, the cells were resuspended in FACS buffer containing 5 $\mu g/10^6$ cells of goat anti-rabbit IgG-Alexa 488 for an additional 30 minutes under the same conditions. These final 2 staining steps resulted in significant amplification of anti-CCR5 fluorescence (data not shown). The cells were then rinsed for a final time, resuspended in 4 mL of FACS buffer, passed through a 5-mL polystyrene tube with a cell strainer cap (Falcon), and sorted into FITC/Alexa 488^+CCR5^+ and FITC/Alexa 488^-CCR5^- populations on a FACS Vantage (Becton Dickinson), gating on the live lymphocyte population by forward and side scatter and gating out any remaining PE-positive events to eliminate residual cells positive for CD8, CD14, or CD19 electronically. The cells were sorted into 15-mL Falcon tubes (Fisher) containing 1 mL of FBS, and the numbers of FITC/Alexa 488^+ and FITC/Alexa $^-$ events were recorded for future calculations. Sorted cells were then rinsed in preparation for further manipulations to determine the average viral output per infected cell (see below). PBMC-derived $CD4^+$ T cells were subjected to the same procedure, with the elimination of steps needed for removal of $CD8^+$, $CD14^+$, and $CD19^+$ cell types, which were performed before infection (see below).

Peripheral Blood Mononuclear Cell–Derived $CD4^+$ T-Cell Isolation and Stimulation

PBMCs were isolated from leukocyte/buffy coats (Stanford Blood Bank) by Ficoll (Sigma) separation. Briefly, 30 mL of buffy coat was diluted 1:5 in sterile PBS and underlaid with 14 mL of Ficoll per 30 mL of diluted cells in 50-mL Falcon tubes (Fisher). The tubes were spun at 1400 rpm in a Beckman GS-6R centrifuge for 30 minutes at room temperature with the centrifuge brake disengaged. The upper layer of liquid was removed, and the PBMC layer was set aside. When needed, 10 mL of ammonium, chloride, potassium buffer (ACK) lysis buffer (Quality Biologic) was added to remove erythrocytes, incubating at room temperature for 5 minutes. The cells were rinsed 3 times in large volumes of PBS and counted on a hemocytometer. $CD4^+$ T cells were negatively selected using a $CD4^+$ T-cell isolation kit (Miltenyi Biotec), as per the manufacturer's instructions, in combination with LS Columns for Midi MACS (Miltenyi Biotec) and the Midi MACS Separation Unit (Miltenyi Biotec). This method typically yielded $CD4^+$ T cells with greater than 95% purity. These cells were then stimulated with 50 $\mu g/mL$ of plate-bound purified anti-CD3 (Zymed) and 100 $\mu g/mL$ of soluble purified anti-CD28 (BD Biosciences) antibodies for 72 hours at 37°C,[25,29–31] and they were then rinsed and plated in 96-well U-bottom plates at 5×10^5 cells/mL. These cells were then infected in the same manner as the HLACs and manipulated as required for individual experiments.

Determination of Average Viral Output per Infected Cell

After isolation of $CD4^+CCR5^+$ and $CD4^+CCR5^-$ T cells, one third of the total were assessed for the presence of intracellular p24 by p24 ELISA assay. The remaining cells were cultured at 37°C for 36 hours, after which the supernatant was harvested and assessed for the presence of p24 by ELISA and for infectious virus by $TCID_{50}$ assay. These values were then used, in combination with the percentage of $CD4^+CCR5^+$ and $CD4^+CCR5^-$ cells infected at the time of sorting (as determined by FACS analysis of intracellular p24) and the absolute number of cells in each sorted population (corrected for the fraction of cells used to assess viral production in each case) to determine the average output of viral protein/infectious virus per infected cell, according to the following equation: average virus production per cell = nanograms of intracellular p24 or p24 produced or $TCID_{50}$s produced/number of infected cells in each subset.

The number of infected cells in each subset could be determined according to the following equation: number of infected cells in each subset = [absolute number of sorted cells

in each subset ($CD4^+CCR5^+$ or $CD4^+CCR5^-$)] × (% of infected cells in each subset, as determined by FACS analysis for intracellular p24) × (fraction of total sorted cells used to assess each measure of viral production [ie, 0.33 for intracellular p24 and 0.67 for supernatant p24 and $TCID_{50}$]).

RESULTS

High Levels of R5 HIV-1 p24 Production in Human Lymphoid Aggregate Cultures

Previous experiments revealed equivalent levels of R5 and X4 HIV-1 production after infection of intact blocks of tonsillar tissue in HLH.[24,32] We further examined these observations in the context of an alternative culture system, the HLAC.[33] In the preparation of such cultures, a fixed number (2×10^6) of dispersed tonsillar cells was plated in each well of the 96-well U-bottom plates and allowed to settle into a high-density aggregate. Thereafter, and in contradistinction to other in vitro systems, HIV-1 replication proceeded in the absence of exogenous stimulation. This in vitro culture system has 2 advantages over HLH: first, the number of cells placed into each well is known; and second, all wells are equivalent. A potential disadvantage of HLAC is that some cells (eg, $CD4^+$ myeloid cells) that are contained in HLH are only poorly represented after transfer of dispersed tonsillar cells into the 96-well plate (data not shown). Such selective loss of macrophage populations was advantageous in the case of these experiments, permitting focused attention on HIV-1 interactions with $CD4^+$ T cells.

HLACs were infected in triplicate with equivalent inputs (0.001 moi) of X4 (NL4-3) or R5 (81A) HIV-1. These viruses are identical except for determinants in the V1 to V3 loops that confer differential tropism for CXCR4 or CCR5, respectively. On each day after infection (day 4 through day 8 or 9), culture supernatants were harvested and the amount of p24 (HIV-1 capsid protein) produced was measured by p24 ELISA. Concomitantly, cells from the infected cultures were permeabilized and stained with antibodies against HIV-1 p24 to determine the percentage of infected cells. As shown by the example of 2 representative donors (n = 19), HIV-1 infection of HLACs resulted in a time-dependent increase in supernatant p24 (Fig. 1). In some cases, represented by donor 1 (see Fig. 1A, left panel), more p24 was produced after R5 HIV-1 infection than after X4 HIV-1 infection; in other cases, represented by donor 2 (see Fig. 1B, left panel), the opposite was observed. In all cases, however, the percentage of $p24^+$ cells after R5 HIV-1 infection was much smaller, in the range of 1% to 15% at day 8 or 9, than that generated after X4 HIV-1 challenge, 30% to 40% at

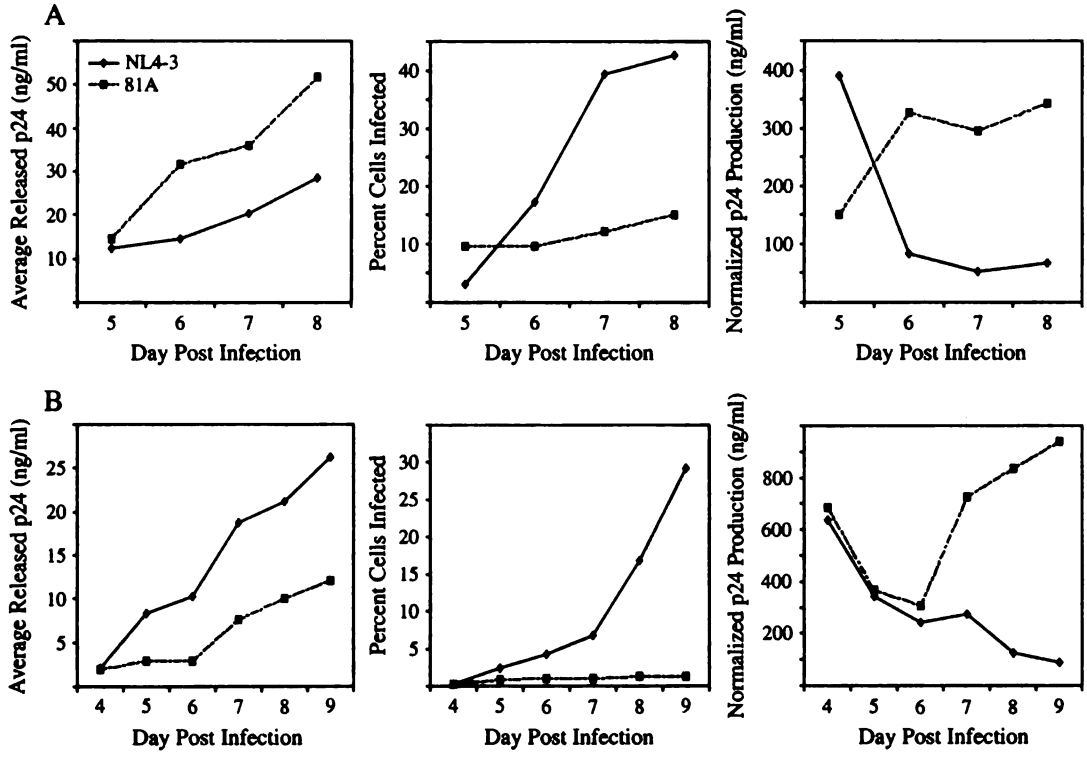

FIGURE 1. Assessment of HIV-1 replication kinetics in HLAC. HLACs were infected at an moi of 0.001 with X4 HIV-1 (NL4-3) or R5 HIV-1 (81A). Data for 2 independent tonsil donors are shown (A, donor 1) and (B, donor 2). Supernatants of the infected cultures were collected between days 4 and 9 and assessed for the amount of released p24 (ng/mL) by p24 ELISA (left panels). In parallel, the percentage of infected $CD3^+CD4^+$ cells was measured by flow cytometry (center panels). Values shown represent the average of triplicate wells. The supernatant p24 levels were then divided by the corresponding fraction of infected cells to yield the normalized supernatant p24 data, expressed as "normalized p24 production (ng/mL)" (right panels). These data are representative of experiments using 19 different donors.

the same time point (see Figs. 1A, B, middle panels). When supernatant p24 levels were normalized for the percentage of cells infected, the average amount of virus (p24) produced after R5 HIV-1 infection was 5- to 10-fold higher than that observed after X4 HIV-1 infection (see Figs. 1A, B, right panels).

This apparent disparity in virus production might be attributable to a virus-intrinsic property (ie, a given target cell might produce more p24 after infection with R5 than with X4 HIV-1). Alternatively, it might be attributable to a cell intrinsic property (ie, R5 and X4 viruses infect different target cells, and those cells infected with R5 HIV-1 are more efficient in replicating and/or releasing HIV-1). In either case, it is also possible that the ratio of replication competent to defective virus released into the supernatant is nonidentical after R5 or X4 infection.

Apparent Replicative Advantage of R5 HIV-1 Is Virus Intrinsic

To discriminate between these possibilities, the amount of infectious virus made per cell after R5 or X4 infection was calculated more precisely. In HLACs, 80% to 100% of $CD3^+CD4^+$ T cells express CXCR4, whereas 5% to 15% express CCR5; notably, nearly all $CD3^+CD4^+CCR5^+$ T cells are also $CXCR4^+$ and therefore permissive for entry by R5 and X4 HIV-1 (Fig. 2A). To determine whether such $CD4^+CCR5^+CXCR4^+$ T cells might produce more virus after R5 HIV-1 infection than after X4 HIV-1 infection, HLACs were infected with equivalent amounts (0.001 moi) of NL4-3 (X4) or the isogenic isolate 49-5 (R5). At an early time point after infection (day 5 or 6), before noticeable levels of cell death in infected cultures (as assessed by CD4/CD8 ratio in infected compared with mock-infected cultures), a small portion of each was analyzed by intracellular p24 staining to determine the percentage of infected ($p24^+$) cells that were $CD4^+CCR5^+$ and $CD4^+CCR5^-$ (see Fig. 2B). The remaining cells were enriched for $CD4^+$ T cells (by bead depletion of cells expressing CD8, CD14, or CD19) and then sort-purified into subpopulations that were negative for these lineage markers and were $CD4^+CCR5^+$ or $CD4^+CCR5^-$ (see Fig. 2C). The sorted populations were divided into 2 portions. One third were lysed immediately to quantitate p24 content (via p24 ELISA). The other two thirds were cultured in vitro for an additional 1.5 days so that viral replication could continue. At the end of this time, supernatants were harvested and assessed for p24 production (by ELISA) and for infectious virus release (by $TCID_{50}$ assay).

The average amount of virus made per cell was calculated by relating these 3 values (intracellular p24 at the time of sort purification and the amount of p24 and $TCID_{50}$ made during the 1.5-day culture period) to the absolute number of infected cells (determined by multiplying the percentage of $p24^+$ cells [$CD4^+CCR5^+$ or $CD4^+CCR5^-$] × absolute number of sort-purified cells in each subset). As shown in Figure 3, significant variability was observed between donors in the absolute amount of p24 made per infected cell. HLACs prepared from multiple independent donors (n = 9) revealed 2 consistent qualitative patterns, however. First, after X4 HIV-1 infection, the average amount of p24 and $TCID_{50}$s produced per $CD4^+CCR5^+$ cell was greater (by 1- to 100-fold) than the average amount made per $CD4^+CCR5^-$ cell. Second, and more strikingly, the average amount of p24 and $TCID_{50}$s made per $CD4^+CCR5^+$ cell was 5- to 10-fold higher after R5 HIV-1 infection than after X4 HIV-1 infection.

Replicative Advantage of R5 HIV-1 Is Observed in Activated $CD4^+$ T Cells

Although HLACs are composed primarily of T cells, myeloid cells permissive for HIV-1 infection may also be present. To exclude measurement of viral production from

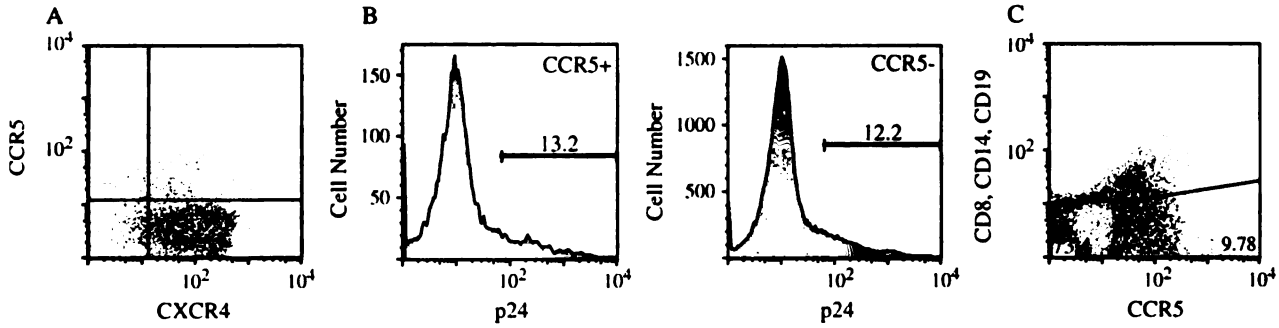

FIGURE 2. Characterization and purification of $CD4^+$ T-cell populations from HLAC. A, Most $CD3^+CD4^+$ T cells that express CCR5 also express CXCR4. Cells from HLACs were first gated on the lymphocyte population by forward and side scatter and then on $CD3^+CD4^+$ T cells by extracellular staining for CD3 and CD4. Shown are representative results for CCR5 and CXCR4 staining on the $CD3^+CD4^+$ T-cell population. B, Representative example of intracellular p24 staining in $CD3^+CD4^+CCR5^+$ and $CD3^+CD4^+CCR5^-$ populations. Cells from HLACs were assessed for expression of viral p24 at the conclusion of HIV-1 infection. In this example, the cultures were infected with NL4-3 at an moi of 0.001. On day 5 after infection, cultures were harvested and assessed for expression of CD3, CD4, CCR5, and p24. Background CCR5 staining was assessed by comparison to a sample stained for CD3, CD4, and p24 only. The percentage of $p24^+$ cells in each population was then determined by comparison to a mock-infected control. C, Purification of $CD3^+CD4^+CCR5^+$ and $CD3^+CD4^+CCR5^-$ cells from HLAC. Cells from HLACs were first depleted of $CD8^+$, $CD14^+$, and $CD19^+$ populations by magnetic bead depletion and then sort-purified into $CCR5^+$ and $CCR5^-$ populations, gating first on lymphocytes and then on events that were negative for the lineage markers CD8, CD14, and CD19. Shown is a representative sample of final gating of $CCR5^-$ and $CCR5^+$ populations. These fractions were collected and analyzed for virus production (see Fig. 3).

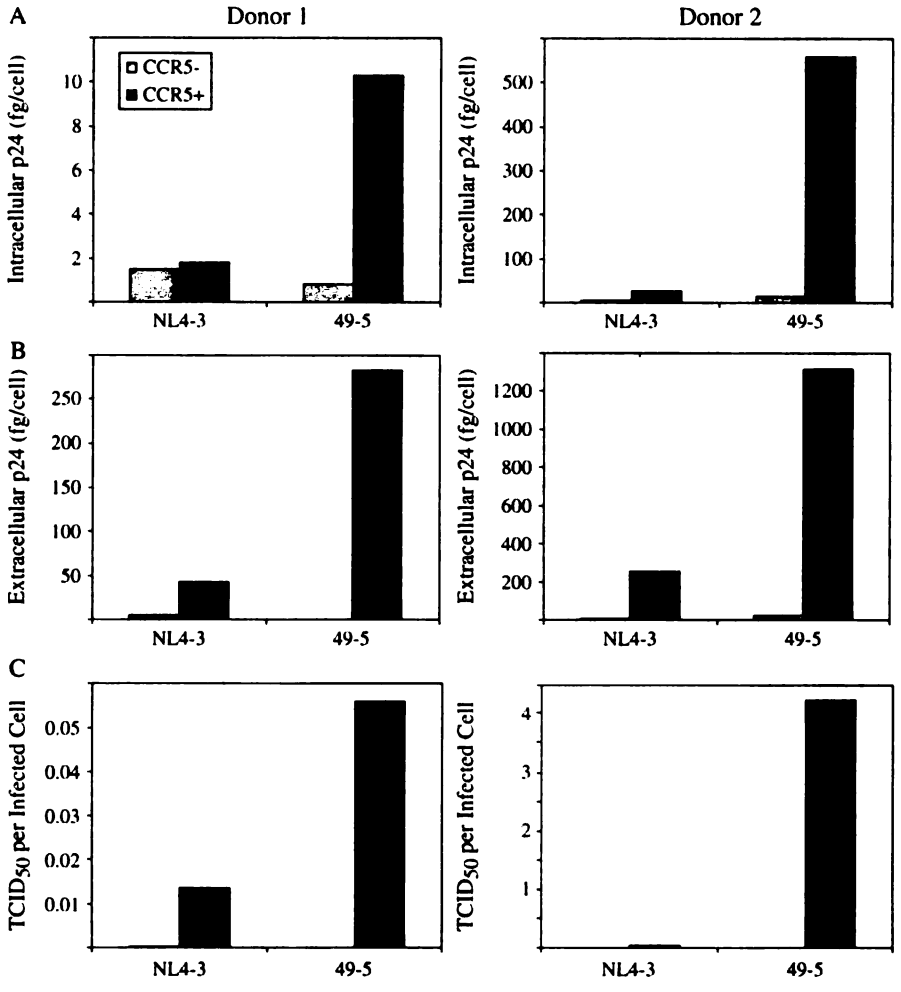

FIGURE 3. HLACs infected with R5 HIV-1 (49-5) yields a higher viral output per infected cell than those infected with X4 HIV-1 (NL4-3). HLACs were infected with NL4-3 or 49-5 at an moi of 0.001 for 5 to 6 days. Cultures were harvested, and $CD3^+CD4^+CCR5^+$ and $CD3^+CD4^+$ $CCR5^-$ cells were purified as described in Fig. 2. In parallel, unsorted HLACs were analyzed to determine the percentage of $CCR5^+$ (FITC)- and $CCR5^-$-infected cells ($p24^+$). The sorted cells were then separated into 2 groups. One third of the cells were analyzed immediately by p24 ELISA to determine their internal p24 concentration. The remaining cells were placed back into culture for 1.5 days to allow viral replication to continue. At the conclusion of this period, supernatants from each well were harvested and measured for p24 production (by ELISA) and for infectious virus (by $TCID_{50}$ assay). As detailed in the Materials and Methods section, these data were used to determine the average intracellular p24 concentration per infected cell (A), the average extracellular p24 concentration per infected cell (B), and the average $TCID_{50}$ per infected cell (C). These data are representative of differences seen in 9 different donors.

such cells, we extended our analysis to in vitro culture conditions designed to stimulate and expand T cells preferentially in a purified culture. $CD4^+$ T cells were purified from PBMCs and incubated with plate-bound monoclonal antibodies against CD3 and with soluble antibodies against CD28. As a function of time thereafter, $CD4^+$ T cells in these cultures showed evidence of stimulation, expressing CXCR4 (98%), CCR5 (10%), and a number of activation markers (eg, 29% were positive for $CD38^+$ and $CD69^+$).[25] A comparable level of activation was seen in HLACs (ie, 20% of $CD4^+$ T cells in HLACs were $CD69^+$ [data not shown]). After 72 hours of stimulation, the cultures were thoroughly rinsed and then infected with X4 HIV-1 (NL4-3) or an R5 isolate (49-5) isogenic in the V3 loop at an moi of 0.001. The average amount of virus made per infected cell was then calculated using the approach employed previously for HLACs. $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 were harvested 6 to 7 days after infection with X4 (NL4-3) or R5 (49-5) HIV-1, stained with antibodies against CCR5, and sorted into $CD4^+CCR5^+$ and $CD4^+CCR5^-$ populations. The sorted populations were assessed for the average amount of intracellular p24 at the time of sort-purification and for the amount of p24 and $TCID_{50}$s produced during 1.5 days of further culture in vitro. As shown in Figure 4 for 2 independent donors, $CD4^+CCR5^+CXCR4^+$ T cells infected with R5 HIV-1 produced an average of fivefold more p24 and fivefold more $TCID_{50}$s per infected cell than $CD4^+CCR5^+CXCR4^+$ T cells infected with X4 HIV-1.

DISCUSSION

We have recently reported[25] that noncytopathic R5 HIV-1 strains are able to replicate more efficiently in costimulated $CD4^+$ T-cell cultures than cytopathic X4 HIV-1 strains over the course of a 10-day infection, and previous studies have described similar observations in HLACs.[24] Here, we have examined this issue more closely, analyzing and comparing the relative replicative capacity of X4 and R5 HIV-1 within human $CD4^+CCR5^+CXCR4^+$ T cells that should be permissive for infection by each type of virus. Two different culture conditions were used: unstimulated HLAC and human peripheral blood–derived $CD4^+$ T cells stimulated with antibodies against CD3 and CD28. Sort-purified $CD4^+CCR5^+CXCR4^+$ T cells from each of these sources were found to make, on average, 5- to 10-fold more infectious virus per infected cell after

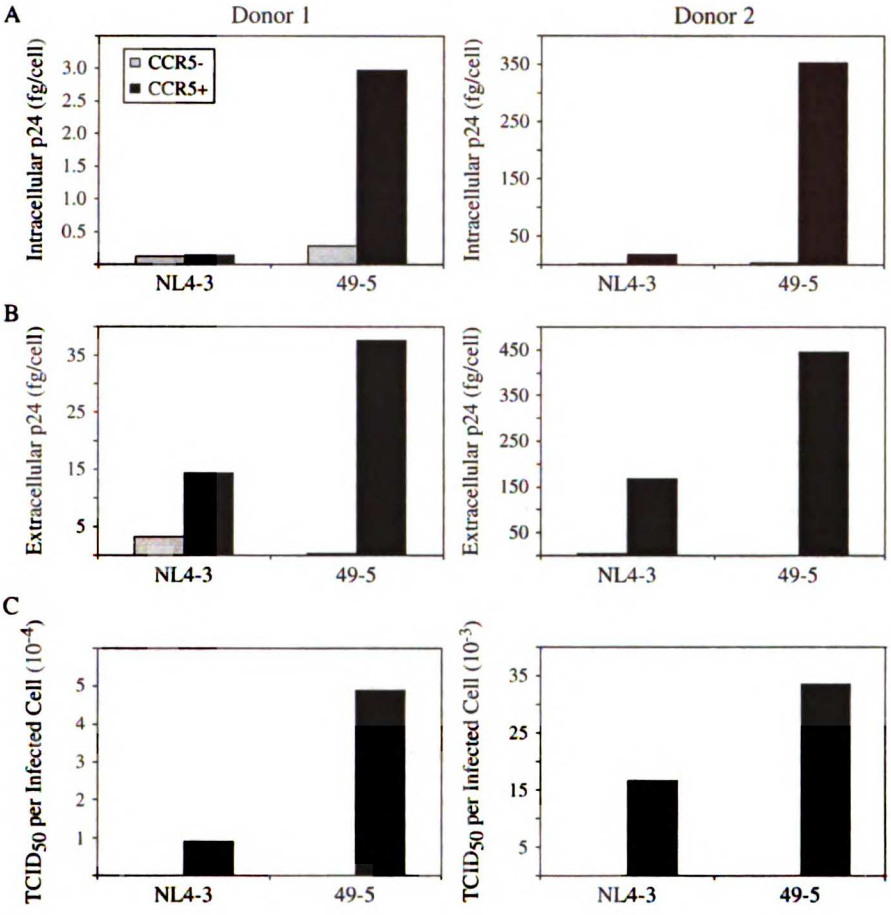

FIGURE 4. PBMC-derived $CD4^+$ T cells infected with R5 HIV-1 (49-5) yield a higher viral output per infected cell than those infected with X4 HIV-1 (NL4-3). $CD4^+$ T cells were purified from human PBMCs, stimulated with plate-bound anti-CD3 and soluble anti-CD28 antibodies (as described in the Materials and Methods section), and then infected (at an moi of 0.001) with NL4-3 or 49-5 for 6 to 7 days. Infected cells were harvested and analyzed as described in Figs. 2 and 3 (except that the removal of $CD8^+$, $CD14^+$, and $CD19^+$ cells via magnetic bead separation was not required, because the $CD4^+$ T cells had already been purified). These data were used to determine the average intracellular p24 concentration per infected cell (A), the average extracellular p24 concentration per infected cell (B), and the average $TCID_{50}$ per infected cell (C). The data shown are from 2 independent PBMC donors.

challenge with R5 HIV-1 than after challenge with X4 HIV-1. This difference was reflected as higher levels per infected cell of intracellular p24 as well as higher levels of p24 and infectious virus particles released into the supernatant. It is interesting to note (see Fig. 3) that in most cases, $CD4^+CCR5^+CXCR4^+$ T cells also generated higher levels of virus than did $CD4^+CCR5^-CXCR4^+$ T cells after challenge with X4 HIV-1. Possibly, $CD4^+CCR5^+CXCR4^+$ T cells are more permissive for viral replication and/or more long-lived after infection than are $CD4^+CCR5^-CXCR4^+$ T cells. In either case, $CCR5^+$ T cells are able to sustain R5 HIV-1 replication to a greater degree than X4 HIV-1 replication.

As shown by experiments using purified $CD4^+$ T cells, the replicative advantage of R5 HIV-1 was not dependent on interaction with other cell lineages (eg, myeloid cells). Given similar results using T cells from multiple donors, this also seems to be a generalized finding. The high average output of R5 HIV-1 per infected cell could be reflective of a cell population that is relatively homogeneous with respect to viral production. Alternatively, the $CD4^+CCR5^+CXCR4^+$ T-cell population may be composed of discrete subpopulations (eg, with varying degrees of activation, in different stages of the cell cycle), some of which are more permissive for viral production than others. Although our current assay system cannot discriminate between these possibilities on the single cell level, it is clear that R5 HIV-1 challenge does result in up to 100-fold higher levels of viral production per cell when averaged across the entire $CD4^+CCR5^+CXCR4^+$ population. This difference in relative production may contribute to the predominance of R5 isolates of HIV-1 in vivo.

Given the finding that R5 output is higher on a single cell basis, what might be the mechanism behind this difference? It may be that R5 but not X4 HIV-1 is able to infect a subpopulation of T cells able to generate a large amount of virus per unit time.[34] Alternatively, the frequency of superinfected cells may be higher in the context of R5 HIV-1 infection as opposed to X4 HIV-1 infection, allowing for a larger number of virions to be produced per infected target cell. Another possibility is that R5 HIV-1 is less cytopathic, allowing cumulatively increased virus production over the lifespan of individual cells. Experiments to discriminate between these possibilities await the development of an assay that can follow single infected cells over time. It seems likely, however, that the last possibility (differential cytopathicity between X4 and R5 HIV-1 viruses) is not playing a major role under the conditions focused on in this study. During the early stages (days

5–7) of infection that we have studied, there seems to be no significant difference in cell viability in the cell cultures infected by the isogenic R5 and X4 HIV-1 isolates (data not shown).

Another possibility is that R5 HIV-1 signaling through CCR5 generates an intracellular environment more suitable for productive viral infection than does X4 HIV-1 signaling through CXCR4. Although these 2 pathways share at least 1 common downstream signaling intermediate,[35] they are likely to be divergent.[36–40] It should be noted that the consequences of coreceptor signaling in HIV-1 infection are a subject of some debate. Whereas several studies have suggested that coreceptor signaling can enhance infection by HIV-1,[41–44] others indicate that coreceptor signaling has no impact on HIV-1 infection.[45–47] The HLAC system may be used as a means of investigating the impact of coreceptor signaling in unstimulated primary human cells, generating a comparison based on viral coreceptor preference. In this manner, we hope to understand better the mechanisms at play behind the differences in viral output observed in this study.

The replicative advantages we have reported of R5 HIV-1 over X4 HIV-1 may have important implications in HIV-1 pathogenesis. In combination with other factors (eg, presence of susceptible cells at sites of transmission, high viral output by infected macrophages, selective uptake by APCs such as follicular dendritic cells, increased immunogenicity of X4 HIV-1),[17–24] the replicative advantage of R5 HIV-1 reported here could underlie the patterns of infection seen in vivo. If so, the question still remains: why is there a switch to the use of CXCR4 in some patients? Some studies have shown that X4 HIV-1 may be capable of viral production equivalent to R5 HIV-1 in a highly activated immune system[43] such as is present in late stages of HIV-1 infection in vivo, and other factors may tip the balance as well. Because such X4 switches are only observed in 50% of those infected with B clade virus,[14–16,25] they are not a necessary precondition for the development of AIDS. Further studies examining the replicative advantage of R5 HIV-1 may shed light on the mechanisms of disease progression and on methods to prevent transmission or to slow progression after infection.

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