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Host and Viral Factors Controlling HIV Pathogenesis

by

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DISSERTATION

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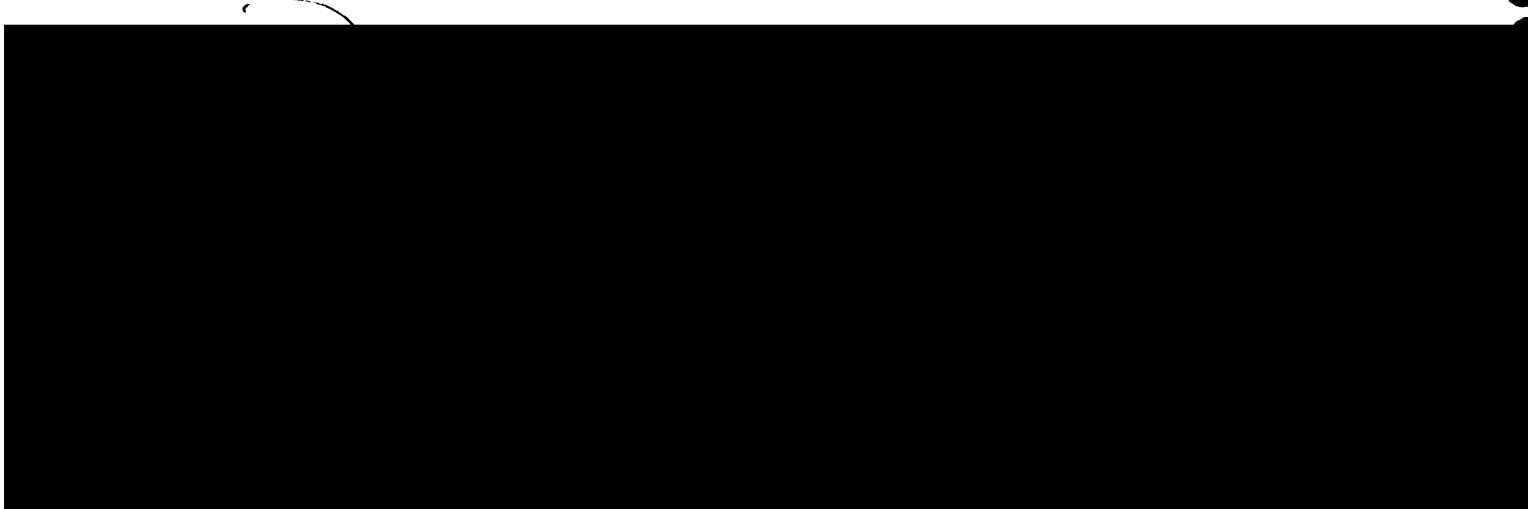

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Throughout graduate school, the input, feedback, and guidance of my advisor, Warner C. Greene, has been invaluable. Warner has the remarkable ability to understand both what an experiment means and what it does not mean. He is highly critical, while remaining supportive. In addition, Warner has always filled his lab with superb scientists who have been the best role models any student could ask for.

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And finally, I must thank Dianne Barber, the discussion section leader from my first-year cell biology class. It was during these discussion sections that I met Valerie Ng whom I would be fortunate enough to marry four years later.

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Contributions of Co-Authors

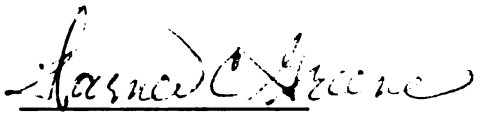
Chapter 3 of this dissertation is based on material published in the *Journal of Virology* (2001) 75: 8842-8847, titled, "Cytopathicity of Human Immunodeficiency Virus Type 1 Primary Isolates Depends on Coreceptor Usage and Not Patient Disease Status," by copyright permission of the American Society of Microbiology. The following co-authors contributed helpful advice or reagents to this work: David Kwa, Birgit Schramm, Verena Trautner, Ruth Connor, Hanneke Schuitemaker, James I. Mullins, Angélique B. van't Wout. This work was supervised by Mark A. Goldsmith.

Chapter 5 of this dissertation is based on material submitted for publication in *The Journal of Experimental Medicine* titled "Select Cytokines Enhance HIV Infection of Naive CD4 T Cells in Lymphoid Tissues by Stimulating High-Molecular-Mass APOBEC3G Complex Formation." The co-author, Wes Yonemoto, contributed helpful advice and experimental assistance. This work was supervised by Warner C. Greene.

Host and Viral Factors Controlling HIV Pathogenesis

by

Jason F. Kreisberg



Warner C. Greene, M.D., Ph.D.

HIV pathogenesis depends critically on both viral and host factors. While much is known about the viral factors, less is understood about the key host factors. We sought to identify novel host factors that control permissivity to HIV infection. To identify positively acting factors, HIV-permissive cells were subjected to retroviral-mediated insertional mutagenesis and screened for HIV-resistant cells. Despite conducting four independent screens of two mutagenized cell lines, no positively acting factors were identified.

We evaluated the role of two viral proteins important in pathogenesis: envelope (Env) and Nef. During late-stage disease in some patients, Env switches from utilizing CCR5 to CXCR4 for entry. This switch correlates with accelerated disease progression. We looked for other properties that might accelerate disease progression but found no evidence of coreceptor-independent increases in cytopathicity. Thus, the increased cytopathicity exhibited by CXCR4-tropic strains seems to reflect only a change in coreceptor utilization.

HIV pathogenesis also critically depends on Nef. Prior studies suggested that Nef activates target cells and thereby provides an improved microenvironment for viral replication and spread. Although HIV-infected cells exhibit increased activation, this phenotype proved independent of Nef expression.

Finally, we refocused on host factors that impede HIV infection. HIV infects resting CD4 T cells in lymphoid tissues but not those in peripheral blood. We explored the role of the tissue microenvironment and its influence on the antiviral factor APOBEC3G (A3G) in regulating permissivity to HIV. We demonstrated that IL-2 and IL-15 production in tissues played a key role in rendering naive CD4 T cells susceptible to HIV. Infection of memory CD4 T cells similarly required endogenous soluble factors but not IL-2 or IL-15. A3G exists as a high-molecular-mass (HMM) complex in permissive, tissue-resident naive CD4 T cells, but as a low-molecular-mass (LMM) form in nonpermissive, blood-derived naive CD4 T cells. After treatment with endogenous soluble factors, these circulating cells became permissive, and LMM A3G assembled into HMM complexes. Thus, endogenous IL-2 and IL-15, acting in concert with other soluble factors in lymphoid tissues, stimulate HMM A3G formation in tissue-resident CD4 T cells, relieving the post-entry restriction block for HIV conferred by LMM A3G.

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Chapter 1

Introduction

The HIV pandemic continues to grow with devastating medical, social, and economic consequences. In 2005, nearly 5 million people were infected, and over 3 million people died (UNAIDS, *AIDS Epidemic Update: December 2005*). In the United States alone in the last 25 years, over half a million people have died from HIV infection (CDC, *HIV/AIDS Surveillance Report, 2004*). In addition to its structural and enzymatic proteins, Gag, Pol, and Env, HIV encodes six additional proteins: Tat, Rev, Nef, Vif, Vpr, and Vpu. Like other retroviruses, the HIV genome is a single-stranded RNA molecule, which, upon infection, is reverse-transcribed into double-stranded DNA and integrated into the host genome. In most cases after integration, viral genes are expressed, and progeny virions are produced, thereby completing the viral life cycle.

Host Factors in HIV Replication

HIV replication critically depends on the interplay of host and viral factors. For example, the process of viral entry requires the viral envelope to interact sequentially with two host factors: the receptor CD4 (1) and then, one of the two coreceptors, CCR5 (2, 3) or CXCR4 (4). Binding to CD4 induces a conformational change in the viral envelope that allows subsequent interactions to one of the two coreceptors (5, 6). Coreceptor engagement induces yet another conformational change that allows the viral envelope to complete the fusion process (7).

Before infection, a conical shell of viral capsid proteins surrounds the viral genome. Upon entry, the viral capsid disassembles in a process termed uncoating (8), and the viral enzymes, reverse transcriptase and integrase, convert the incoming viral genome into a double-stranded DNA molecule that is integrated into the genome of the host, often near actively transcribed genes (9). Although the steps between viral entry and

integration are largely governed by viral factors, some studies have suggested an important role for host factors as well. For example, the host protein LEDGF/p75 binds strongly to integrase (10, 11) and is likely important for targeting integration events near actively transcribed genes (12-14), although other host factors also probably influence site selection. Cytoskeletal components are also important. Intact actin filaments increase the efficiency of reverse transcription (15), and microtubules are used for transporting virion contents from the cell periphery to the nucleus (16). Finally, host factors likely are also probably important for actively transporting viral genomes through the nuclear pore complex.

Viral gene expression and subsequent virion production require an array of host factors. Transcription factors, such as NF-κB, Sp1, and AP1, bind to sequences in the viral promoter (17) and recruit RNA polymerase II which can initiate transcription but fails to elongate. High level elongation commences when Tat recruits two host proteins, cyclin T and cdk9, that in turn phosphorylate the C-terminal domain of RNA polymerase II (18-20). Initially, only fully-spliced transcripts encoding Tat, Rev, and Nef, reach the cytoplasm. For expression of structural and enzymatic viral genes that are required for production of infectious virions, singly-spliced or unspliced transcripts need to be exported from the nucleus. These events are triggered by the action of Rev, which binds to an RNA stem loop structure termed the Rev response element present in either singly-spliced or unspliced transcripts (21-23). Rev facilitates nuclear export of these RNAs by binding to the host nuclear export factor, Crm1 (24, 25). Later in the viral life cycle, the host protein HP68 plays a key role in promoting capsid assembly (26), although the molecular mechanisms of this action are not yet clear. Finally, virion release requires Gag

recruitment of the host vesicular trafficking protein Tsg101 (27) and other members of ESCRT family (28, 29).

Although many host factors are necessary for completion of the viral life cycle, HIV and other retroviruses must also successfully overcome a number of innate anti-viral host factors. The anti-viral factors, Fv1 and TRIM-5α, block the viral life cycle after entry but before integration (30, 31). Resistance to these factors is conferred by mutations in CA. Fv1 is expressed in mice and blocks infection of the murine leukemia virus (MLV). Interestingly, Fv1 is homologous to retroviral capsids (30), but precisely how it inhibits MLV infection is unknown. In contrast to Fv1, the anti-viral activity of TRIM-5α acts most efficiently across species boundaries: TRIM-5α from Old World monkeys inhibits HIV infection (31), whereas human TRIM-5α inhibits MLV but has only weak effects against HIV (32-35). TRIM-5α contains four domains: a RING, B-box, coiled-coil, and B30.2 (SPRY) domain (31, 36). None of these domains share homology with retroviral capsids. The anti-viral activity of TRIM-5α appears related to its ability to accelerate uncoating.

HIV also encounters a potent anti-viral factor within human cells: apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like-3G (A3G) (37). After incorporation into budding virions, A3G, a cytidine deaminase, induces hypermutation of single-stranded DNA (ssDNA) formed during reverse transcription (38-41). However, these potent antiviral effects of A3G are countered by the HIV Vif protein which targets A3G for accelerated proteasome-mediated degradation and partially blocks its synthesis (42-46).

A3G can also inhibit HIV infection in a Vif-insensitive manner (47). In resting CD4 T cells from the peripheral blood, A3G exists in a low-molecular-mass (LMM) form; conversely, enzymatically inactive high-molecular-mass (HMM) A3G complexes occur in either stimulated CD4 T cells or CD4 T cell lines. CD4 T cells containing HMM A3G are permissive to HIV infection, but CD4 T cells from the peripheral blood containing LMM A3G are not. Depletion of LMM A3G from these resting cells relieves this block, thereby demonstrating that LMM A3G inhibits HIV infection. It is not clear if this anti-viral activity of LMM A3G requires cytidine deamination or not.

Naive and Memory CD4 T Cells During HIV Infection

CD4 T cells are one of the principal targets of HIV infection. These cells develop in the thymus and emerge as naive cells that circulate between the blood and secondary lymphoid organs, such as the spleen and lymph nodes. Upon entry into secondary lymphoid organs, naive cells survey peptides displayed on antigen-presenting cells (APC). The combined actions of both antigen and costimulation from the APC activate the naive cells. After activation, naive cells massively proliferate. Most cells become short-lived effectors cells, but some become long-lived memory cells (48-50).

Memory CD4 T cells circulate between the blood and secondary lymphoid organs and also migrate to peripheral tissues. Memory cells are both more sensitive to stimulation and respond more vigorously than naive cells. As shown for CD8 cells in mice, memory cells in secondary lymphoid organs constitutively express high levels of cytokine mRNA, which facilitates rapid cytokine secretion upon stimulation (51). Also when stimulated, memory cells rapidly divide many more times than naive cells (52). Intense investigation seeks to explain the molecular mechanisms underlying these

differences in naive and memory T cells. Current hypotheses include differences in lipid raft composition (53, 54), chromatin structure (55), and Lck localization (56, 57).

Both naive and memory CD4 T cells are permissive to HIV infection *in vivo* (58-61). CCR5 expression is restricted to a subset of memory cells (62, 63) while CXCR4 is expressed on both naive and memory CD4 T cells (64-66). Thus, memory cells can be infected by either X4- or R5-tropic strains, but only X4-tropic strains can infect naive cells. In HIV-infected patients, given that R5-tropic viruses can be detected throughout the course of infection and are often the prominent viral quasispecies (67-70), most HIV-infected CD4 T cells in patients are memory cells (61). Importantly, these cells also give rise to a latently infected population of cells (71, 72). In addition, approximately 50% of all HIV-infected patients develop X4-tropic strains, the appearance of which is correlated to rapid disease progression (69, 70, 73). Both X4- and R5- tropic viruses can be isolated from naive cells from these patients (58-60).

Chapter 2

Attempted Identification of Host Factors Necessary for HIV-1 Infection after Receptor Engagement

Introduction

Like all viruses, HIV exploits various cellular factors to complete its replicative life cycle. For example, HIV entry requires the presence of the CD4 receptor (1), and one of two coreceptors, either CXCR4 (4) or CCR5 (2, 3). Similarly, the expression of viral genes after integration not only requires RNA polymerase II but also the action of both cdk9 and cyclin T to enable polymerase processivity (18-20). Late in the viral life-cycle, HP68 is a necessary cofactor during capsid assembly (26), while Tsg101 is necessary for virion release (27). Nevertheless, in many steps of the viral life cycle, such as uncoating, reverse transcription, nuclear localization, and integration, the identity and function of host genes remains a mystery. To identify novel host factors necessary for events after viral entry but before integration, we employed an experimental approach involving somatic mutagenesis.

Somatic mutagenesis is a largely unbiased approach to candidate gene discovery. Although this technique has been powerfully exploited to identify key molecules in various biological pathways, such as the essential roles of Lck in TCR signaling (74, 75) and the JAK/STATs in the IFN response (76-79), this approach has met with limited success in virology. A notable exception is the finding that overexpression of fasciculation and elongation protein ζ-1 (FEZ1) induces a post-entry block to retroviral infection (80). The role of FEZ1 was identified by characterizing a mutagenized cell line selected for resistance to retroviral infection (81).

Two techniques are commonly used to introduce random mutations throughout the genome: DNA-damaging chemicals (82-86) and retroviral insertion. When chemical mutagens are used, the genetic basis of interesting mutants can be identified by screening

a cDNA library for transcripts that rescue the observed block (87). The assumption underlying this approach — that the defect is a loss of function of a single gene —is not always true, as was the case for FEZ1 (80). Only after analysis of highly overexpressed transcripts in the mutagenized, retrovirus-resistant cell line was FEZ1 found to protect these cells from retrovirus infection.

As an alternative to chemical mutagens, retroviruses can be used as insertional mutagens, or "gene traps" (88-91). This approach has two features designed to aid identification of the gene of interest. First, the mutagenized gene is marked by the retrovirus, now a unique and well-defined element in the genome. Second, because the mutagenic retroviral stocks can be titered, the dose can be tightly controlled to ensure that the majority of mutagenized cells in a culture contain only a single proviral insertion.

In our efforts, we employed an MLV-based gene trap, known as a RET vector, which contains a number of important features (Fig. 2.1A) (92). At the 3′ end of the integrated provirus, a constitutive promoter (P2 in Fig. 2.1A) is present that drives the expression of a neomycin-resistance gene that ends not with a signal for polyadenylation but rather with a splice donor. Therefore, for an infected cell to acquire neomycin resistance, the integrated provirus must integrate both within a host gene and in the proper orientation to find a downstream splice acceptor and polyadenylation site. Another key feature of the RET vector is the presence of loxP sites in place of the viral LTRs. Treatment of mutants with cre recombinase leads to proviral excision and phenotypic reversion. The RET vector also contains a constitutive promoter (P1 in Fig. 2.1A) driving the expression of the herpes simplex virus (HSV) thymidine kinase (tk) gene which allows for both positive and negative selection. Viral stocks can be titered on tk-deficient

cells by selection in medium containing hypoxanthine, aminopterin, and thymidine (HAT); cre recombinase-mediated revertants can be selected by growth in ganciclovir. Finally, a strong splice acceptor followed by a polyadenylation signal is located at the 5′ end of the integrated provirus. This will ensure that whatever gene the provirus has integrated into is effectively disrupted. In summary, RET vectors are a useful tool for efficient and reversible insertional mutagenesis.

GFP-expressing, single-cycle HIV reporter viruses have desirable features for screening mutagenized cells for resistance to HIV infection. The genomes of the viruses chosen for this screen contain a CMV promoter constitutively driving GFP expression to mark infected cells (93, 94), thereby eliminating the need for either of the Tat cofactors, cdk9 and cyclin T (18-20). In addition, because GFP fluorescence can be easily detected in living cells, uninfected cells (GFP–) can be separated from infected cells (GFP+) by flow cytometry. Multiple rounds of screening can be performed to enrich mutagenized cells displaying strong resistance to HIV infection. Additionally, these HIV reporter viruses can be pseudotyped with different viral envelopes, each utilizing different host receptors. In screens aimed to identify cellular genes required for HIV infection after surface receptor engagement, pools of reporter viruses pseudotyped with different envelopes can be employed.

Finally, one of the most important parameters for the success of such a screen is the choice of the target cell line. For this screen, the ideal target cell should be readily infected by both MLV and HIV, lack endogenous tk so that the RET vectors can be titered accurately, and be susceptible to phenotypic changes due to disruption at a single locus. Although no natural cell line has all three of these properties, derivatives of either

HeLa or CHO cells represent attractive options. Upon expression of CD4 and CCR5, both cell types are highly permissive for infection by MLV and HIV (95, 96). In addition, there is a tk-deficient subclone of HeLa cells (97) but not CHO cells. Whereas HeLa cells have an average 3n chromosome content (98), CHO cells are functionally hemizygous at many loci (99-102). Two HIV-permissive HeLa-based cells lines were initially mutagenized with RET vectors and screened for permissivity to GFP-expressing HIV reporter viruses. Unfortunately, no refractory cell lines were identified in four independent screens, each employing a different combination of pseudotyped viruses. Reasoning that CHO cells might be more amenable to insertional mutagenesis due to their functional hemizygosity, we turned our focus to these cells. We generated an HIV-permissive CHO cell line, and we performed preliminary experiments attempting to establish an HIV-based selection strategy.

Results

Using a RET Vector for Insertional Mutagenesis

To maximize production of MLV virions containing RET vectors, we tested the following packaging cell lines: PT67 (Clontech), Phoenix-Ampho (kindly provided by G. Nolan, Stanford University), and Plat-E (103). Viral titers, measured in HAT-resistant colony-forming units (cfu), were typically determined on tk-deficient HeLa cells (97) but occasionally on other tk-deficient cells lines, such as Rat2 (104) or L-M tk(-) (105). For packaging cell lines expressing the ecotropic MLV envelope (Env) (106), PT67 and Plat-E, the amphotropic (ampho) MLV Env (107) was cotransfected along with the RET vector to allow infection of human cells. The glycoprotein (G) from VSV was also occasionally cotransfected during virion production to expand viral tropism to a broader range of cells (108, 109). Regardless of the target cell, Plat-E routinely produced the highest viral titers (data not shown).

To confirm that a single retroviral insertion was sufficient to render cells resistant to HAT selection, HeLa tk(-) cells were infected at a low multiplicity of infection (moi), and HAT-resistant colonies analyzed by Southern blotting for proviral insertions. Parental HeLa tk(-) cells served as a negative control while a polyclonal culture of RET-infected HAT-resistant HeLa tk(-) cells was used as a positive control. In both clones, only a single proviral band was detected, indicating that each resulted from a single insertion (Fig. 2.1B). The bands were specific for the provirus, as neither was detected in the parental HeLa tk(-) cells. In the polyclonal population, a smear of bands was present, consistent with the notion that insertion of a single provirus in cells at different loci resulted in RET-containing fragments of various sizes.

RET vectors were subsequently used to mutagenize two cells lines: HeLa cells stably expressing CD4 and CCR5 (HeLa CD4/CCR5) (96) and a subclone of this cell line, H11 (see below for isolation and characterization of H11).

Characterizing and Optimizing Infections by HIV Reporter Viruses

Because we sought to identify host factors required after viral entry but before gene expression, it was important to ensure that the single-round reporter viruses used in the screen mark only cells containing an integrated provirus. To this end, we produced GFP-expressing HIV reporter virions containing either wild-type integrase or a mutant, D64E, incapable of integrating but competent at all other steps in the viral life cycle (110-112). HeLa cells infected with virions containing wild-type integrase stably expressed high levels of GFP, whereas cells infected with the IN mutant transiently expressed lower levels of GFP (Fig. 2.2). Thus, cells containing an integrated provirus could be identified by high-level expression of GFP after 5 days.

Key to the success of this project was an efficient process to screen for permissivity to HIV infection. Some groups have reported that spinoculation, infecting cells during centrifugation, greatly increases the number of infected cells. Although reports have appeared that target cells may become more permissive when subjected to higher gravitational forces (113, 114), others have claimed that the increase in the number of infected cells is simply due to increased deposition of virions on the cell surface (115-117). The cationic polymer, polybrene, also can be used to increase viral infectivity, likely because it can neutralize the negative charges on the virion surface and on the cell membrane (118). The effects of these two treatments were tested both separately and in combination. Regardless of the envelope used for pseudotyping, the

combination of spinoculation and polybrene led to more infected HeLa CD4/CCR5 cells than either treatment alone or no treatment at all (Fig. 2.3A). Thus, nearly all infections for this project, both with GFP-expressing pseudotyped HIV reporter virions and RET-containing MLV virions, were performed by spinoculation in the presence of polybrene.

To optimize the permissivity of HeLa CD4/CCR5 cells, we isolated subclones of this cell line by limiting dilution and tested each for surface expression of CD4 and CCR5 and permissivity to GFP-expressing reporter viruses. Subclone 11 was highly permissive to GFP-expressing reporter viruses pseudotyped with the BaL-strain HIV Env or the ampho MLV Env (Fig. 2.3B). Like the parental cell, subclone 11 continued to express high levels of surface CD4 and CCR5 (data not shown). Interestingly, subclone 6 was highly permissive to virions pseudotyped with the ampho MLV Env but not with the BaL HIV Env. Although this subclone still expressed high levels of surface CD4, CCR5 expression was greatly reduced, accounting for its resistance to HIV Env but not MLV pseudotypes.

Screening Insertionally Mutagenized Cell Lines for Cells Resistant to HIV Infection

As we optimized infection conditions, we began to observe increased levels of cell death which could not be inhibited by the reverse transcriptase inhibitor AZT at doses that blocked GFP expression (data not shown). Thus, it is likely that either entry or steps soon thereafter can trigger cell death. Of note, such toxicity was observed following exposure to virions pseudotyped with VSV-G or the HIV Envs JRFL or ADA but not the BaL HIV or ampho MLV Env (data not shown).

Four screens were performed simultaneously, three with mutagenized HeLa CD4/CCR5 subclone 11 (RH11) cells and one with parental HeLa CD4/CCR5 (RH4R5)

cells. VSV-G pseudotyped virions were used to screen either RH11 or RH4R5 cells. In addition, RH11 was screened with cocktails of virions pseudotyped with different envelopes. One screen used virions pseudotyped with VSV-G, BaL HIV Env, or ampho MLV Env, and another with virions pseudotyped with BaL HIV Env or ampho MLV Env. Each batch of virus used for these screens was titered to achieve high levels of infection (60% to 80%) but minimal levels of toxicity.

Unfortunately, none of the four screens identified an HIV-resistant cell line (see "Materials and Methods" for details on how the screens were performed). Three screens proceeded through at least 10 rounds of infection, isolation of GFP– cells, and rescreening. The fourth screen only proceeded through five rounds due to technical difficulties. The screen using VSV-G pseudotyped virions and RH5R5s proceeded through 12 rounds of screening without ever isolating a resistant cell line (Fig. 2.4). Cells from each round of each screen remain in cryogenic storage.

CHO Cells Expressing CD4, CCR5, and Cyclin T Are Permissive to HIV Infection and Suitable for Insertional Mutagenesis and Screening

CHO cells can support all steps in the viral life cycle except for entry or gene expression (95). As CHO cells might be more susceptible to the mutagenic effects of retroviral insertion than HeLa cells in view of their functional hemizygosity at multiple loci, these cells were engineered to express human CD4, CCR5, and cyclin T (CHO CD4/CCR5/CycT) by retroviral transduction of three expression vectors, followed by sorting for cells with high levels of surface expression of CD4 and CCR5. The resulting culture was sorted three more times to isolate cells stably expressing high levels of the transgenes. This procedure only selected for CD4 and CCR5. To determine if the isolated

culture expressed functional cyclin T, both CHO and CHO CD4/CCR5/CycT cells were infected with VSV-G-pseudotyped reporter viruses expressing luciferase under the control of the HIV LTR. The CHO CD4/CCR5/CycT cells expressed 100-fold higher levels of luciferase than the parental CHO cell lines (data not shown), confirming that these cells did express functional human cyclin T that can efficiently transactivate the viral LTR.

To identify a more highly permissive CHO CD4/CCR5/CycT cell line, subclones were isolated by limiting dilution and tested for surface expression of CD4 and CCR5 and for permissivity to HIV infection. Of the eight subclones identified, subclone 4 performed best in all assays. Surface expression levels of CD4 and CCR5 were slightly higher and more uniform in subclone 4 than in the parental CHO CD4/CCR5/CycT cells (Fig. 2.5A). Importantly, subclone 4 was highly permissive to either VSV-G or BaL HIV Env pseudotyped GFP-expressing reporter viruses (Fig. 2.5B) and no toxicity was observed (data not shown).

As they express the necessary host factors, CHO CD4/CCR5/CycT cells should be permissive to a spreading infection by HIV. To test this notion, both CHO CD4/CCR5/CycT (subclone 4) and HeLa CD4/CCR5 (subclone 11) cells were inoculated with a low dose of 49-5, a CCR5-tropic HIV molecular clone (119). To determine whether the amount of soluble p24 production detected was due simply to virion binding and release, AZT was added to some of the infected cultures at the time of infection. In both the HeLa and CHO cultures, soluble p24 was undetectable in the presence of AZT (Fig. 2.5C). To determine the level of soluble p24 production due to a single round of infection, AZT was added one day after inoculation to allow completion of one round of

the viral life cycle while preventing subsequent rounds of infection. Some soluble p24 was produced under these conditions, more from the CHO than the HeLa cultures. Finally, in the absence of AZT, high levels of p24 were produced from both the CHO and HeLa cultures (Fig. 2.5C). As these levels are clearly above what was produced in a single round, this demonstrates that these cells can support all steps in the viral life leading to a spreading infection.

Discussion

Despite considerable effort, our screening strategy was unsuccessful. Without a successful screen, it is difficult to be certain why this approach failed but two reasons are likely. First, only 60% to 80% of the HeLa cultures could be infected without excessive toxicity. A similar but much more efficient approach led to the identification of the anti-viral host factor, TRIM-5α (31). In this screen, a cDNA library from a cell line refractory to HIV infection was expressed in an HIV-permissive cell line to identify the factor capable of protecting the cells from a GFP-expressing HIV reporter virus. After seven rounds of screening in which 99% of the cells were initially infected, TRIM-5α was identified as the responsible anti-viral factor. This study makes clear that a screen operating at 60% to 80% infection levels would be unlikely to efficiently identify an HIV-resistant cell line.

Second, the HeLa cells might not have been sufficiently mutagenized. From measures of chromosome content, most loci in HeLa are present in two to three copies (98). Thus, reducing the copy number of a gene by one when there are still one or two other copies present might be inadequate to generate the desired functional change. Although there are some genes where this might be true, this likely represents a skewed subset of the overall genome.

CHO cells represented an attractive option to address both of the above concerns as they are highly permissive and resistant to the type of cell death seen in highly infected HeLa-based cultures. In addition, CHO cells are functionally hemizygous at many loci (99-102), meaning that a single retroviral insertion would be much more likely to have a functional outcome than in HeLa cells. Unfortunately, a tk-deficient version of CHO cells

does not exist for titering virus. One future approach to this problem would be to replace the tk-expression cassette in the RET vectors with another marker, such as GFP or dsRed2, which would allow for rapid determination of viral titers on a wide variety of cell lines. Alternatively, a tk-deficient CHO cell line could be generated by growing cells in bromo-deoxyuracil (97, 105).

Instead of a screen based on GFP expression, a selection based on the killing of infected cells might be an easier and more powerful approach. A selection could be designed in many ways. One approach would be to select for cells resistant to the cytopathic effects of wild-type HIV infection (120). However, under certain conditions, HIV can kill uninfected, bystander cells (121). It is unknown if bystander killing occurs in CHO cells. If it does, selection with wild-type HIV would kill both infected and uninfected cells, meaning that survivors would have to be resistant to both direct and indirect forms of cell death. A more targeted approach to ensure death of only HIV-infected cells would be to use a single-cycle reporter virus expressing a cytotoxic protein, such as the diphtheria toxin subunit A (DTA), which can be expressed from a single open reading frame and is very potent (122). Expression of DTA under lineage-specific promoters has been used *in vivo* to study the consequences of eliminating various cell types (123, 124).

In an effort to generate DTA-expressing single-round reporter viruses, we performed some preliminary experiments. Plasmids were constructed, and attempts were made to produce virions. Unfortunately, DTA expression during virion production in 293T cells appears highly toxic. To protect the virus-producing cells from DTA, we generated 293T cells stably expressing a mutant form of elongation factor-2, the host

Materials and Methods

Production and Titration of MLV Virions Containing RET Vector

MLV virions containing RET vectors were produced by calcium phosphate transfection of packaging cell lines grown in DMEM and containing 10% FCS and 1% penicillin/streptomycin (Gibco). For cotransfections with the ampho Env, plasmids were combined in a ratio of 3 μg of RET DNA to 1 μg of ampho Env. For cotransfections with VSV-G, a ratio of 3.6 μg to 0.4 μg was used. The medium was changed approximately 16 h after transfection, and 32 h later, the virion-containing supernatant was harvested, sterile filtered, and stored in the vapor phase of a liquid nitrogen storage-unit. In some control experiments, the MLV-based tk-expression plasmid, G1TK1SVNA, was also used.

To titer virus stocks, 2.5×10^4 tk-deficient target cells per well, often HeLa BU25, were seeded in a 12-well tissue-culture plate and spinoculated the next day. For spinoculation, 1 ml of virus, neat or diluted, was added to 0.5 ml of medium and polybrene (5 μg/ml). Plates were centrifuged at 1200 *g* (2500 rpm) for 90 min at 32°C and immediately returned to tissue-culture incubators. For either HAT (Gibco) or G418 (600 μg/ml) selection, medium was replaced with selection medium 2 days later and changed every 3–4 days thereafter. HAT-resistant colonies were counted after 6-10 days of selection. Culture medium was aspirated, colonies were fixed in 10% formaldehyde and stained with methanol blue (1% coomassie blue in methanol) for counting. Typically, the titer of MLV-virions containing the 1031 RET vector produced from Plat-E cells cotransfected with ampho Env was 10^4 to 10^5 CFU/ml when tested on HeLa BU25 cells.

When tested on different tk-deficient cells lines, Rat2 and L-M tk– cells generated 1.5- to 2-fold more colonies than HeLa BU25 cells.

Southern Blot for Integrated RET Vector

HeLa-based cell lines were grown to confluency in a 75-cm^2 flask, detached with trypsin, and the cell pellet lysed in 600 λ of 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, 10 mM NaCl, 0.5% SDS, and 1 mg/ml proteinase K. After incubation for 16 h at 37°C, 1.8 ml of 150 mM NaCl in ethanol was added and the samples were incubated at room temperature for 30 min. The resulting DNA precipitate was washed with 70% cold ethanol prior to resuspension in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Genomic DNA (10 μg) was digested with *Hind*III before separation by agarose gel electrophoresis and transfer to Zeta Probe membrane (BioRad). An end-labeled DNA probe was prepared with a fragment from the RET vector corresponding to the neomycin resistance open reading frame (800-bp fragment after a *Bam*HI digest) and incubated with the membrane overnight before detection by phosphoimager.

Production of and Infection with GFP-Expressing HIV Reporter Viruses

GFP-expressing pseudotyped reporter viruses were produced by calcium phosphate transfection of 293T cells with the following ratio of plasmids: 1 μg packaging construct, 2 μg genome, and 1 μg envelope. One of the packaging constructs, ΔR8.2, expressed all of the HIV accessory proteins, and another, ΔR8.91, only expressed the accessory proteins Tat and Rev (93). The ΔR8.91 plasmid containing the D64E mutation in integrase was kindly provided by Brian North (Dr. Eric Verdin's Lab). Virion production and infectivity was similar between the two packaging vectors. Two different

GFP-expressing genomes were used. Most experiments, including the screen, were performed with a construct containing a CMV-promoter driving GFP expression (pHR CMV GFP) (94). In some experiments, a construct containing a PGK-promoter driving GFP expression that also contained the central polypurine tract (PPT GFP) (126, 127) was used.

For infection with these viruses, 4–8 × 10^4 adherent target cells per well were seeded on a 24-well tissue-culture plate and infected the next day by replacing the culture medium 0.5–2 ml of virus and incubating for 1–2 h. In most infections, polybrene (5 μg/ml) was added and the plates were centrifuged (spinoculation) at 1200 *g* for 1–2 h at 32°C. After infection, the inoculum was replaced with 1 ml of fresh medium, and samples were assessed for GFP expression 2–3 days later.

Flow Cytometric Analysis of GFP Content or Surface Proteins

For analysis of either surface markers (CD4 or CCR5) or GFP expression, adherent cell lines were detached by incubation in TEN buffer (40 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl) for 5–10 min at 37°C. Detached cells were centrifuged and resuspended in staining buffer (PBS containing 2% FBS). Samples were immunostained for 20–30 min at 4°C before flow cytometric analysis on a FACSCalibur (BD Biosciences) and analysis (FlowJo, Treestar).

Mutagenesis of HeLa-Based Cell Lines

Approximately 10^7 HeLa CD4/CCR5 or HeLa CD4/CCR5 subclone 11 cells were infected with 10^6 HAT CFU/ml. After the protocol devised for titration, 2.5 × 10^4 cells per well were seeded in a 12-well tissue-culture plate and spinoculated the next day with

2.5×10^3 HAT CFU/ml. The plates were immediately returned to tissue-culture incubators. To mutagenize 10^7 cells, 40 12-well plates were infected by spinoculation. Two days later and every three days after that, the cells were washed with PBS and fresh medium containing G418 (600 μg/ml) was added. Twelve days after infection, G418-resistant colonies (approximately 50–200 per well) were collected and subsequently cultured in the absence of G418 while screening for HIV-resistant cell lines.

Screens of RET-Mutagenized HeLa-Based Cell Lines

Each round of the screen sought to inoculate 5×10^6 mutagenized HeLa-based cells and to sort out uninfected cells 5 days later. In parallel, approximately 10^6 non-mutagenized HeLa-based cells were also infected and sorted. As the infections were performed by spinoculation, 8×10^4 cells per well were seeded in 24-well tissue-culture plate and infected the next day. The culture medium was replaced with titered virus stocks so to infect 60% to 80% of the culture in the absence of any toxicity. To check the titer of the virus stocks during each round of the screen, a separate culture of non-mutagenized non-screened HeLa-based cells were also spinoculated at the same time. Three days after infection, cultures were collected from the 24-well plates and transferred to appropriate tissue-culture flasks to continue growing. In addition, a small portion of samples was collected for GFP measurement by flow cytometric analysis. Typically 4–6 days after infected, GFP– cells were sorted from both the mutagenized and non-mutagenized culture on a FACSVantage. These cells were then returned to culture until there were enough cells for re-screening, typically 3–5 days later.

Generation of HIV-Permissive CHO-Based Cell Lines

CHO cells cultured in Ham's F12 medium (Gibco) containing 10% FCS and1% penicillin/streptomycin were spinoculated with VSV-G-pseudotyped HIV-reporter viruses expressing CD4, CCR5, or cyclin T (pHR CMV CD4, pHR CMV CCR5, or pHR CMV cyclin T, respectively, courtesy of Dr. Oliver Keppler). Three days after infection, cells were sorted for high levels of CD4 and CCR5 expression. After 3–4 days, this process was repeated for a total of four times. Once a culture stably expressing CD4 and CCR5 was established, limiting dilution was performed to isolate permissive clones.

Spreading Infection Assay of CHO-Based Cell Lines

Either CHO- or HeLa-based cell lines (1×10^4 cells per well) were seeded on a 24-well tissue-culture plate and inoculated the next day with 650 $TCID_{50}$ (128) 49-5 in 1ml of medium for 24 h. Medium was changed the next day and every other day for the next 7 days. On each day that medium was changed, a sample of the supernatant was saved to assay for p24 Gag production by ELISA (Perkin Elmer). AZT (50 μM) was added to some of the wells at the time of infection and to others 24 h after inoculation.

Figure Legends

Figure 2.1. The RET vector used for insertional mutagenesis and two clonal cell lines containing single insertions. (A) Constructed on an MLV retroviral backbone, the RET vector contains many of its functional features on the negative strand so as not to interfere with viral RNA production. A loxP site is located in place of the viral transcriptional enhancers in the 3′ LTR. During reverse transcription, this part of the 3′ LTR is duplicated resulting in an integrated provirus that lacks any transcriptional activity from the viral LTRs but is instead flanked by loxP sites which allows for proviral excision upon treatment with cre recombinase. The strong bcl-2 splice acceptor (SA) and subsequent polyadenylation signal is positioned so to block the expression of any gene into which the vector has inserted. At the other end of the vector, a constitutive promoter (P2) drives transcription of the gene for neomycin resistance followed by a splice donor (SD) but lack a polyadenylation signal. For a cell to become resistant to neomycin, the neo transcript must find a subsequent SA and polyadenylation signal. Finally, the RET vector contains an HSV-tk expression cassette (P1) allowing virus production to be titered on a tk-deficient cell line after HAT selection. (B) HeLa tk(-) were infected with RET containing virions at a low moi and subjected to HAT selection. From this culture, two subclones were isolated by limiting dilution. Genomic DNA was isolated, digested with *Hind*III, and probed for the neomycin resistance gene present in the RET vector by southern blot.

Figure 2.2. Integration is required for stable GFP expression from an HIV reporter virus. HeLa CD4/CCR5 were spinoculated with VSV-G pseudotyped GFP-expressing HIV

reporter viruses containing either wild-type (wt) or D64E integrase (IN). GFP expression was monitored 3, 5, and 7 days after infection by flow cytometry.

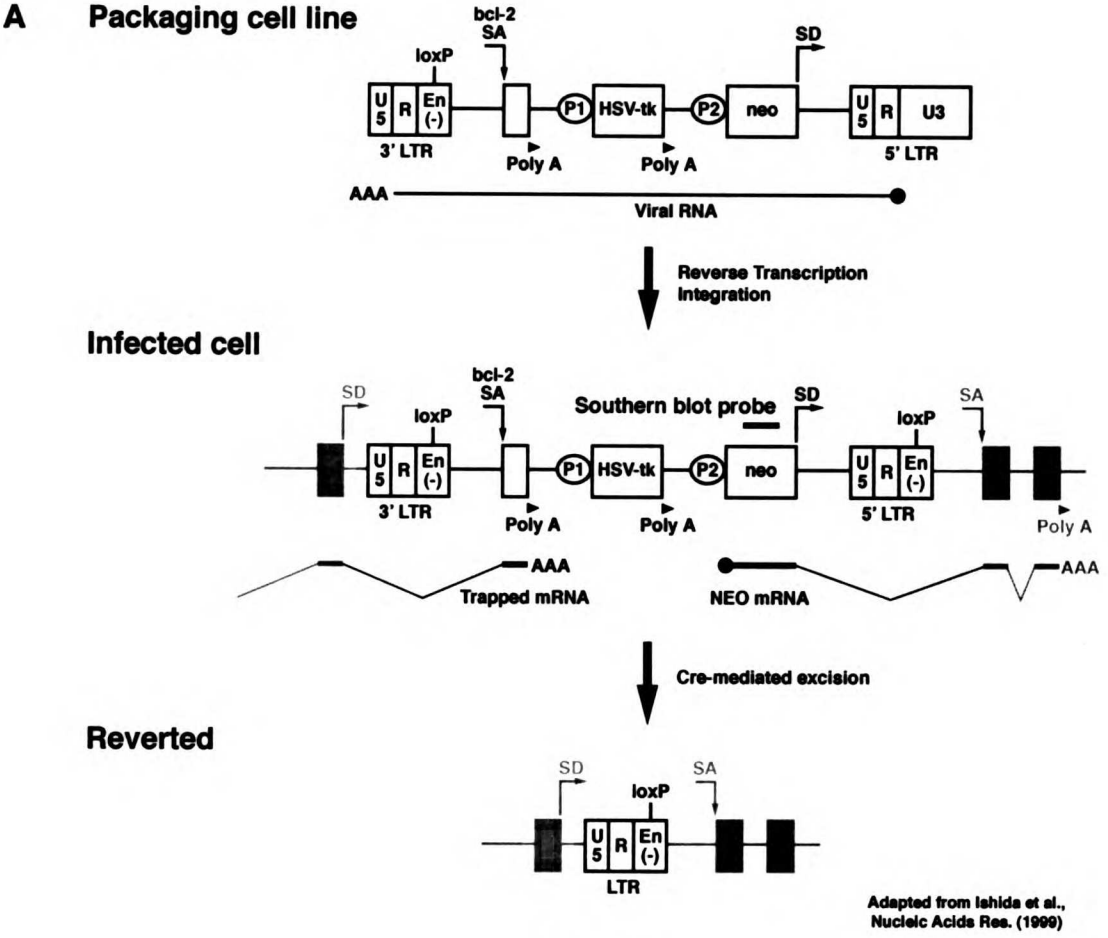
Figure 2.3. Increased reporter virus infectivity due to spinoculation and polybrene and the isolation of a highly permissive HeLa CD4/CCR5 subclone. (A) HeLa CD4/CCR5s were infected with GFP-expressing reporter virions containing no envelope, VSV-G, the BaL HIV Env, or the amphotropic MLV Env. Infections were performed under standard tissue-culture conditioned or by spinoculation. Polybrene (5 μg/ml) was added to some of the infections. (B) Parental and HeLa CD4/CCR5 subclones were tested for permissivity to GFP-expressing reported viruses containing either no envelope, the BaL HIV Env, or the amphotropic MLV Env. Shown are means with error bars depicting standard deviations of the spinoculations performed in triplicate.

Figure 2.4. No outgrowth of uninfectable cells in mutagenized HeLa CD4/CCR5s after twelve rounds of screening with VSV-G pseudotyped GFP reporter viruses. Each round of screening consisted of 3 infections. Mutagenized or non-mutagenized HeLa CD4/CCR5s were infected with VSV-G pseudotyped GFP-expressing reporter viruses and GFP- cells isolated by flow cytometry for subsequent re-screening. In parallel, naive HeLa CD4/CCR5 were infected during each round to monitor infection conditions (titer). Shown are the percentages of GFP+ cells after each infection.

Figure 2.5. CHO cells expressing CD4, CCR5, and cyclin T are highly permissive to HIV infection. (A) Surface expression levels of CD4 and CCR5 were determined by flow cytometry on the original CHO cells, the parental CHO CD4/CCR5/CycT cells or subclone 4. (B) GFP expression in HeLa CD4/CCR5 (subclone 11) or CHO

CD4/CCR5/CycT (subclone 4) was measured in either uninfected cultures or cultures infected with GFP-expressing HIV reporter virions pseudotyped with either VSV-G or the BaL envelope of HIV. (C) Soluble p24 production from either HeLa CD4/CCR5 (subclone 11) or CHO CD4/CCR5/CycT (subclone 4) was measured after inoculation or not with HIV-1 (49-5, an R5 molecular clone). In some of the inoculated cultures, AZT (50 μM) was added either at the time of infection or one day later. Soluble p24 was measured by ELISA with a lower limit of detection of 3 pg/ml.

Figure 2.1



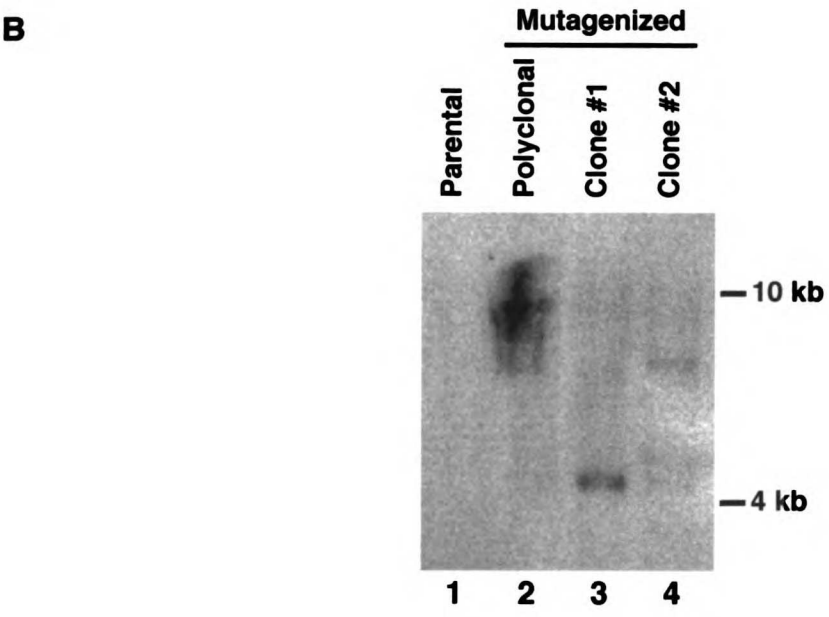


Figure 2.2

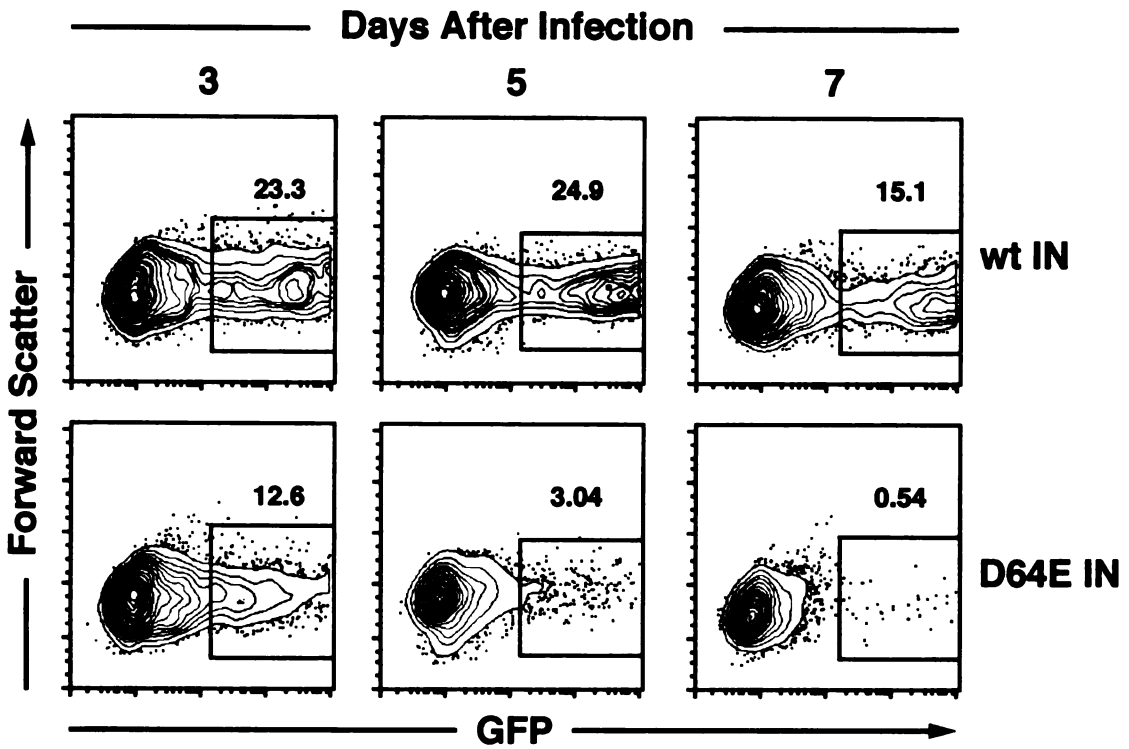


Figure 2.3

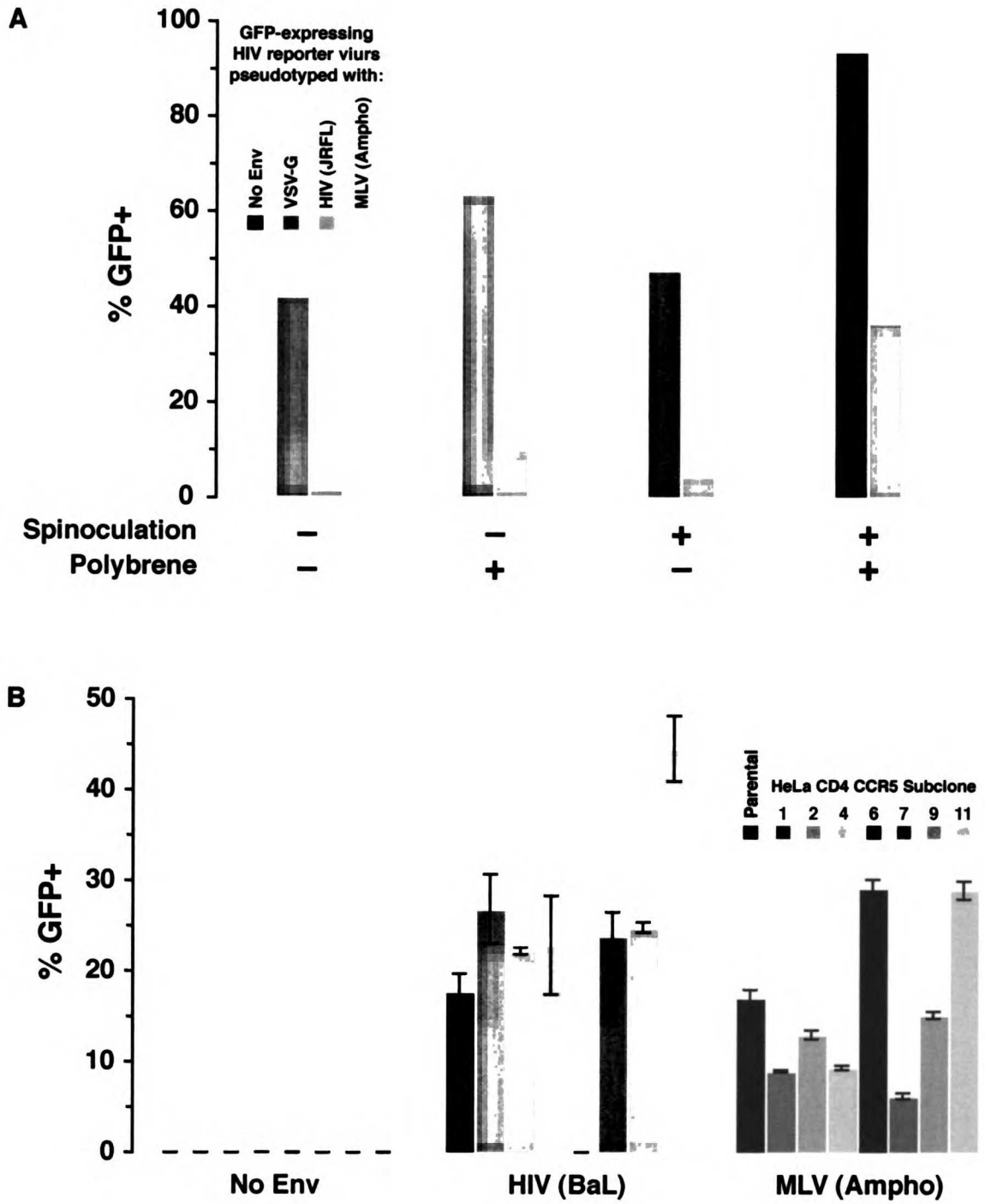


Figure 2.4

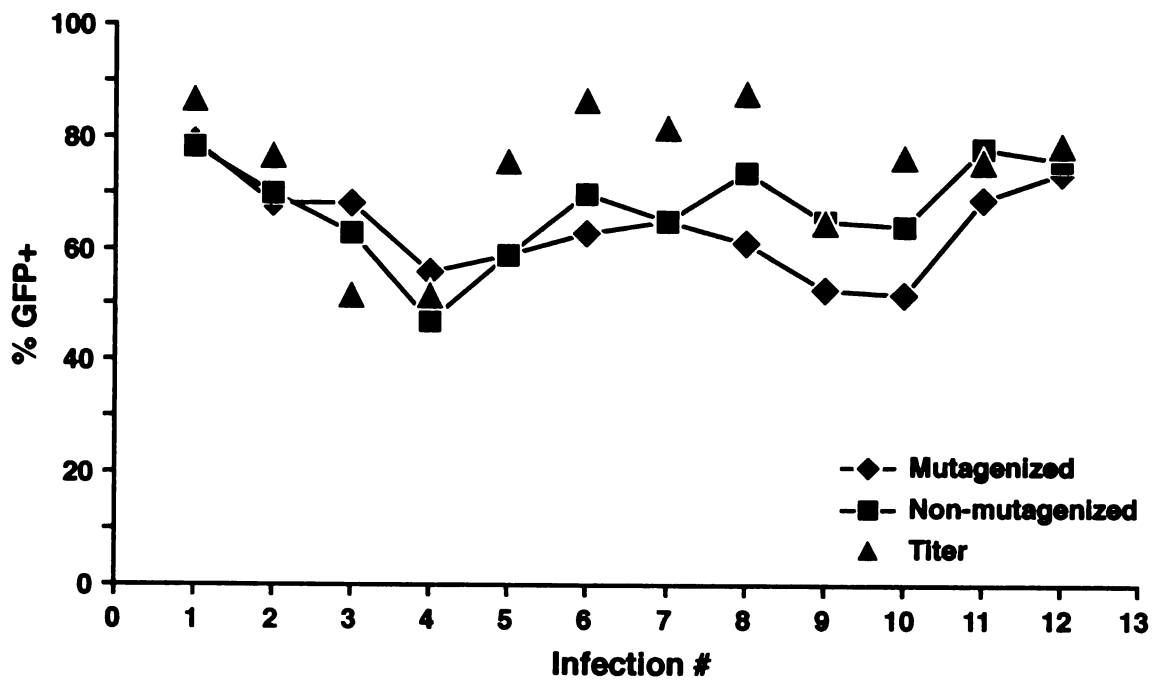
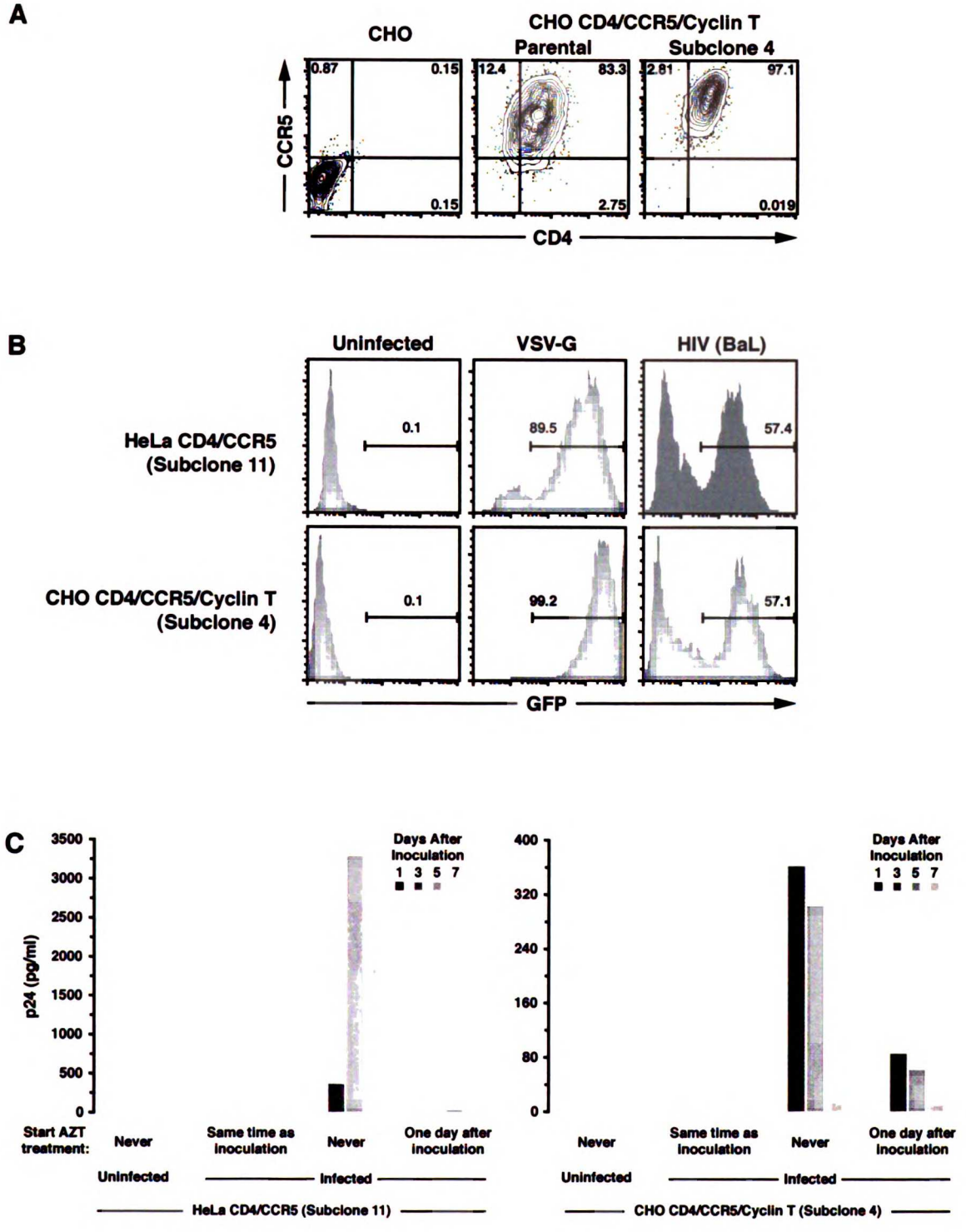


Figure 2.5



Chapter 3

Cytopathicity of HIV-1 Primary Isolates Depends on Coreceptor Usage and Not Patient Disease Status

Introduction

Human immunodeficiency virus type 1 (HIV-1) is known to evolve throughout the course of disease in infected individuals (129, 130). To compare the cytopathicity and replication kinetics of clinical isolates from early and late stages of disease, various cell line-based assays have been used to show that late viruses typically are more cytopathic and can replicate faster *in vitro* (69, 70). The identification of HIV-1 coreceptors and their expression on various cell lines has shed new light on these data. Virtually all HIV-1 viruses isolated from patients use one or both of two chemokine receptors, CCR5 (2, 3) or CXCR4 (4), as major coreceptors along with CD4 (1) for entry into target cells (reviewed in ref (131)). Viruses isolated early in the course of disease typically use CCR5 as a coreceptor (R5 viruses) whereas viruses isolated late in the course of disease commonly can use either CXCR4 alone (X4 viruses) or both CCR5 and CXCR4 (R5X4 viruses) (73). Typically, cell lines used for *in vitro* characterization express high levels of CXCR4 and low levels of CCR5 (63), and these facts explain why late X4 viruses characteristically replicate more vigorously and have greater cytopathic effects in such experiments. Likewise, using a novel experimental system based on *ex vivo* human lymphoid histocultures, it has been established that X4 viruses are more cytopathic than R5 viruses (65, 66, 132) and specifically that late X4 viruses are more cytopathic relative to early R5 viruses (133). An important question remains whether primary isolates from different stages of disease differ in their cytopathicity independently of coreceptor preferences.

Results

To determine whether HIV-1 cytopathicity corresponded to the stage of HIV disease, we tested a variety of primary isolates and biological clones derived from HIV-1-infected patients using an *ex vivo* human lymphoid histoculture system (65, 66, 132-134). These experiments were carried out in either blocks (66, 132, 133) or dispersed cultures (135) of human tonsil specimens, and similar results were obtained in both assays. HIV-1 isolates or clones were first expanded and then titered by end-point dilution on PHA-activated PBMCs pooled from 2–4 normal donors. The inoculum size was either 20 $TCID_{50}$ per tissue block or 50 $TCID_{50}$ per well of dispersed tissue. Histoculture infections were carried out typically for 2 weeks, with culture media changes the day after infection and every 3 days thereafter. At the end of the experiment, the tissue was harvested and split into 2 equal samples for immunostaining and analysis by fluorescence activated cell sorting (FACS). One sample was stained with antibodies to CD3, CD4, CD8, and CCR5 for analysis of depletion of both total $CD4^+$ $CD3^+$ lymphocytes (referred to hereafter as CD4 T cells) and $CCR5^+$ and $CCR5^-$ subsets of CD4 T cells. The other sample was stained with antibodies to CD3, CD4, CD45RA, and CD62L for measurement of depletion of naive and memory CD4 T cells (136, 137).

We first sought to determine whether X4 viruses present early after infection in some individuals differ in their cytopathicity from X4 viruses isolated late in disease. We compared the $CD4^+$ T-cell depletion potential of an R5X4 isolate from a patient obtained within 90 days of infection (Fig. 3.1, Patient X; Table 3.1) to that of an R5X4 isolate derived from a patient 6.5 years after seroconversion (Fig. 3.1, Patient W). As a positive control, we also asssayed depletion by a highly cytopathic X4 molecular clone, NL4-3

(65, 66, 132, 133, 138). We found that the early R5X4 isolate depleted CD4 T cells as potently as did both the late R5X4 isolate and the control virus, NL4-3 (Fig. 1A); each virus led to 85–90% depletion of all CD4 T cells relative to uninfected samples. Furthermore, subsetting of CD4 T cells into $CCR5^+$ or $CCR5^-$ fractions revealed thorough depletion of both subsets by all viruses tested here (Fig. 3.1A). In addition, depletion analysis of naive and memory subsets of CD4 T cells was performed. As observed previously with other X4 viruses, severe depletion of both $CD4^+$ T-cell subsets was observed with all X4 and R5X4 viruses tested here (Fig. 3.1B). Finally, robust viral replication kinetics were observed for these viruses based on measurements of HIV-1 p24 in the culture supernatant (Fig. 3.1C). These results, which are consistent with the fact that nearly all CD4 T cells in human tonsils express CXCR4 (65, 66) and are thus potential targets for HIV-1, demonstrate that cytopathicity correlates well with the coreceptor preference of X4 isolates. Indeed, we have detected very little variability in the depletion behavior of a wide range of X4 or R5X4 isolates (data not shown).

We next sought to determine if the behavior of R5 viruses was similarly independent of patient status. We compared the cytopathicity of an early R5 biological clone to a late R5 biological clone derived from the same patient (Fig. 3.1, Patient Z). In addition, we tested whether these clones differed from two other R5 isolates: one isolate was derived from a patient within 90 days of infection (Fig. 3.1, Patient Y), and the other was isolated from a patient 5 years after seroconversion who was asymptomatic at the time (Fig. 3.1, Patient W). As a positive control, we also tested a previously characterized R5 molecular clone, 81A (119). All five R5 viruses were found to deplete CD4 T cells equally. Each depleted approximately 15% of total CD4 T cells (Fig. 3.1A) but nearly all

$CCR5^+$ CD4 T cells (Fig. 3.1A). As demonstrated previously, the apparent decreased cytopathicity of R5 viruses compared with X4 viruses is due to a decreased target pool size resulting from the limited expression of CCR5 compared with that of CXCR4 (65, 66). Moreover, subsetting CD4 T cells into memory or naive revealed that R5 viruses depleted a portion of memory CD4 T cells, presumably the CCR5-expressing fraction, but did not deplete naive CD4 T cells (Fig. 3.1B) due to very low CCR5 expression (62, 63, 139). To establish that there was nothing unusual about the patient from whom these biological clones originated, we also tested an R5X4 biological clone that was isolated late in disease from Patient Z at the same time as the previously tested late R5 biological clone. Indeed, the depletion profile of this R5X4 biological clone was similar to that of all other X4 and R5X4 viruses (Fig. 3.1A & B). Substantial replication was evident for all R5 viruses and the control R5X4 clone (Fig. 3.1C). In summary, the results thus far revealed no evidence that any viral trait other than coreceptor preference regulates cytopathicity of primary isolates in *ex vivo* cultures of human tonsils.

It is possible that the late R5 biological clone tested above was not especially cytopathic because it had experienced no selective pressure to acquire greater cytopathic properties in the context of highly cytopathic R5X4 viruses that were already systemic in the individual. To address this issue, we tested a panel of biological clones isolated longitudinally from four patients who exhibited significant disease progression but never developed detectable X4 viremia or were treated with antiretrovirals. Two patients were homozygous for the wild-type allele of CCR5 (Fig. 3.2, Patients A and B) and progressed to AIDS within ~4 years of seroconversion, whereas the two other patients were heterozygous for the Δ32 allele of CCR5 (Fig. 3.2, Patients C and D) and progressed to

AIDS in ~8–10 years. The depletion patterns of these eight viruses were analyzed with particular interest in differences between early and late viruses within a given patient or between viruses from genotypically different patients. However, the results from these eight viruses showed no such differences. Each infection yielded moderate depletion of CD4 T cells with profound depletion of the $CCR5^+$ subset of CD4 T cells and sparing of the $CCR5^-$ subset (Fig. 3.2A). Moreover, memory CD4 T cells were depleted moderately by each of these isolates, while the naive CD4 T cells were not (Fig. 3.2B). Again, substantial viral replication kinetics were seen throughout the course of the experiment among these isolates (Fig. 3.2C). These results demonstrate that R5 viruses isolated in either the absence or presence of systemic X4 viremia are equally cytopathic, even among viruses causing severe disease progression in the absence of evolution to X4 phenotype. These data argue against a model of HIV-1 evolution that posits selective pressure on HIV-1 during the course of disease to acquire cytopathic traits other than expanded target cell range via coreceptor evolution.

Discussion

In summary, we have shown that the cytopathicity for tissue lymphocytes of a diverse set of primary isolates from various stages of disease is entirely restricted by coreceptor utilization, and does not typically display coreceptor-independent evolution during the progression of disease. This finding likely has implications for disease pathogenesis, but the possibility that there may be subtle, coreceptor-independent evolution of pathogenicity *in vivo* that is not reflected in this *ex vivo* culture system cannot be excluded. Likewise, we cannot exclude the possibility that propagation of virus isolates may have diminished virulence differences, a potential problem with any functional survey of primary isolates. Given the range of sources of viruses and the uniformity of our results, this report nonetheless establishes the general principle that the ability of HIV-1 to deplete CD4 T cells in histoculture is a predictable event based on coreceptor usage of the virus and coreceptor expression of the target tissue.

Our results indicating equal cytopathicity of early and late R5 viruses from patients who progressed to AIDS but lack X4 viremia are in agreement with one but not another study of similar isolates tested in the SCID-hu Thy/Liv xenotransplant model (140, 141). Berkowitz *et al.* analyzed two late stage R5 biological clones, including one from Patient A, and did not find increased cytopathicity relative to control viruses. In contrast, Scoogins *et al.* tested the cytopathicity of biological clones from early, middle, and late stage disease derived from some of the same patient isolates we tested here and found significant depletion of $CD4^+CD8^+$ thymocytes in some implants with a single late stage clone but not with clones from earlier in disease. It is important to note the differences between the SCID-hu model and histoculture when interpreting the above

results. The human tissue in the SCID-hu xenografts originates from thymic tissue and thus represents a system to test the effects of HIV-1 on immature and developing thymocytes (142, 143). In fact, the bulk of these tissues are $CD4^+CD8^+$ thymocytes, of which more than 90% would be eliminated by thymic selection normally. In contrast, the experimental explants used in the present studies are derived from mature lymphoid tissue that is populated by T-cells that have survived thymic selection. Thus, the depletion properties observed here are indicative of the cytopathic capabilities of HIV-1 for mature CD4 T cells.

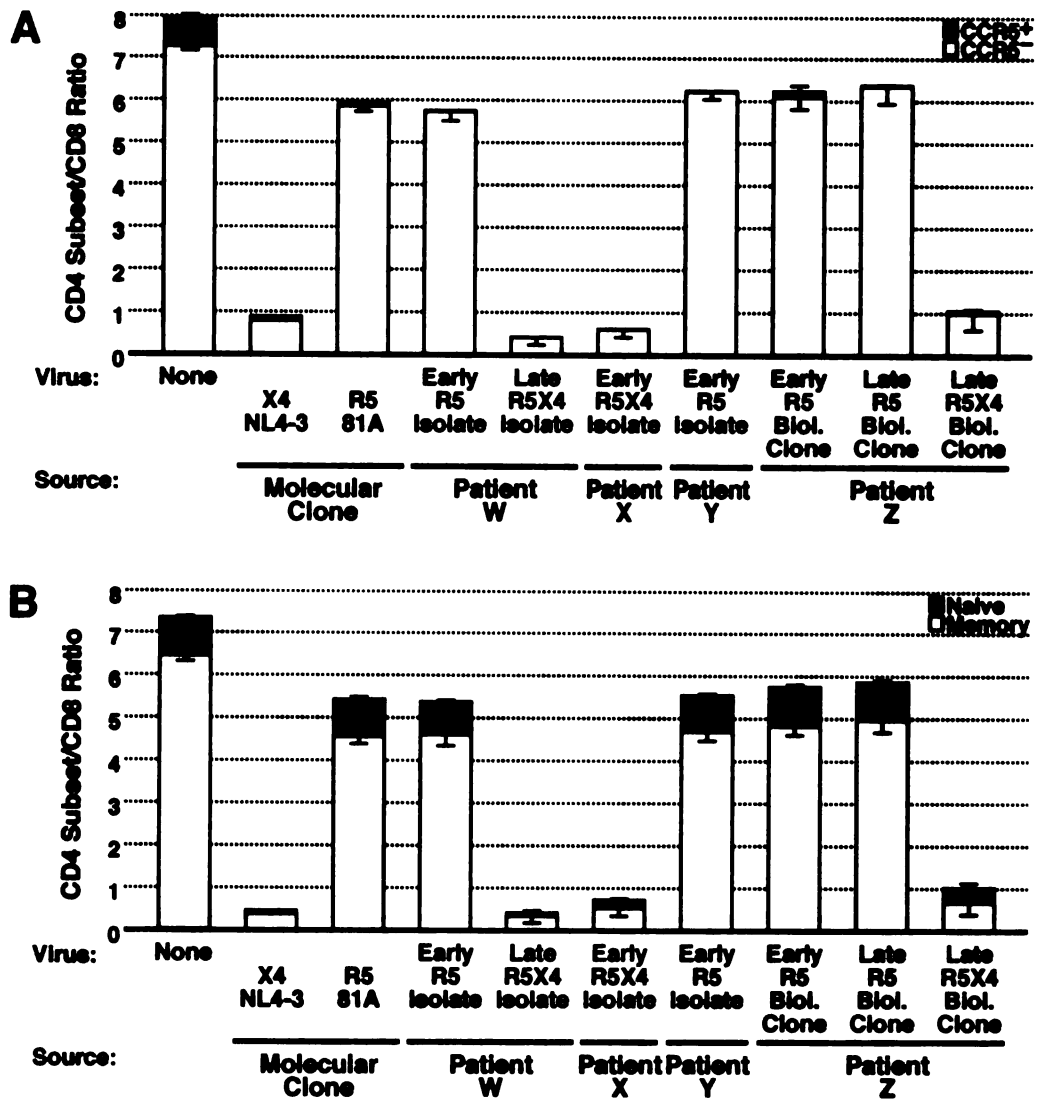
In the context of disease progression, these data regarding the cytopathicity of early and late R5 viruses indicate that HIV-1 need not experience an increase in its cytopathicity over time for it to cause severe disease in infected people. An R5 virus that successfully infects and eliminates the entire CCR5-expressing pool of CD4 T cells is apparently cytopathic enough to deplete the immune system sufficiently to cause AIDS, presumably through attrition of cells that dynamically express CCR5 at various stages of the cellular life cycle (62). We hypothesize that as the immune system seeks to replenish the CCR5-expressing fraction of CD4 T cells to restore homeostasis in the context of peripheral destruction of such cells, an R5 virus will continually find new target cells until too few $CD4^+$ cells remain to maintain a functional immune system.

Figure Legends

Figure 3.1. Cytopathic potential of primary HIV-1 isolates tracks with coreceptor utilization but not stage of disease. (A) Dispersed human tonsil tissue was inoculated in replicate microtiter wells (n=3 for experimental viruses, n=2 for NL4-3), with the indicated viruses (see Table 3.1). Tissue was harvested, immunostained, and analyzed by FACS 13 days after infection as described (66, 133). The total height of the bar in the graph represents the ratio of CD4 T cells to CD8 T cells. The $CCR5^+$ and $CCR5^-$ subsets are represented by the black and white sections of the bar graph, respectively. Standard error of the mean is represented by the error bars. (B) The samples shown in panel A were analyzed for depletion of memory and naive cells. Naive CD4 T cells were defined as CD4 T cells that were $CD45RA^+$ $CD62L^+$, and all other CD4 T cells were defined as memory CD4 T cells (136, 137). The white portion of the bar graph represents the memory subset and the black portion represents the naive subset. (C) Culture supernatant was assayed for HIV-1 p24 by ELISA to monitor viral replication. Experiments with different donor specimens were conducted twice in dispersed cultures and once in tissue blocks, and are representative data from one experiment.

Figure 3.2. Primary R5 HIV-1 isolates from advanced disease without X4 viremia retain selective cytopathicity for $CCR5^+$ T-cells. Experiments were performed with the indicated viruses (see Table 3.1) as described in the legend of Figure 1. (A) Depletion of CD4 T cells, including total CD4 T cells, and $CCR5^+$ and $CCR5^-$ subsets. (B) Depletion of memory and naive subsets of CD4 T cells. (C) HIV-1 replication kinetics. Experiments

Figure 3.1



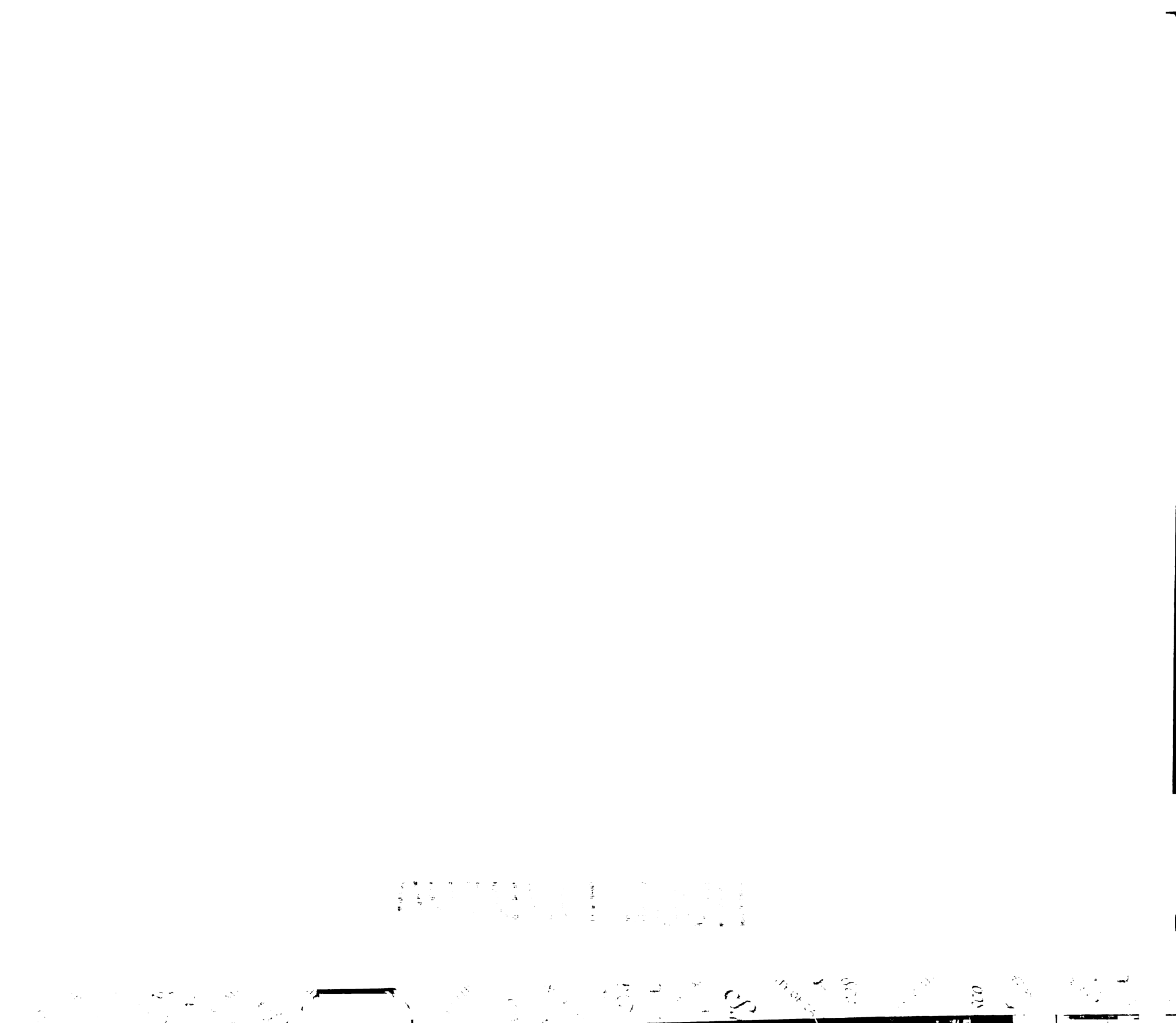


Figure 3.2

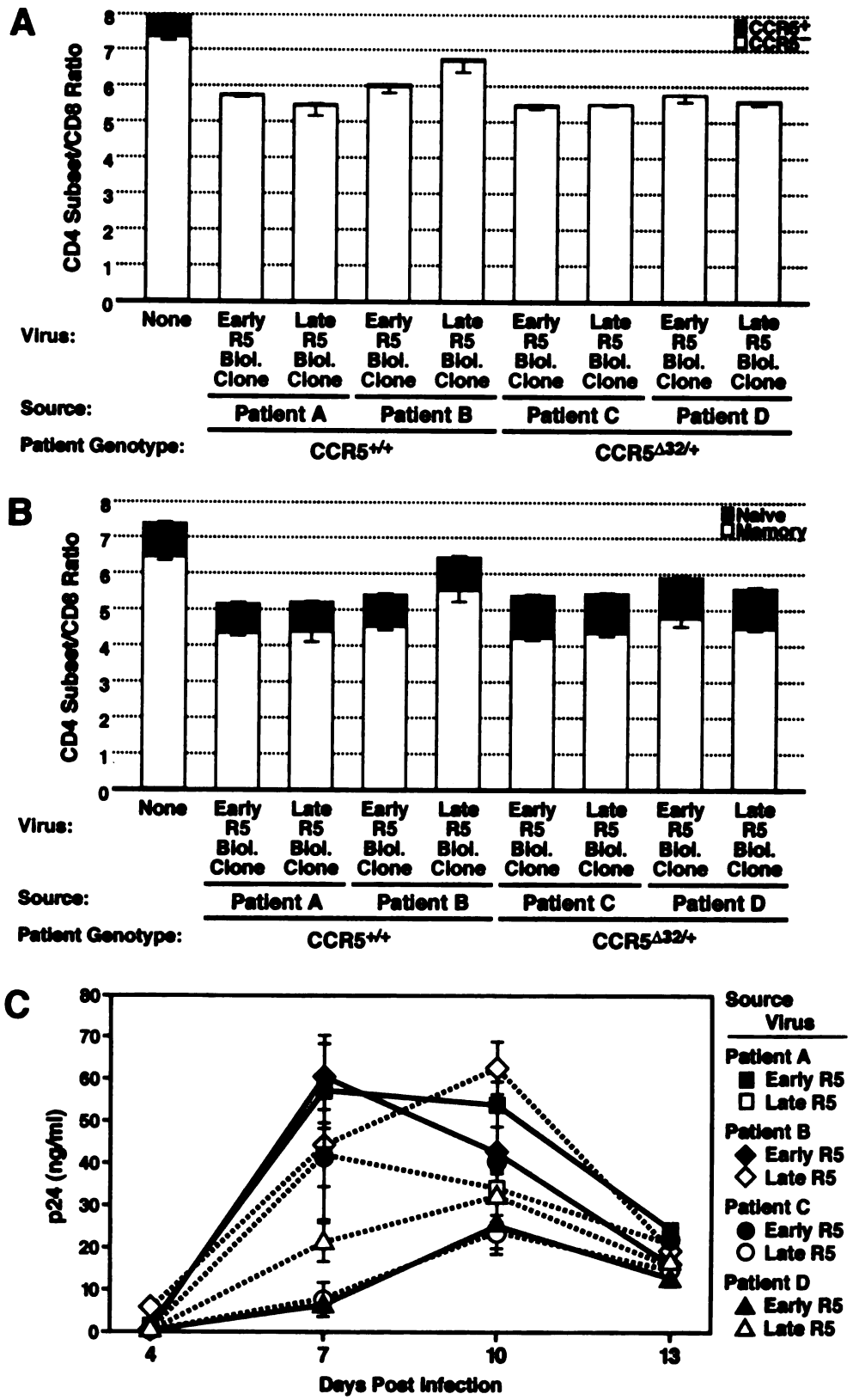


Table 3.1. Summary of Primary HIV-1 Viruses Tested

Figure	Source	Virus	Literature Reference(s)	Patient Designation in Literature
1	Patient W	Early R5 isolate	(70, 73, 133)	Patient C (1/85)
	Patient W	Late R5X4 isolate	(70, 73, 133)	Patient C (7/86)
	Patient X	Early R5X4 isolate	None	None
	Patient Y	Early R5 isolate	(144)	MJM
	Patient Z	Early R5 biological clone	(130)	Patient 8
	Patient Z	Late R5 biological clone	(130)	Patient 8
	Patient Z	Late R5X4 biological clone	(130)	Patient 8
2	Patient A	Early R5 biological clone	(129, 140, 141, 145, 146)	ACH 424
	Patient A	Late R5 biological clone	(129, 140, 141, 145, 146)	ACH 424
	Patient B	Early R5 biological clone	(146)	ACH 537
	Patient B	Late R5 biological clone	(146)	ACH 537
	Patient C	Early R5 biological clone	(145, 146)	ACH 38
	Patient C	Late R5 biological clone	(145, 146)	ACH 38
	Patient D	Early R5 biological clone	(145, 146)	ACH 617
	Patient D	Late R5 biological clone	(145, 146)	ACH 617

Chapter 4

HIV-Infected Primary CD4 T Cells Are Hyper-Responsive Independent of Nef

Introduction

CD4 T cells are constantly being infected and killed in HIV-infected individuals where the half-life of HIV-infected CD4 T cells is 1–2 days (147-149). The majority of infection occurs in lymphoid organs, either in solid tissues such as lymph nodes (150-152) or in gut-associated lymphoid tissue (61, 153-156). In these tissues, HIV-infected CD4 T cells are frequently non-dividing, resting cells (153, 157), which is consistent with *in vitro* studies of HIV-infected lymphoid organ cultures (135). Since HIV replication is sensitive to the activation status of infected cells, these findings raise the possibility that there might be a replicative advantage to increasing target cell activation during the limited time available for virion production.

A key HIV protein thought to modulate the activation state of HIV-infected cells is Nef, a 27 kDa, myristoylated protein expressed early in the viral life cycle. Although not absolutely essential for viral replication, Nef is critical for high levels of viral replication both *in vitro* (158, 159) and *in vivo* (160, 161). In the absence of Nef, progression to AIDS is delayed in both HIV-infected humans (161) and SIV-infected rhesus macaques (160). Although many functions have been proposed for Nef, it mainly appears to serve as an adaptor between molecules either at or just proximal to cellular membranes. These actions have numerous consequences that often appear to be cell-type, expression level, or strain specific. Among its more frequently studied properties, Nef can down-regulate CD4 (162, 163), MHC I (164), or other cell-surface markers (165-167); extend the life of HIV-infected cells (168, 169); and increase the activation status of HIV-infected cells (170-173). Recent studies have demonstrated that either by direct (174) or indirect (175) interactions, Nef inhibits upstream inhibitory pathways, thereby

promoting downstream cellular activation. In both cases, the ability of Nef to activate cells is compromised when the myristoylation site in Nef is mutated.

These studies sought to understand the consequences of HIV-infection on the activation status of primary CD4 T cells and to determine if Nef played a role in this process. Whereas many previous studies have marked HIV-infected cells through the use of strains engineered to express a reporter gene (176-179), we chose to use replication-competent molecular clones of HIV that express endogenous viral genes at levels recapitulating those present in natural infection (119, 180). To identify HIV-infected cells, we performed immunostaining for intracellular Gag along with other intracellular and cell-surface markers. Because it is present in both virions and infected cells, Gag is not the ideal viral protein to uniquely identify HIV-infected cells. However, this procedure is well established and accepted for *in vitro* studies (135, 177, 181), although its use for the analysis of samples from HIV-infected patients has been controversial (182, 183).

To accurately assess the role of HIV infection on cellular activation, and Nef expression in particular, it is important to employ a system that recapitulates as closely as possible events occurring *in vivo* such as avoiding overexpression of viral constituents or the addition of strong T cell activators. Studies from both HIV- or SIV-infected individuals have shown that within tissue, resting CD4 T cells are permissive for infection (153, 157). Two experimental systems closely model these finding: PBLs cultured in IL-7 (184-187) or human lymphoid aggregate cultures (HLAC) (135). In both of these systems, naive and memory CD4 T cells are permissive to HIV infection in the absence of marked cellular activation or cellular division.

Results

HIV-Infected Cultures Contain Two Populations of CD8– T Cells That Stain Positively for Gag

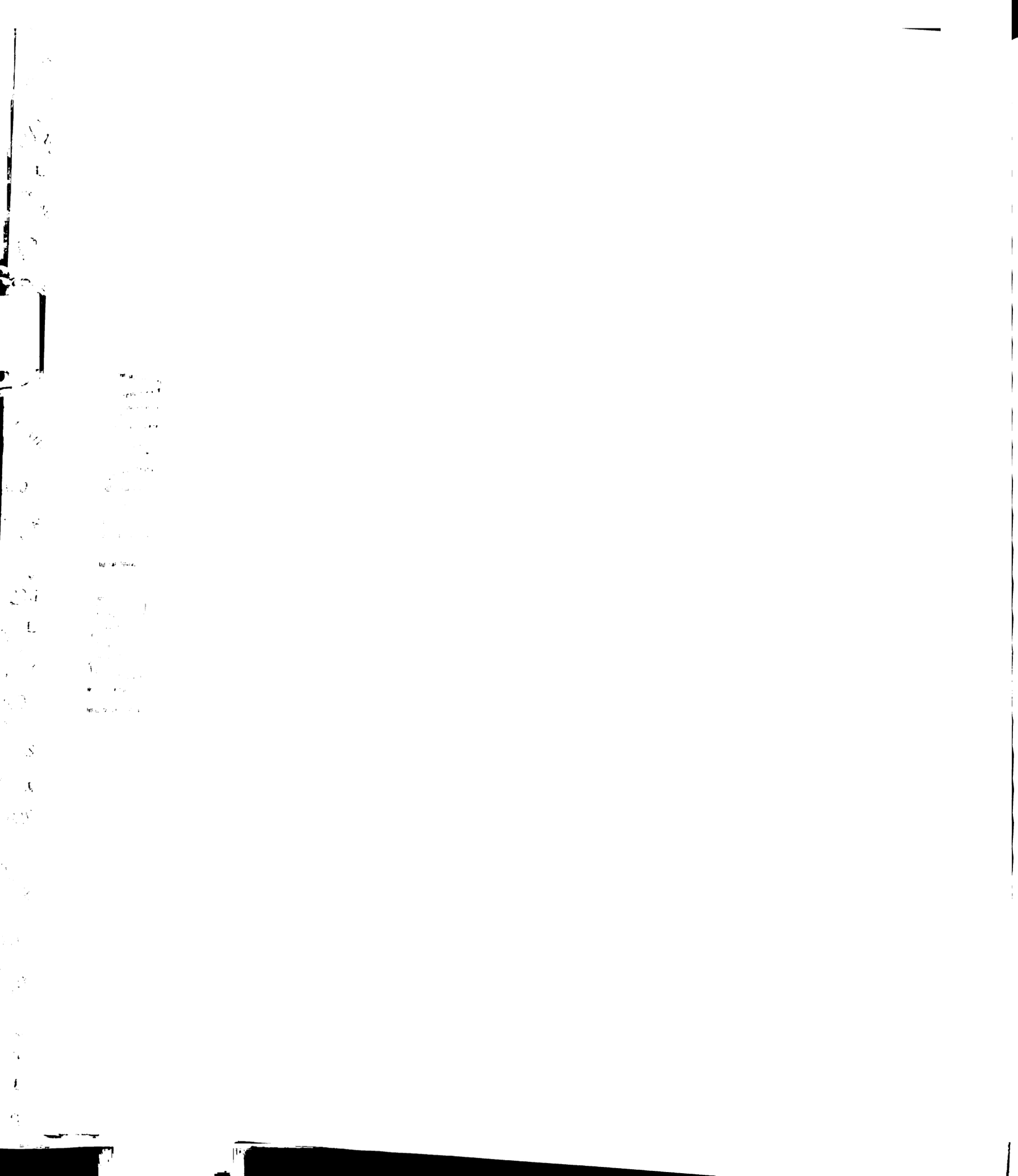
Isogenic X4- (NL4-3) and R5-tropic (81A) molecular clones of HIV were used to infect either IL-7-treated cultures of PBLs or HLAC (119). To study the role of Nef, NL4-3- or 81A-based viruses containing or lacking Nef were tested (180). The ΔNef constructs only expressed the first 35 amino acids of Nef. In most experiments, immunostaining for both surface and intracellular antigens followed by flow cytometric analysis was performed every 2–4 days to ensure observations at the peak of infection. To ensure that both wild-type and ΔNef viruses reached maximal infection at the same time, ΔNef-infected cultures were inoculated with higher concentrations of virus. In our initial experiments, we quickly realized that there were two populations of Gag+ T cells, specifically both CD4– and CD4+ cells that stained positively for intracellular Gag (Fig. 4.1). Interestingly, the CD4– subset of cells typically contained more Gag than CD4+ cells. The loss of CD4 expression in cells infected with wild-type HIV likely reflected the combined action of Nef, Env and Vpu, each of which is known to down-regulate CD4. The same pattern was observed in HIV-infected HLAC cultures (Fig. 4.1B and C). Importantly, Gag+ CD4– cells were also detected in ΔNef-infected primary CD4 T cell cultures, suggesting that Env and Vpu was sufficient to achieve CD4 down-regulation.

However, we considered the possibility that intracellular Gag immunostaining in these cultures reflected staining of both endogenously produced Gag and surface-bound virions or intracellular capsids. A better approach to the identification of infected cells would be based on a different viral protein not contained in the virion such as Tat or Rev.

Unfortunately, all antibodies against Tat and Rev proved inadequate to detect HIV-infected cells (data not shown).

The absence of surface CD4 expression in a subset of Gag-containing cells could reflect CD4 degradation by Vpu (188, 189) or a selective loss of CD4 at the cell membrane, perhaps due to increased retention, mediated by Env (190, 191), or internalization, mediated by Nef (162, 163). To determine if an internalized pool of CD4 is still present in these cells, cells were permeabilized and stained with antibodies reacting with CD4. Importantly, preparation of cells for intracellular staining did not interfere with the ability of this antibody to recognize CD4 (data not shown). Addition of antibodies recognizing CD4 during intracellular staining did not reveal CD4 expression in Gag+ cells (Fig. 4.1D), indicating that CD4 is potentially absent in this population.

In NL4-3 ΔNef-infected cultures, the Gag+ CD4– population typically expressed higher levels of CD4 than the other infected cultures (Fig. 4.1A–C). Although this might reflect the contribution of Nef to the down-regulation of CD4, this explanation is not consistent with the low levels of CD4 in the Gag+ CD4– population in 81A ΔNef-infected cultures (Fig. 4.1A and C). In NL4-3- but not 81A-infected cultures, bystander killing involving the interaction between gp120, CD4, and CXCR4 plays a significant role in cell death (121). These same pathways might mediate direct as well as bystander killing (192-196). We hypothesized that blocking this pathway would enable NL4-3 ΔNef-infected cells to survive long enough to fully down-regulate CD4, as in 81A ΔNef-infected cultures in which killing does not occur. To test this possibility, infected cultures were treated with AMD3100 which inhibits both infection (197) and bystander killing (121). Samples were immunostained four days later to measure CD4 expression levels



and intracellular Gag content. Preliminary experiments showed that addition of AMD3100 did not change the pattern of CD4 expression or Gag content in the NL4-3-infected culture but led to the appearance of Gag+ cells with fully down-regulated CD4 in the NL4-3 ΔNef-infected culture (Fig. 4.1E). Importantly, addition of AZT did not change the CD4 expression levels on the Gag+ cells (data not shown), suggesting that extending the life span of these cells allows for the appearance of cells that have fully down-regulated CD4.

In HIV-Infected Cultures, All Gag+ CD4– Cells Are Infected Whereas CD4+ Cells Are a Mixture of Infected and Uninfected Cells

Next, we determined if both Gag+ populations in HIV-infected cultures represent infected cells. Labeled with the fluorescent dye CFSE, CD4 T cells were added to HIV-infected cultures, and surface CD4 expression and intracellular Gag content were monitored for 3 days. Some of the target cells and subsequent cocultures were treated with inhibitors of HIV infection. In cultures without inhibitors of HIV infection, we can monitor the phenotype of CD4 and Gag expression in a population of susceptible target cells. In contrast, cultures in the presence of HIV-inhibitors allow us to monitor phenotypic changes that occur in target cells independent of infection.

After addition to NL4-3 infected cultures, CD4 T cells become Gag+ within 24 h (Fig. 4.2) regardless of whether inhibitors of HIV infection were present or not. In some experiments, Gag+ cells were evident within 10 h. Both of these findings suggest that in these HIV-infected cultures, uninfected CD4 T cells can stain positive with the anti-p24 Gag antibody either as a result of passive virion attachment to the surface of cells or internalization of virions. Conversely, Gag+ CD4– cells only appeared in the absence of

HIV-inhibitors (Fig. 4.2) supporting the notion that all of these cells are infected. Since these cells express high levels of Gag and have had sufficient time to down-regulate CD4, it is likely that they represent cells in which the virus has progressed rather late into its life cycle.

This experimental system, the addition of permissive or non-permissive CFSE-labeled uninfected CD4 T cells to HIV-infected cultures, is identical to those designed to investigate the role of bystander killing (121). In experiments interrogating bystander killing, the question is not the Gag content and CD4 expression of the CFSE-labeled cells but the survival of these cells. Analysis of cell survival revealed that both untreated and AZT-treated labeled cells died rapidly upon addition to NL4-3-infected cultures (Fig. 4.2B). Typically, 50% of cells were eliminated within 2–3 days. In contrast, AMD3100-treated cells were completely protected. These findings confirm the prior work of Jeckle *et al.* who showed the same pattern of killing present after 6 days of co-culture. Our experiments extend these findings by demonstrating that bystander killing of uninfected cells can occur rapidly within these cultures.

We performed similar experiments to compare the rate at which cells become Gag+ CD4– when added to cultures infected with different viral strains. CFSE-labeled cells added to NL4-3-infected cultures become Gag+ CD4– within 36–48 h (Fig. 4.2C), whereas preliminary results suggest the same cells added to an NL4-3 ΔNef- or 81A-infected culture required 3 days to become Gag+ CD4–.

We next sought to determine how quickly CD4+ cells turn into Gag+ CD4– cells and for how long Gag+ CD4– cells survive. CD4+ and CD4– T cells were isolated from an HIV infected culture, labeled with CFSE, and added to an uninfected culture to mimic

the cellular environment from which these cells were initially isolated. Consistent with the hypothesis that some CD4+ cells in the infected culture are infected but have not yet down-regulated CD4, a small percentage of CD4+ T cells from an HIV-infected culture rapidly became Gag+ CD4– (Fig. 4.2D). In addition, Gag+ CD4– disappeared rapidly, consistent with a short half-life for these cells late in the viral life cycle. These experiments underscore the dynamic nature of these HIV-infected cultures.

Gag+ CD4– Cells Express a Novel Pattern of Naive and Memory T Cell Markers

Since Gag+ CD4– cells in HIV-infected cultures are all infected whereas CD4+ cells, regardless of Gag content, are a heterogeneous population of infected and uninfected cells, we sought to determine if Nef affects the properties of infected cells or the type of cell infected. X4-tropic strains of HIV can infect both memory CD4 T cells, which typically expresses moderate levels of activation markers, and naive CD4 T cells, which express low levels of activation markers. We hypothesized that in the absence of Nef, naive CD4 T cells would be refractory to infection due to the lack of cellular stimulation mediated by Nef.

To test this hypothesis, we determined the expression pattern of naive and memory markers on infected cells in cultures infected with NL4-3, 81A, or NL4-3 ΔNef. NL4-3 is X4-tropic so this virus is able to infect both naive and memory CD4 T cells, whereas the isogenic virus 81A is R5-tropic and can only infect a subset of memory CD4 T cells expressing CCR5. Naive CD4 T cells express CD45RA, CD62L, and CD27 but not CD45RO (Fig. 4.3A). In contrast, memory CD4 T cells express CD45RO but not CD45RA; expression of CD62L and CD27 is variable. All these markers, in addition to

those necessary to identify HIV-infected cells, can be measured simultaneously by flow cytometric analysis.

In NL4-3-infected cultures, the majority of Gag+ CD4– cells were memory cells similar to those in uninfected cultures except that they all expressed low levels of CD62L (Fig. 4.3A). Also present was a population of cells with markers of naive cells, CD45RA+ CD27+, as well as markers of non-naive cells. These cells all had intermediate levels of CD62L, and a subset of these also expressed CD45RO. Both phenotypes were rare in uninfected cultures. In 81A-infected cultures, nearly all Gag+ CD4– cells were memory cells that, like the NL4-3 infected cultures, expressed low levels of CD62L. Gag+ CD4– cells in NL4-3 ΔNef-infected cultures were mostly memory cells expressing low levels of CD62L; however CD45RA+ CD27+ cells were also present that all expressed low levels of CD62L and also expressed CD45RO. In HLAC cultures, similar patterns were observed (Fig. 4.3B). In addition, time-course studies demonstrated that the first cells to appear Gag+ CD4– were memory cells expressing low levels of CD62L followed later by the CD45RA+ expressing cells with intermediate levels of CD62L. From these experiments, it is unclear if these CD45RA+ cells also expressing non-naive markers represent infected naive or memory CD4 T cells.

CD62L Is Shed or Down-regulated in HIV-infected Cells

To identify the source of these novel cells, CD4+ target cells were again purified, labeled with CFSE, and added to HIV-infected cultures. Some of the cells were pre-treated with anti-viral compounds to control for changes not related to HIV infection. In addition, HIV-inhibitors were eventually added to all the cultures to prevent bystander killing and viral spread. Most importantly, unlike previous experiments where bulk CD4

T cell cultures were sorted, in these experiments CD4 T cells were further characterized according to their expression of CD45RA or CD62L. After addition to infected cultures, CFSE-labeled cells were immunostained for Gag, CD4 and the naive and memory markers CD45RA, CD62L, and CD45RO. In a control experiment, we determined that none of the antibodies used for sorting blocked the binding of the antibodies used for subsequent analyses. Immunostaining and analysis was performed days 2, 3, 4, and 5 after mixing with similar results found at each time point.

When cells that were originally CD45RA+ CD62L+ were added to either NL4-3- or NL4-3 ΔNef-infected cultures, Gag+ CD4– cells appeared demonstrating that these cells are permissive to infection in the presence or absence of Nef (Fig. 4.4B). Interestingly, these Gag+ CD4– cells expressed a very different pattern of naive and memory markers than the CD4+ cells from uninfected or infected cultures. Whereas the CD4+ all continue to express CD45RA and CD62L while lacking CD45RO, the Gag+ CD4– express CD45RA but only moderate levels of CD62L and considerably higher levels of CD45RO (Fig. 4.4A). These findings suggest that CD62L might be shed or down-regulated whereas expression of CD45RO is increased when naive CD4 T cells are infected with HIV. The presence or absence of Nef does not impact this phenotypic change. Similarly, the memory cells that were initially CD45RA– CD62L+ and became Gag+ CD4– also exhibited decreased levels of CD62L expression (Fig. 4.4B) suggesting this marker was also shed or down-regulated in infected memory CD4 T cells. These findings suggest that Gag+ CD4– cells expressing CD45RA but intermediate levels of CD62L or increased levels of CD45RO likely were infected as naive CD4 T cells.

Tissue-Derived Naive CD4 T Cells Are Permissive to HIV Infection

These findings suggest that purified cultures of naive CD4 T cells should be capable of supporting a spreading infection in the presence of NL4-3 and NL4-3 ΔNef viruses. Naive CD4 T cells were purified from HLAC and cultured either in medium alone or medium containing cytokines that promote infection of otherwise unstimulated CD4 T cells (186). These cultures were inoculated with either NL4-3 or NL4-3 ΔNef and culture supernatants were monitored for Gag content indicating levels of virion production. Although neither virus replicated in these cultures in medium alone, both cultures replicated in the presence of IL-2, IL-4, IL-7, or IL-15 (Fig. 4.5). As expected, NL4-3 replicated to higher levels than NL4-3 ΔNef. This finding confirmed that naive CD4 T cells are permissive to HIV infection in the absence of Nef.

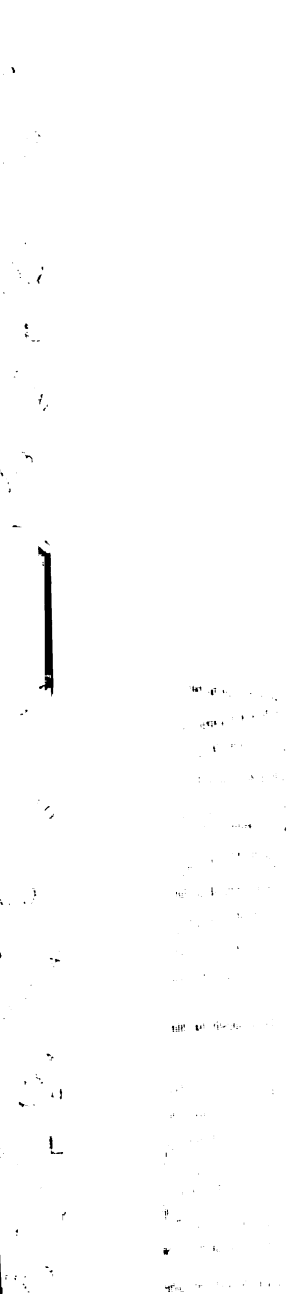
HIV-Infected Cells Express Increased Levels of Activation Markers and Are Hyper-Responsive to SEB Stimulation

Since Nef does not appear to play a role in determining the permissivity of naive and memory CD4 T cells to HIV infection, we performed experiments to determine if HIV-infected cells are in fact hyperactivated and if so, whether this activation is related to Nef expression. CD69 is a marker expressed on activated cells that is present in about 50% of CD4 T cells in HLAC although typically undetectable in PBLs cultured with IL-7 (data not shown). To assess hyperactivation, we compared the expression level of CD69 in different populations present in HIV-infected HLAC. CD69 expression was found in approximately 50% of CD4+ T cells from both uninfected and infected culture cultures but was expressed in nearly all of the Gag+ CD4– cells indicating that these HIV-infected cells display a more activated phenotype.

Is there a functional consequence of the hyperactivated state of HIV-infected CD4 T cells? Preliminary experiments were performed to determine if Nef played a role in how strongly infected cells respond to stimuli. PBL cultures were infected with NL4-3 and 81A, in the presence and absence of Nef. In addition, we also tested a variant of NL4-3 in which the endogenous Nef was replaced with Nef from the SF2 viral strain. SF2 Nef exhibits several unique properties including increased activation of PAK-2 (198). Cultures were either stimulated or not with staphylococcal enterotoxin B (SEB) and immunostaining performed to identify both HIV-infected and cytokine-producing cells. In the absence of SEB stimulation, no cytokine-producing cells were identified in uninfected or infected cultures (data not shown). In contrast, stimulation of uninfected cultures resulted in cells expressing IL-2, TNF-α, or IFN-γ (Fig. 4.6). In infected cultures, the pattern of response by the CD4+ cells was similar to the CD4+ cells in the uninfected culture. However, Gag+ CD4– cells were nearly twice as likely to express either IL-2 or IFN-γ regardless of the strain used for infection. Interestingly, similar percentages of Gag+ CD4– and CD4+ cells expressed TNF-α. Similar results were observed in infected HLAC cultures although the percentage of responding cells was greatly reduced (data not shown).

Nef Prevents Up-regulation of MHC I on HIV-Infected Cells

Finally, we sought to determine if infected PBLs show any of the described changes in cell-surface markers attributed to the action of Nef. Although MHC I (164) did not appear to be down-regulated in Gag+ CD4– cells from either NL4-3 or 81A infected cultures, MHC I expression was increased slightly in this population in the absence of Nef. These findings suggest that Nef does not down-regulate MHC I in HIV-



infected cells but instead prevents MHC I up-regulation upon HIV infection. In contrast, the expression level of another marker claimed to be down-regulated by Nef, CD28 (167), was similar in Gag+ CD4– or CD4+ cells, regardless of whether the CD4+ cells were from an uninfected or infected culture.

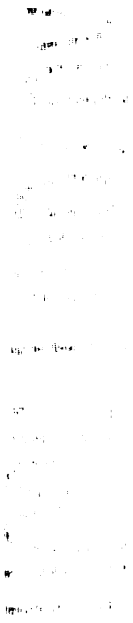
Discussion

Although immunostaining for intracellular Gag expression is commonly used to identify HIV-infected cells, CD4 down-regulation is not always taken into account (135, 181). In these studies, an opportunity to characterize a population that consists exclusively of HIV-infected cells was missed. In other studies, cells containing Gag but lacking surface expression of CD4 have been identified in HIV-infected cultures using both wild-type (177, 178) or reporter gene carrying viruses (176). From this, some have claimed that the Gag-containing cells that express CD4 are HIV-infected cells early in the viral life cycle. This claim is not consistent with our finding that when uninfectable CD4 T cells are added to an HIV-infected culture, Gag+ cells can be rapidly detected. Instead, it is more likely that CD4+ cells in HIV-infected cultures, regardless of intracellular Gag content, are a heterogeneous mix of uninfected cells, some of which score positive for Gag due to virion binding or internalization, and infected cells early in the viral life cycle.

The actions of three viral genes are known to alter CD4 expression levels: Nef (162, 163), Vpu (188, 189), and Env (190, 191). In primary CD4 T cells, over-expression of Nef or the combined activity of Env and Vpu eliminates CD4 from the cell surface (176, 178). The presence of Gag+ CD4– in cultures infected with NL4-3 ΔNef or 81A ΔNef is consistent with the hypothesis that Env and Vpu are sufficient to produce CD4 down-regulation. The appearance of Gag+ CD4– is also likely related to the life-span of infected cells; if infected cells die too quickly, as in NL4-3 ΔNef-infected cultures, they fail to fully down-regulate CD4. The rate of Gag+ CD4– appearance in cultures infected with NL4-3 or NL4-3 ΔNef in our studies is consistent with the half-life of infected cells *in vivo*. *In vivo*, HIV-infected cells have a half-life of 1–2 days (147-149) in the presence

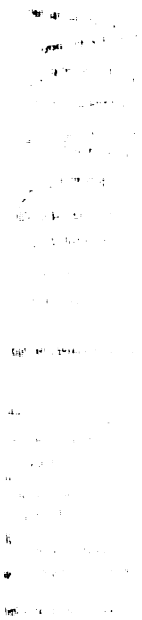
of Nef and 3–4 days in its absence (199). The competition between viral gene expression and cell killing likely contributes to the levels of gene expression and CD4 down-regulation in HIV-infected cells.

The mechanism(s) by which HIV-infected cells die in these cultures is not clear. Interactions between gp120, CD4, and CXCR4 may underlie both bystander (121) and direct killing (192-196). Although we found that CD4 is undetectable both by surface and intracellular staining (Fig. 4.1D), the antibody we used for these studies (RPA-T4, BD Biosciences) binds to the same epitope as gp120 so it remains possible that gp120-CD4 complexes are present in these cells. If so, just as this interaction underlies bystander killing, it could also mediate the killing of HIV-infected cells. To determine if CD4 is still present in these cells, the anti-CD4 antibody OKT4 could be used since it recognizes CD4 even when bound to gp120 (200, 201). Assuming this antibody detects both surface and intracellular CD4, it can be used to determine whether the cells described as Gag+ CD4– express any CD4. If these cells do not express any CD4, it is likely due either to proteosomal degradation mediated by Vpu (202, 203) or lysosomal degradation mediated by Nef (204). As these pathways can be blocked by different inhibitors, it might be possible to clarify which pathway contributes most significantly to the disappearance of CD4 from HIV-infected cells. Alternatively, to test whether or not CD4 complexed with gp120 plays a role in direct killing, the half-life of infected cells in the presence of or absence of AMD3100 could be compared in experiments similar to those shown in Fig. 4.2D. The presence of CD4 complexed to gp120 in HIV-infected cells could also be used to purify a population of consisting entirely of infected cells based on expression of CD3 and OKT4 but not RPA-T4.



Decreased expression of CD62L in CD4 T cell has been shown in response to CD4 cross-linking (205) or infection by an HIV reporter-virus (176). However, it was not clear in these studies if the lack of CD62L expression was due to shedding by infected cells or preferential infection of CD62L– cells. By infecting purified populations of CD4 T cells with known expression levels CD62L, our studies demonstrated that HIV infection leads to decreased CD62L expression. In addition, infection of CD45RA-expressing cells often led to co-expression of CD45RO, a rare phenotype in uninfected cells. By characterizing these cells both before and after infection, we know with certainty that HIV-infection is the underlying cause of both these changes. In contrast, our results that show increased expression of CD69 on HIV-infected cells could reflect preferential infection or increased expression in infected cells. Further experiments will be required to clarify this issue.

The finding that HIV-infection renders cells hyper-responsive to stimulation is consistent with two other reports. In one, HIV-infection increased cytokine production independently of exogenous stimuli (181). These results are difficult to interpret, as infected cells were identified based solely on Gag immunostaining. In addition, cytokine production was notably quite low. Our finding that cytokine production only occurs in response to stimulation is consistent with results reported by Janardhan *et al.* (206), who also found that Nef does not appear to play a role is determining how strongly cells respond. However, a dose-response study of various stimuli were not performed in our study or theirs. Just as the effect of Nef on the growth of HIV in stimulated cultures is most dramatic during moderate stimulation (158, 159), it may be worthwhile to evaluate Nef under conditions of suboptimal stimulation. Regardless, it is striking how much more



responsive HIV-infected cells are than uninfected or bystander cells. However, as with the findings on CD69 expression, it is unclear if this increased responsiveness is due to preferential infection of cells with a higher capability to respond or a bona fide consequence of HIV infection.

In addition to down-regulating CD4, Nef may reduce the expression of other surface proteins such as MHC I (164), CD28 (167), and the coreceptors CXCR4 (165) and CCR5 (166). However, a shortcoming of many of these studies is the lack of study of Nef at levels of expression characteristic of HIV infection in tissue. Instead, Nef is often over-expressed from reporter viruses in highly activated, dividing cultures of primary cells. Using the techniques described here, it is possible to perform these experiments on permissive but not actively dividing CD4 T cells using wild-type virus. Our findings confirm a role for Nef in modulating the expression level of MHC I but not CD28 in HIV-infected cells. Our finding on MHC I expression, however, must be interpreted with caution because we have measured the expression of all MHC I molecules, and Nef appears to primarily target HLA-A and -B but not HLA-C (164).

Materials and Methods

Primary CD4 T Cell Cultures

HLAC cultures were prepared as previously described (135, 207). Additional details are provided in Chapter 6. PBLs were isolated from buffy coats (Stanford Blood Bank) by Ficoll-Hypaque density gradient separation of mononuclear cells, followed by depletion of monocytes (Miltenyi; anti-CD14 Microbeads). Cells were cultured in RPMI containing 10% FBS, 1% penicillin/streptomycin (Gibco), 2 mM L-glutamine (Gibco), and 10 ng/ml recombinant human IL-7 (R&D Systems) in 96-well U-bottom polystyrene plates (5×10^5 cells per well).

HIV Viral Stocks and Infections

Viral stocks were prepared by calcium phosphate transfection of 293T cells with the following molecular clones: NL4-3, NL4-3 ΔNef (Xho), NL4-3 SF2 Nef, 81A, or 81A ΔNef (Xho). Virus-containing supernatants were collected 48 h later, filtered, and stored at –80°C. The concentration of all viral stocks were determined by measuring p24 Gag levels by ELISA (Perkin Elmer) or FLAQ (208).

For Nef-containing viruses, PBLs or HLAC were inoculated with 0.5–5 ng p24 Gag per well; in the absence of Nef, inoculums ranged from 5–200 ng p24 Gag per well. Twenty-four h after inoculation, cells were washed three times and cultured with subsequent medium changes every 2–4 days.

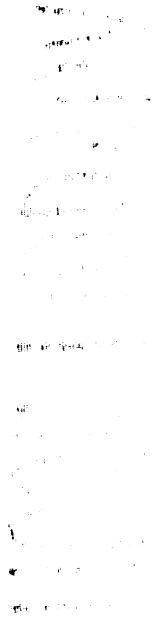
FACS Analysis of HIV-Infected Cultures

Immunostaining for surface proteins was performed in PBS containing 2% FBS and 2 mM EDTA. The following antibodies were routinely used to recognize surface

proteins: anti-CD3 APC-Cy7 (BD Bioscience), anti-CD19 PE-Cy5.5 (Caltag), anti-CD4 PE-Cy7 (BD Bioscience), and anti-CD8 APC Cy5.5 (Caltag). For simultaneous identification of multiple naive and memory markers, the following antibodies were used: anti-CD45RA PE (BD Bioscience), anti-CD45RO ECD (Caltag), anti-CD27 APC-Cy7 (BD Bioscience), and purified CD62L (BD Bioscience) labeled with Alexa 700 (Invitrogen; Zenon Labeling Kit). In other experiments, anti-CD69 APC, anti-MHC-1 FITC, and anti-CD28 APC (all from BD Bioscience) were used. When ethidium monoazide (EMA) was used to exclude dead cells, samples were stained (1 μg/ml) for 5 min at 4°C with direct exposure to a bright light. For intracellular anti-p24 Gag immunostaining, cells were fixed in 1% paraformaldehyde and stained with an anti-p24 Gag antibody (Coulter; KC57) in PBS containing 0.1% saponin (Sigma) and 2% FBS. FACS data were analyzed with FlowJo software (Treestar). Compensation was performed in FlowJo with single-stained compensation beads (BD Biosciences) prepared at the same time as the samples.

Tracking the Fate of Target Cells in HIV-Infected Cultures

One day before isolation of CD4 T cells, 3×10^7 HLAC cells were pre-treated with AZT (125 μM) or AMD3100 (6.25 μM). CD4 T cells were isolated from either 4×10^7 untreated or 3×10^7 treated HLAC cells by immunostaining with CD8-PE, CD19-PE, and CD14-PE. Depletion with anti-PE microbeads (Miltenyi) yielded approximately 1×10^7 CD4 T cells. After washing with PBS, cells were labeled with 1 μM CFSE (Molecular Probes) in PBS for 5 min at room temperature and then washed in serum containing-medium to quench any unbound CFSE. Approximately 5×10^5 labeled-CD4 T cells were added per well of either uninfected or NL4-3-infeceted HLAC culture.

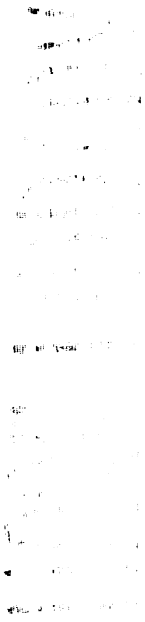


Tracking the Fate of Cells from HIV-Infected Cultures

Uninfected or NL4-3-infected PBLs in IL-2 (20 ng/ml) were immunostained with antibodies recognizing CD8 (FITC), CD3 (PerCP), and CD4 (APC; BD Bioscience) before isolation of CD3+ CD4+ CD8– or CD3+ CD4– CD8– on a FACS Vantage (BD Biosciences). Sorted cells were labeled with CFSE and approximately 8×10^4 were co-cultured with uninfected wells in the presence of AMD3100 (250 nM) and AZT (50 μM). Samples were collected for analysis 1, 2, 3, and 4 days after co-culture. These samples were immunostained for 30 min at 4° C with the following antibodies: anti-CD8 ECD (Coulter) and anti-CD4 PE-Cy7 (BD Biosciences). Samples were fixed in 1% PFA at 4°C overnight before intracellular immunostaining with anti-p24 Gag FITC (Coulter) before analysis on a FACS Vantage (BD Biosciences).

Tracking the Fate of Target Naive and Memory Cells

One day before isolation, 4×10^7 PBLs in IL-7were pre-treated with AMD3100 (250 nM), AZT (50 μM), and 3TC (10 μM). To isolate naive and memory CD4 T cells, uninfected PBLs were stained with anti-CD4 Pacific Blue, anti-CD45RA FITC, anti-CD8 PE, and anti-CD62L APC (all antibodies from BD Bioscience) before sorting on a FACS DiVa for CD4+ CD8– cells that were either CD45RA+ CD62L+, CD45RA– CD62L+, or CD45RA– CD62–. Sorted cells were labeled with CFSE and 6×10^4 sorted cells added to either an uninfected culture or a culture infected with NL4-3, NL4-3 ΔNef, or 81A. For samples pre-treated with HIV-inhibitors, these compounds were again added to the cultures. For cultures untreated at the time of mixing, AMD3100 (250 nM) was added 1 day after mixing; AZT (50 μM) and 3TC (10 μM) were both added 2 days after mixing. Samples for analysis were collected 2, 3, 4, and 5 days after co-culture. They were



immunostained for 30 min at 4° C with the following antibodies: anti-CD45RO ECD (Coulter), anti-CD45RA PE-Cy7 (BD Bioscience), anti-CD62L APC-Alexa750 (Caltag), and anti-CD4 PE-Alexa700 (Caltag). Samples were then fixed in 1% PFA at 4°C for 24–48 h before intracellular immunostaining with anti-p24 Gag FITC (Coulter). Samples were analyzed on an LSR II (BD Biosciences).

Purification and Infection of Naive CD4 T Cells

Naive CD4 T cells in or HLAC were first enriched by Ficoll-Hypaque density gradient separation of mononuclear cells. These cells were then sorted for CD4+ CD45RA+CD62L+ (naive) or CD4+ CD45RA– (memory) with a FACS DiVa (BD Biosciences). After isolation, naive CD4 T cells from tissue ($1–2 \times 10^5$ in 96-well U-bottom polystyrene plates) were cultured with medium alone or medium supplemented with recombinant IL-2, IL-4, IL-7 or IL-15 (R&D Systems). Cells were incubated with NL4-3 (75 ng of p24 Gag) or NL4-3 ΔNef (200 ng of p24 Gag) for 24 h, washed extensively, and cultured; the medium was replaced every 3 days and p24 Gag content at each time measured by FLAQ (208).

Cytokine Flow Cytometry on HIV-Infected Cells

Uninfected or HIV-infected cultures were stimulated for 6 h with SEB (10 μg/ml) in the presence of BFA (10 μg/ml) during the last 4 h. Samples were immunostained for 30 min at 4° C with the following antibodies: anti-CD3 Cascade Blue (BD Biosciences), anti-CD19 PE-Cy5.5 (Caltag), anti-CD4 APC-Cy7 (BD Biosciences), and anti-CD8 ECD (Coulter). After washing, the samples were fixed in 1% PFA and incubated at 4°C overnight. After another wash, intracellular staining was performed using the following

antibodies: anti-p24 Gag FITC, anti-IL-2 PE, anti-TNF-α APC, and anti-IFN-γ PE-Cy7 (all antibodies recognizing cytokines are from BD Biosciences). Samples were analyzed on an LSR II (BD Biosciences).

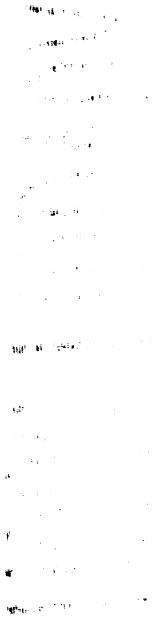


Figure Legends

Figure 4.1. HIV-infected PBL and HLAC cultures contain two populations of CD8– T cells that stain positively for intracellular Gag. (A) PBLs cultured in IL-7 (10 ng/ml) were either left uninfected or infected with NL4-3, NL4-3 ΔNef, 81A or 81A ΔNef. After 11 days, immunostaining for surface markers and intracellular Gag was performed. (B and C) Uninfected or NL4-3, NL4-3 ΔNef, 81A or 81A ΔNef-infected HLAC were immunostained as in (A) either 11 (B) or 9 (C) days after infection. Different time points are shown to demonstrate the peak of infection. (D) PBLs culture in 10 ng/ml IL-7 were left uninfected or infected with NL4-3 or NL4-3 ΔNef. After 7 days, the cultures were divided in half for immunostaining. Both samples were stained with antibodies against surface markers before fixation. Intracellular staining was then performed with antibodies recognizing Gag in the presence or absence of antibodies recognizing CD4. (E) Intracellular Gag staining was performed on HLAC cultures split in half 5 days after infection; one culture was treated with AMD3100 (250 nM) and the other was not. Four days later, cultures were immunostained for surface markers and intracellular Gag. In (A), (B), (C) and (E), the shown population was gated as follows: CD3+ CD8– CD19– lymphocytes (cells with small forward and side scatter). In (D) CD8– lymphocytes are shown.

Figure 4.2. In HIV-infected cultures, all intracellular Gag+ CD4– cells are infected whereas CD4+ cells, regardless of intracellular Gag content, are a mix of infected and uninfected cells. (A) CD4 T cells from uninfected HLAC pre-treated (or not) with HIV inhibitors (125 μM AZT or 6.25 μM AMD3100) for 24 h were purified, labeled with

CFSE, and added to uninfected or NL4-3-infected HLAC in the presence or absence of the same inhibitors. Immunostaining was performed every day for the next 3 days to monitor the Gag and CD4 levels in CFSE+ cells. (B) From an experiment similar to (A), the relative number of CFSE+ cells remaining (% Bystander Alive) at each time point was calculated as a ratio of CFSE+ cells to CFSE- CD8+ lymphocytes. The ratios were normalized to the uninfected culture 1 day after mixing. (C) CFSE-labeled uninfected CD4 T cells were added to either uninfected HLAC or HLAC infected with NL4-3, NL4-3 ΔNef, or 81A. Surface CD4 and intracellular Gag expression was measured in the CFSE+ cells at various times after mixing. (D) CD4+ T cells from an uninfected PBL culture or both CD4+ and CD4– T cells from an NL4-3-infected PBL culture were sorted by flow cytometry, CFSE labeled, and returned back to an uninfected PBL culture. Surface CD4 and intracellular Gag expression was measured in the CFSE+ cells each day for the next three days.

Figure 4.3. Intracellular Gag+ cells in HIV-infected cultures express an altered pattern of naive and memory T cell markers. (A) PBLs cultured in IL-7 (10 ng/ml) were left uninfected or infected with NL4-3, NL4-3 ΔNef, or 81A and immunostained 13 days later. The left panels show the expression of CD4 and intracellular Gag on cells gated as follows: CD3+ CD8– CD19– lymphocytes. For the uninfected culture, the expression of naive and memory markers on CD4+ cells is shown on the right as contour plots. In the infected cultures where there are both Gag+ CD4–and CD4+ cells, the expression pattern of naive and memory markers of both populations are shown as dot plots with the Gag+ CD4– population (black) overlaid on the CD4+ cells (gray). The values next to the gates are the percentages of Gag+ CD4– (black) contained within that gate. (B) HLAC was

either not infected or infected with NL4-3 or NL4-3 ΔNef and immunostained at 5, 7, 9, or 11 days after inoculation. The expression levels of CD45RA and CD62L in both Gag+ CD4– and CD4+ cells are displayed as in (A).

Figure 4.4. HIV infection changes the expression pattern of CD62L and CD45RO. (A) CD4 T cells expressing both CD45RA+ and CD62L+ were purified from uninfected PBLs cultured with IL-7 (10 ng/ml); some cells had been pre-treated (Pre-Tx) with a cocktail of HIV inhibitors (50 μM AZT, 10 μM 3TC, and 250 nM AMD3100). Sorted cells were labeled with CFSE and added to cultures either uninfected or infected with NL4-3 or NL4-3 ΔNef. For cells pre-treated with HIV-inhibitors, this was continued during further culture (Post-Tx). For the cultures untreated at the time of sorting and mixing, AMD3100 (250 nM) was added after 1 day of co-culture and both AZT (50 μM) and 3TC (10 μM) were added the next day (Only Post-Tx). Three days after mixing, immunostaining was performed to identify infected CFSE-labeled cells and to ascertain the changes in cell-surface markers on these cells. The use of contour plots and dot plots is the same as Fig. 4.3. (B) Similar to above, CD45RA– CD62L+ CD4 T cells were sorted, added to uninfected or infected cultures, and immunophenotyped.

Figure 4.5. NL4-3 ΔNef can replicate in purified cultures of naive CD4 T cells from HLAC. Purified naive CD4 T cells from HLAC were cultured in medium alone or medium containing recombinant cytokines and inoculated with NL4-3 (75 ng of p24 Gag) or NL4-3 ΔNef (200 ng of p24 Gag). After extensive washing, virus-containing supernatants were monitored for p24 Gag content after 3, 5, 8, or 11 days of culture.

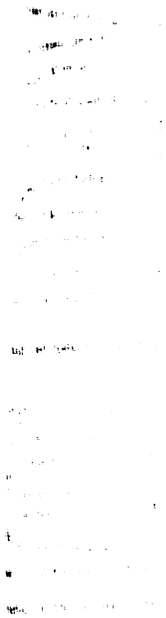


Figure 4.6. HIV-infected cells are hyperactivated and hyper-responsive to stimuli by SEB. (A) HLAC were left uninfected or infected with NL4-3, NL4-3 ΔNef, 81A or 81A ΔNef and immunostained 5 days later. Shown are histograms depicting the expression of the activation marker, CD69, on either Gag+ CD4– (black) or CD4+ (grey) cells. (B) PBLs cultured in IL-7 (10 ng/ml) were left uninfected or infected with NL4-3, NL4-3 ΔNef, 81A or 81A ΔNef. After 11 days, cultures were stimulated for 6 h with SEB (10 μg/ml) in the presence of BFA (10 μg/ml) during the last 4 h. Immunostaining was then performed first for cell surface markers and then for intracellular Gag, IL-2, TNF-α, and IFN-γ. In the top panel, the expression of CD4 and intracellular Gag is displayed on cells previously been gated as follows: CD3+ CD8– CD19– lymphocytes. The lower panels show the cytokine expression profiles of either the Gag+ CD4– cells (black) or CD4+ cells (gray). (C–E) Shown are the percentages of Gag+ CD4– (black bars) or CD4+ cells (gray bars) also expressing IL-2, TNF-α, or IFN-γ.

Figure 4.7. Nef-dependent changes in HIV-infected T cells. PBLs cultured in IL-7 (10 ng/ml) were left uninfected or infected with NL4-3, NL4-3 ΔNef, 81A, or 81A ΔNef and immunostained 9 days later. The top panel shows the expression of CD4 and intracellular Gag on cells gated as follows: CD3+ CD8– CD19– lymphocytes. Below are histograms showing the expression of either MHC I or CD28 on either Gag+ CD4– (black) or CD4+ (gray) cells.

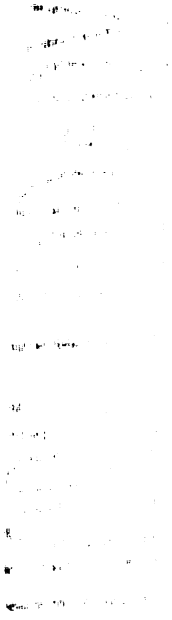


Figure 4.1

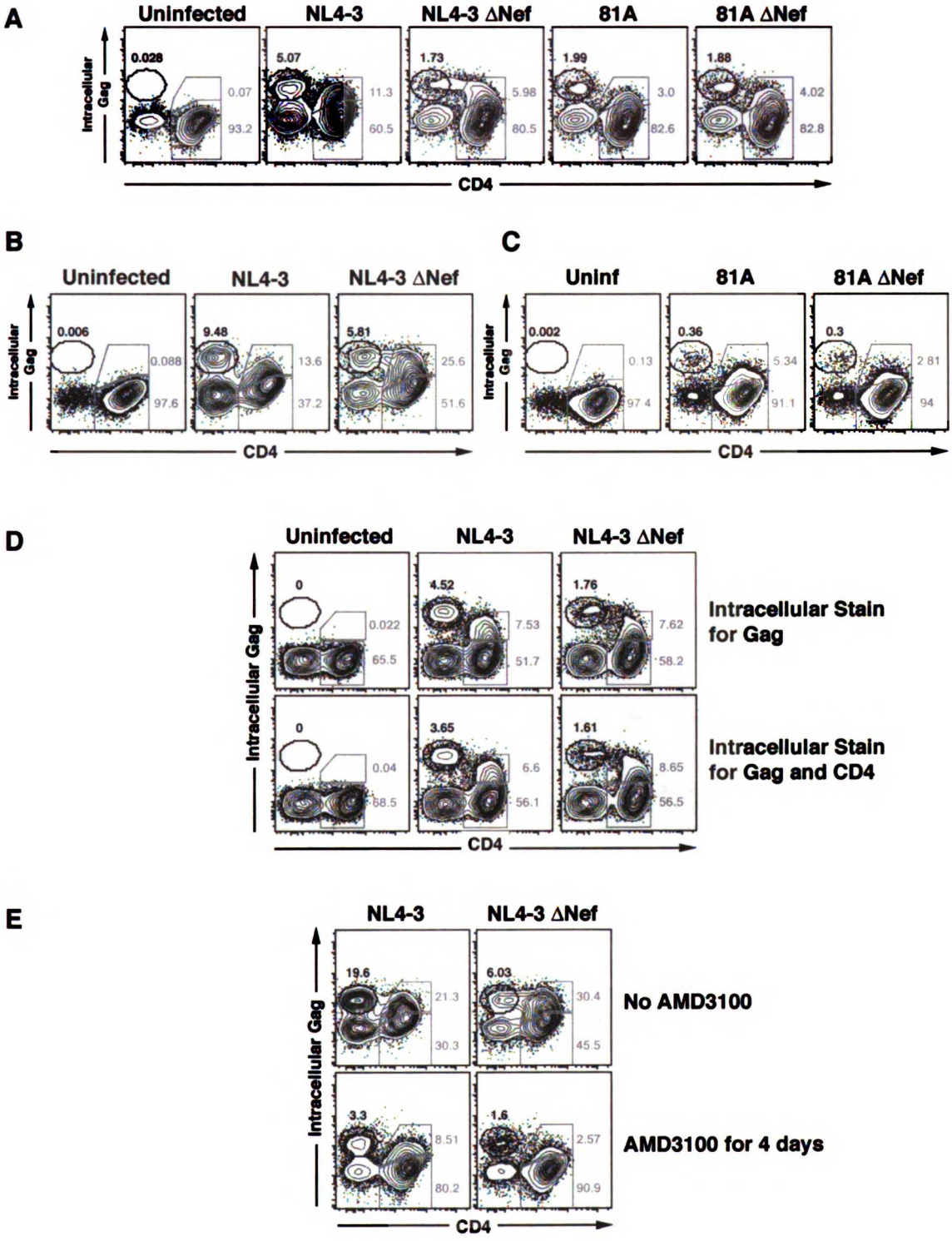

A
Uninfected
NL4-3
NL4-3 ΔNef
81A
81A ΔNef
Intracellular Gag
CD4
0.028
0.07
93.2
5.07
11.3
60.5
1.73
5.98
80.5
1.99
3.0
82.6
1.88
4.02
82.8
B
Uninfected
NL4-3
NL4-3 ΔNef
0.006
0.088
97.6
9.48
13.6
37.2
5.81
25.6
51.6
C
Uninf
81A
81A ΔNef
0.002
0.13
97 4
0.36
5.34
91.1
0.3
2 81
94
D
Uninfected
NL4-3
NL4-3 ΔNef
Intracellular Gag
0
0.022
65.5
4.52
7.53
51.7
1.76
7.62
58.2
Intracellular Stain for Gag
0
0.04
68.5
3.65
6.6
56.1
1.61
8.65
56.5
Intracellular Stain for Gag and CD4
E
NL4-3
NL4-3 ΔNef
Intracellular Gag
19.6
21.3
30.3
6.03
30.4
45.5
No AMD3100
3.3
8.51
80.2
1.6
2.57
90.9
AMD3100 for 4 days

Figure 4.2

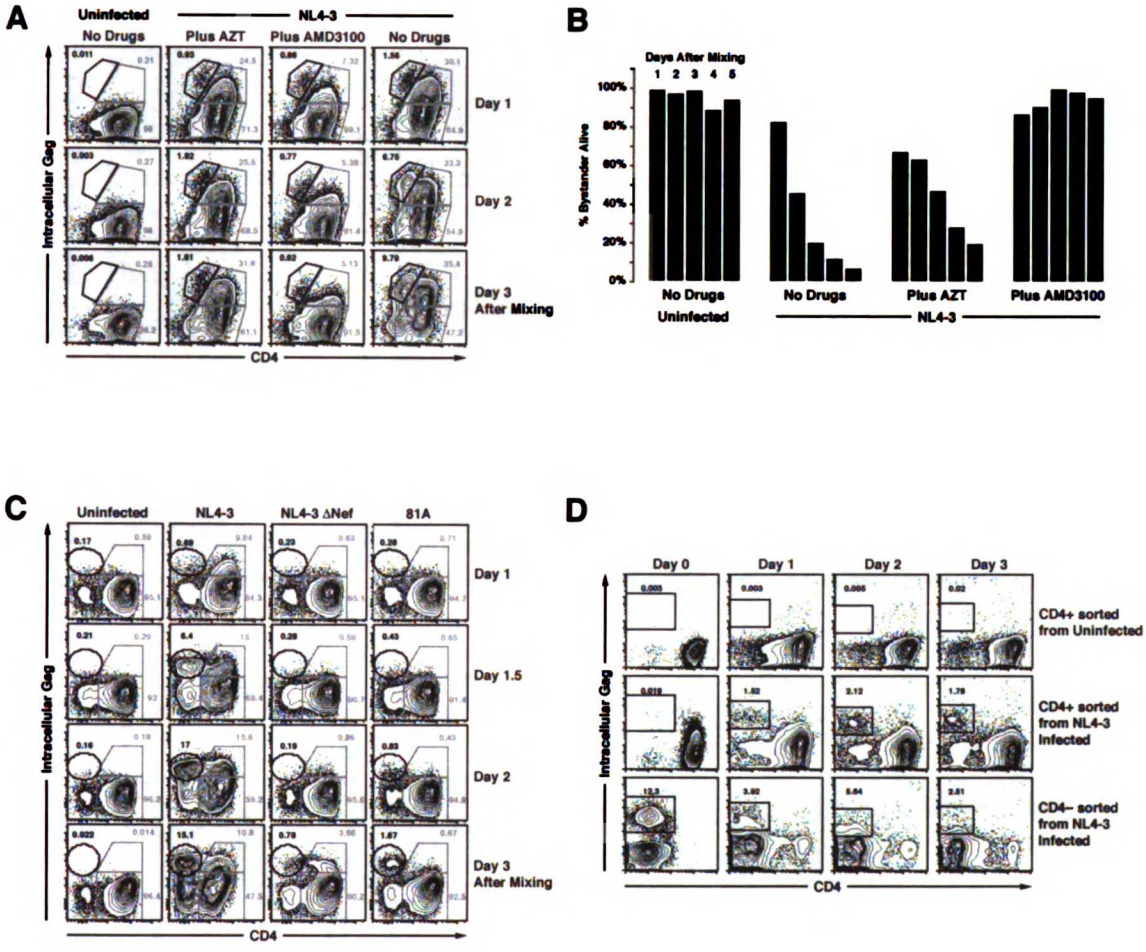
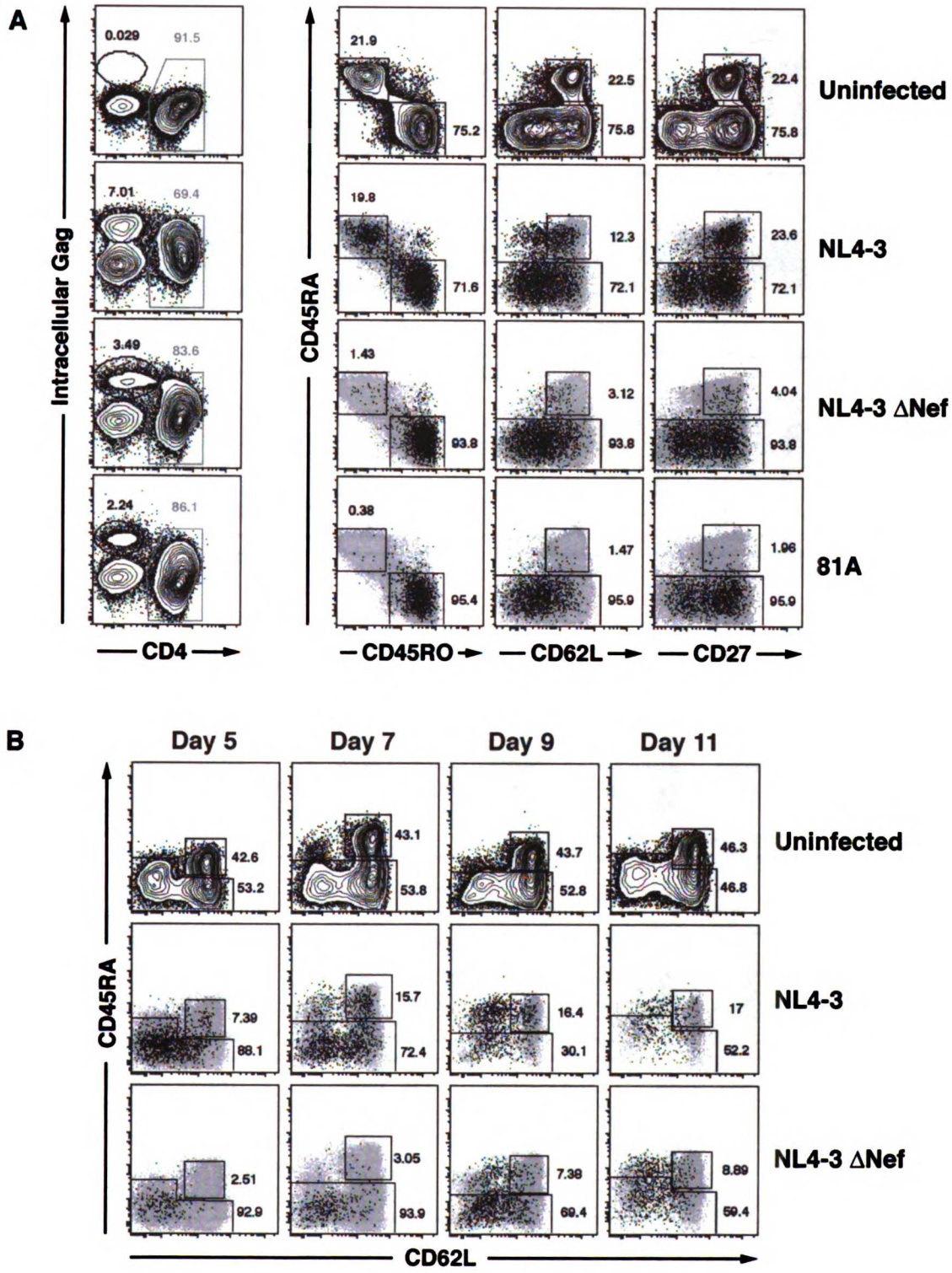

A
Uninfected
NL4-3
No Drugs
Plus AZT
Plus AMD3100
No Drugs
Day 1
Day 2
Day 3
After Mixing
Intracellular Gag
CD4
B
Days After Mixing
1 2 3 4 5
% Bystander Alive
100%
80%
60%
40%
20%
0%
No Drugs
Uninfected
No Drugs
Plus AZT
Plus AMD3100
NL4-3
C
Uninfected
NL4-3
NL4-3 ΔNef
81A
Day 1
Day 1.5
Day 2
Day 3
After Mixing
Intracellular Gag
CD4
D
Day 0
Day 1
Day 2
Day 3
CD4+ sorted
from Uninfected
CD4+ sorted
from NL4-3
infected
CD4– sorted
from NL4-3
infected
Intracellular Gag
CD4

Figure 4.3


A
Intracellular Gag
CD4
CD45RA
CD45RO
CD62L
CD27
0.029
91.5
21.9
75.2
22.5
75.8
22.4
75.8
Uninfected
7.01
69.4
19.8
71.6
12.3
72.1
23.6
72.1
NL4-3
3.49
83.6
1.43
93.8
3.12
93.8
4.04
93.8
NL4-3 ΔNef
2.24
86.1
0.38
95.4
1.47
95.9
1.96
95.9
81A
B
Day 5
Day 7
Day 9
Day 11
CD45RA
CD62L
42.6
53.2
43.1
53.8
43.7
52.8
46.3
46.8
Uninfected
7.39
88.1
15.7
72.4
16.4
30.1
17
52.2
NL4-3
2.51
92.9
3.05
93.9
7.38
69.4
8.89
59.4
NL4-3 ΔNef

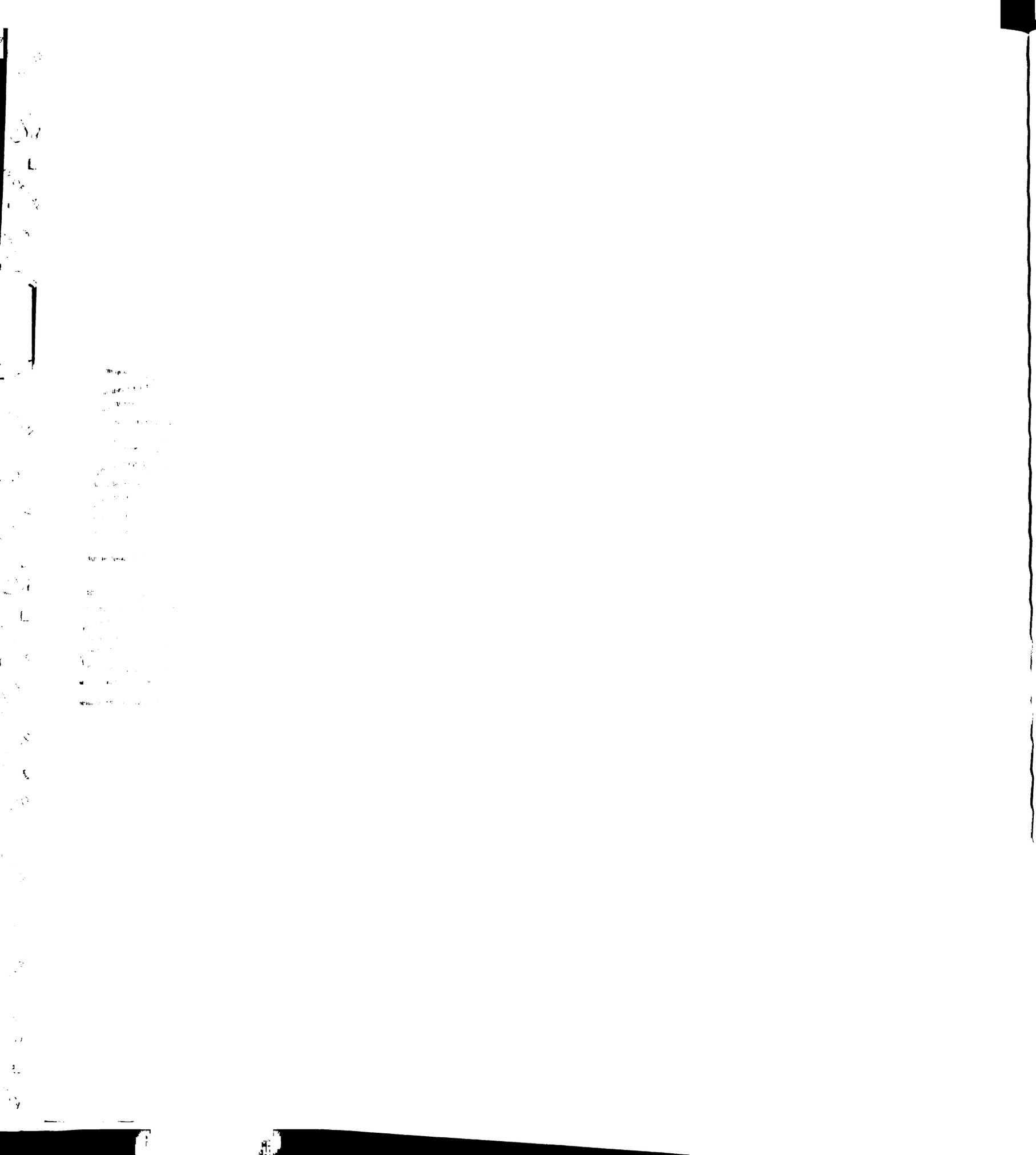


Figure 4.4

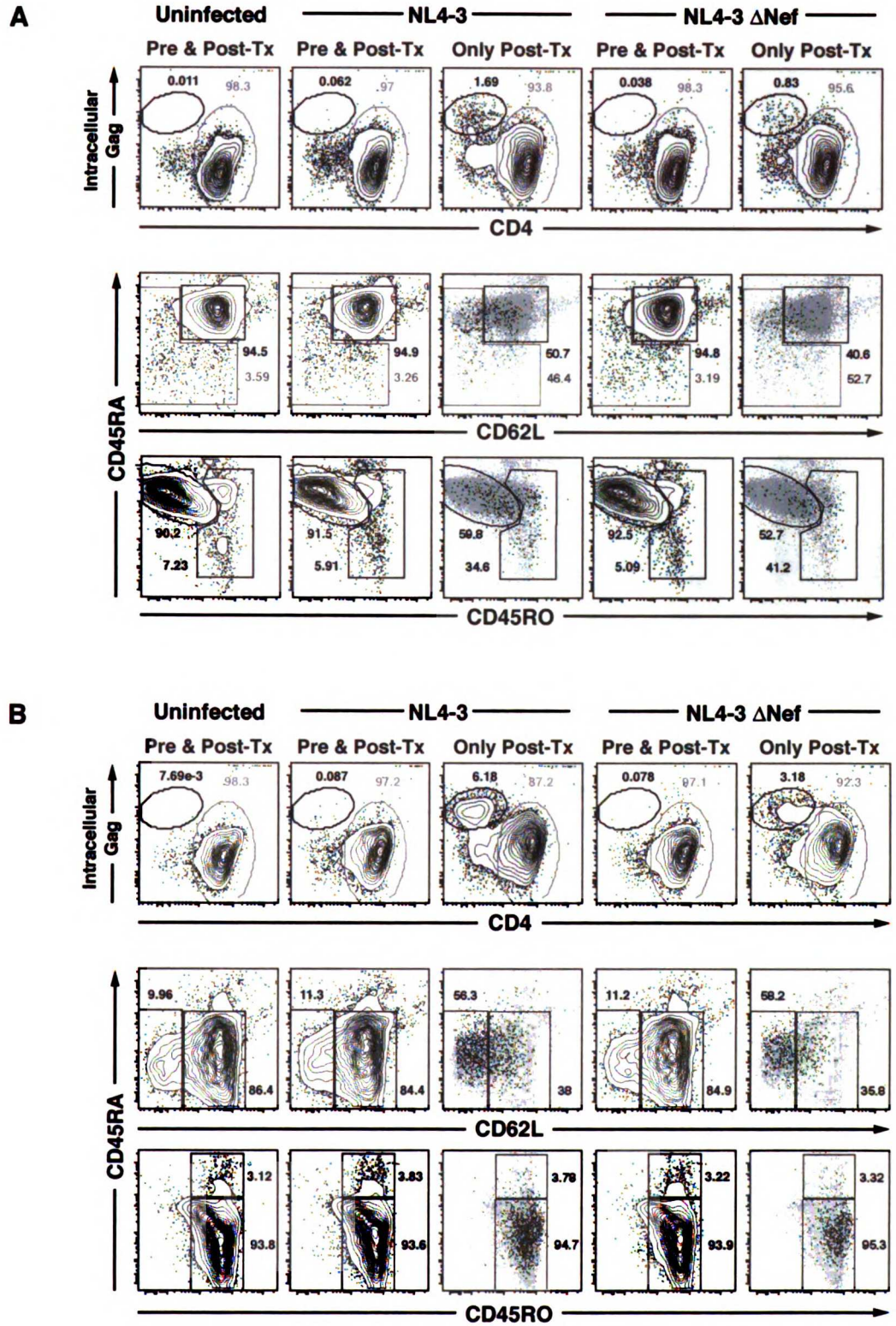
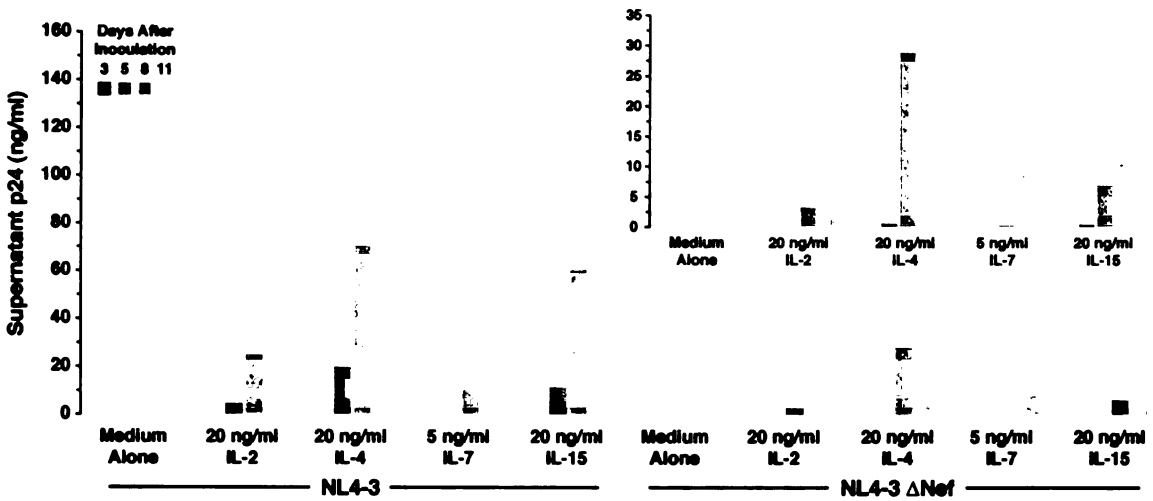

A
Uninfected
NL4-3
NL4-3 ΔNef
Pre & Post-Tx
Pre & Post-Tx
Only Post-Tx
Pre & Post-Tx
Only Post-Tx
Intracellular Gag
0.011
98.3
0.062
.97
1.69
93.8
0.038
98.3
0.83
95.6
CD4
CD45RA
94.5
3.59
94.9
3.26
50.7
46.4
94.8
3.19
40.6
52.7
CD62L
90.2
7.23
91.5
5.91
59.8
34.6
92.5
5.09
52.7
41.2
CD45RO
B
Uninfected
NL4-3
NL4-3 ΔNef
Pre & Post-Tx
Pre & Post-Tx
Only Post-Tx
Pre & Post-Tx
Only Post-Tx
Intracellular Gag
7.69e-3
98.3
0.087
97.2
6.18
87.2
0.078
97.1
3.18
92.3
CD4
CD45RA
9.96
86.4
11.3
84.4
56.3
38
11.2
84.9
58.2
35.8
CD62L
3.12
93.8
3.83
93.6
3.78
94.7
3.22
93.9
3.32
95.3
CD45RO

Figure 4.5


Supernatant p24 (ng/ml)
Days After Inoculation
3 5 8 11
Medium Alone
20 ng/ml IL-2
20 ng/ml IL-4
5 ng/ml IL-7
20 ng/ml IL-15
NL4-3
NL4-3 ΔNef

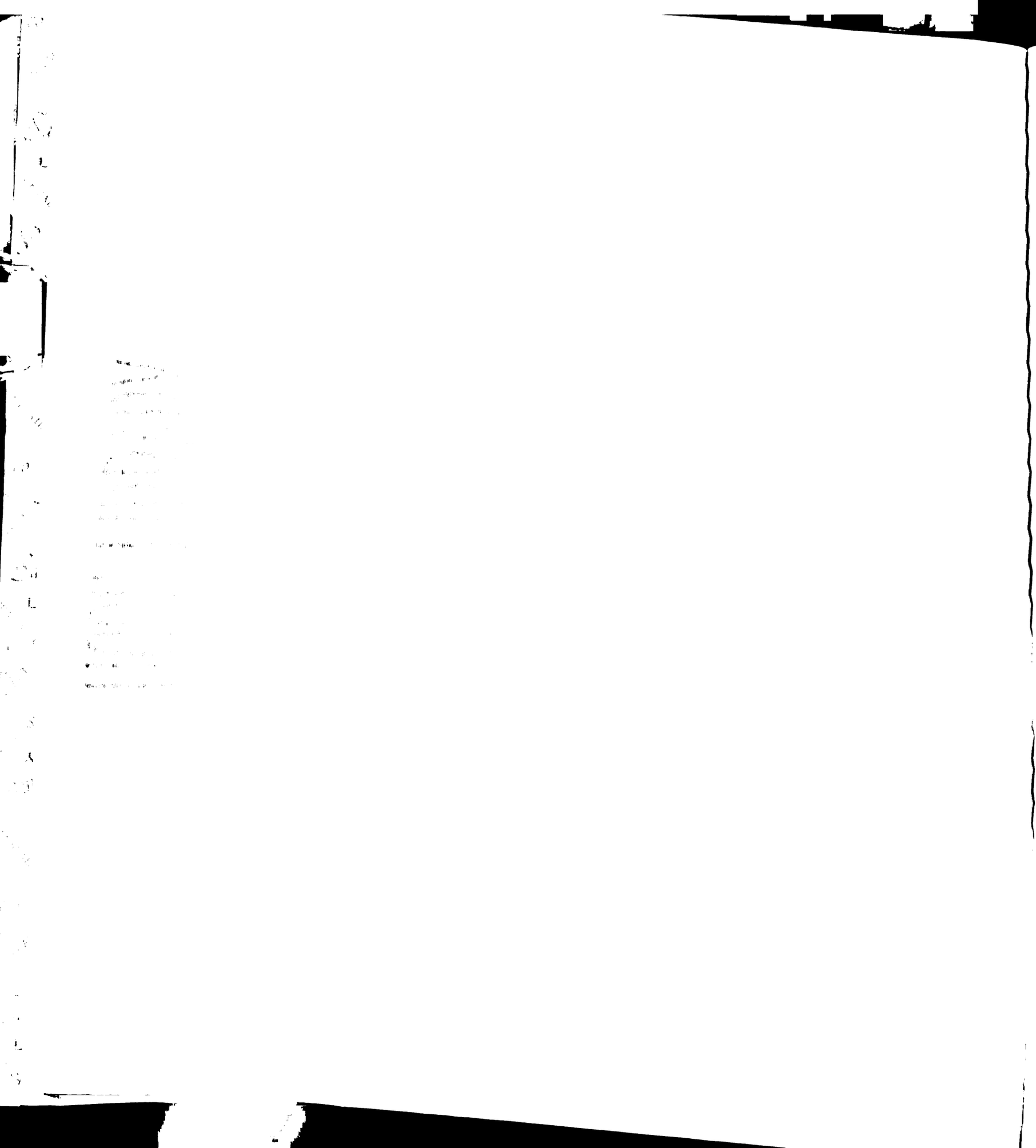


Figure 4.6

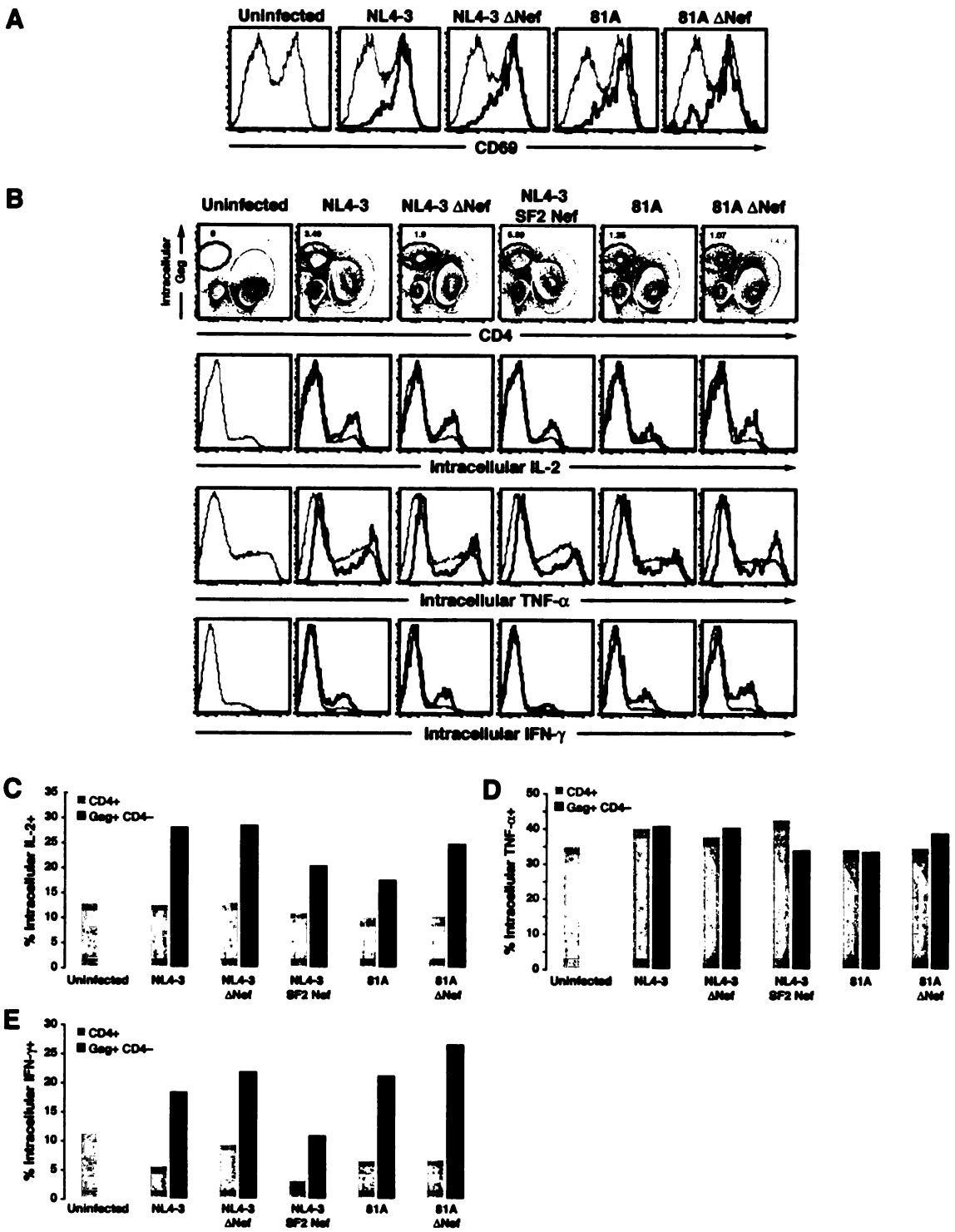
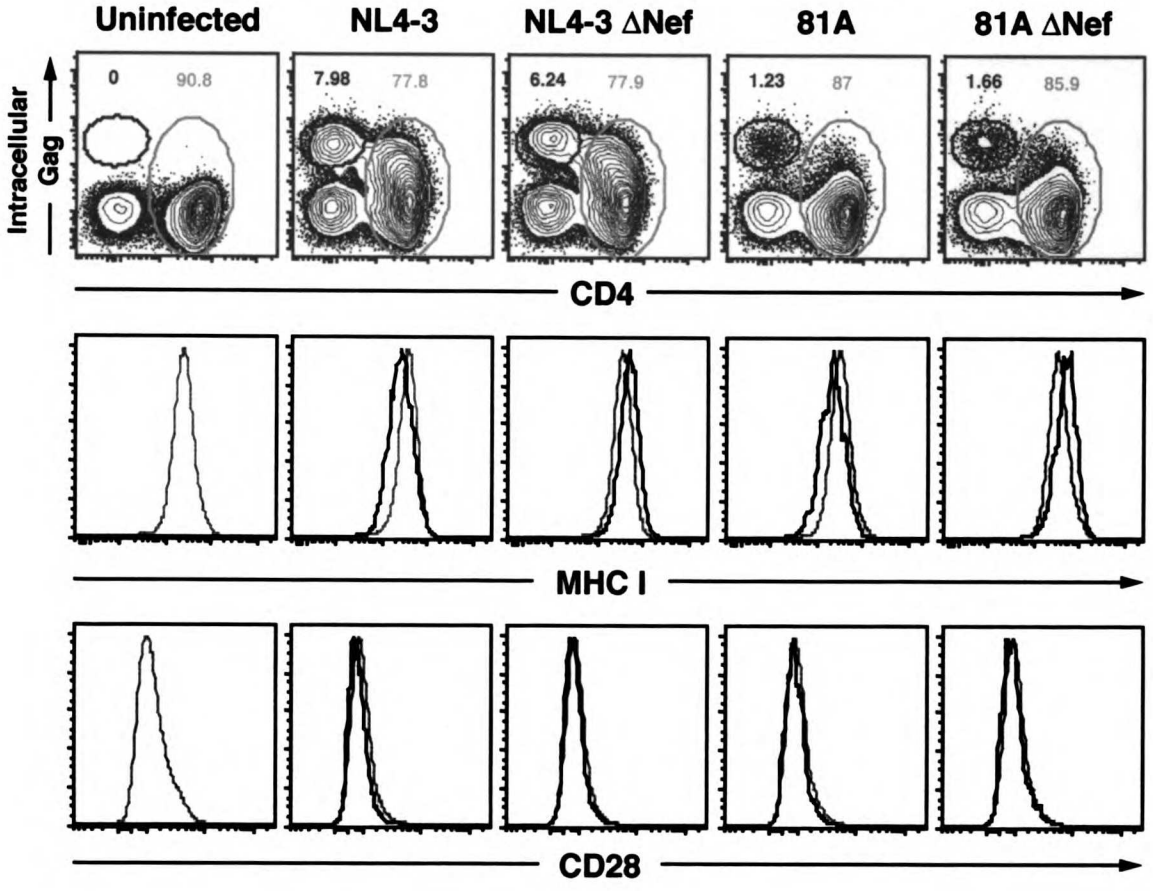

A
Uninfected
NL4-3
NL4-3 ΔNef
81A
81A ΔNef
CD69
B
Uninfected
NL4-3
NL4-3 ΔNef
NL4-3 SF2 Nef
81A
81A ΔNef
Intracellular Gag
CD4
Intracellular IL-2
Intracellular TNF-α
Intracellular IFN-γ
C
% Intracellular IL-2+
CD4+
Gag+ CD4–
D
% Intracellular TNF-α+
E
% Intracellular IFN-γ+

Figure 4.7


Uninfected
NL4-3
NL4-3 ΔNef
81A
81A ΔNef
Intracellular Gag
0
90.8
7.98
77.8
6.24
77.9
1.23
87
1.66
85.9
CD4
MHC I
CD28

Chapter 5

Select Cytokines Enhance HIV Infection of Naive CD4 T Cells in Lymphoid Tissues by Stimulating High-Molecular-Mass APOBEC3G Complex Formation

Introduction

Cellular activation has long been considered a requirement for HIV infection of CD4 T cells, since HIV fails to infect resting CD4 T cells from peripheral blood. Infection is aborted either during reverse transcription (209) or before nuclear import of the viral preintegration complex (210). In contrast, recent studies show that resting CD4 T cells residing in tissue, analyzed either *in vivo* or *ex vivo,* are permissive for HIV infection (135, 153, 157, 211). Since the vast majority of CD4 T cells are present in lymphoid tissues (212), most resting CD4 T cells are permissive for HIV infection. Over half of all memory CD4 T cells are infected and killed during acute SIV infection in rhesus macaques (153, 154). Similarly, during acute SHIV infection, resting naive CD4 T cells are a principal target for infection and emerge as primary virus-producing cells (211). The molecular mechanisms underlying the permissivity differences between resting CD4 T cells in tissue and blood remain unknown.

Ex vivo lymphoid cultures are an attractive system to address this question, as resting CD4 T cells, both naive and memory, are permissive to HIV infection independent of exogenous stimuli (135). Although well-recognized for memory CD4 T cells (213, 214), HIV infection of naive CD4 T cells was not fully appreciated until recently. In some studies, naive CD4 T cells from peripheral blood are refractory to HIV infection *in vitro* (136, 215), but the physiological relevance of this finding is uncertain because circulating HIV-infected naive CD4 T cells are detectable *in vivo* (58, 60).

The antiviral factor A3G plays a key role in regulating the permissivity of CD4 T cells to HIV infection. Recently, we reported that resting CD4 T cells in peripheral blood are protected from HIV infection through the action of LMM A3G (47). siRNA-mediated

"knockdown" of A3G expression rendered these cells permissive to infection, as did stimulation with mitogens that promoted the recruitment of LMM A3G into inactive HMM A3G ribonucleoprotein complexes. These findings raised the possibility that in HIV-permissive, resting CD4 T cells from tissue, A3G might be present in HMM complexes. In this study, we utilized lymphoid organ cultures to explore this possibility and the role of the tissue microenvironment in regulating HIV permissivity of these cells.

Results and Discussion

Soluble factors in HLAC-conditioned medium render tissue-derived naive CD4 T cells permissive to HIV infection

We first sought to identify the mechanisms underlying the permissivity of naive CD4 T cells in lymphoid tissues. To determine if cell-cell contact or soluble factors produced in lymphoid tissue are required for HIV infection of tissue-resident naive CD4 T cells, these cells were purified from human lymphoid aggregate cultures (HLAC) prepared from human tonsils (135, 207) and tested under different culture conditions. Infected cells were identified by intracellular Gag (anti-p24) immunostaining and flow cytometric analysis. Naive CD4 T cells cultured in isolation in medium alone could not be infected by HIV (Fig. 5.1, left panels). However, when the cells were returned to autologous HLAC cultures, HIV infectivity was restored (Fig. 5.1, middle panels). To determine if endogenously produced soluble factors contributed to the acquisition of HIV permissivity, the cells were cultured in HLAC-conditioned medium. Again, the cells became permissive for HIV infection (Fig. 5.1, right panels). Addition of AZT blocked detection of intracellular Gag immunostaining demonstrating that this signal was dependent on *de novo* reverse transcription. Of note, the viability of naive CD4 T cells in these cultures was comparable (data not shown), suggesting that the infection results reflect differences in their state of permissivity rather than overall health. In addition, the activation status of both cultures was similar; each contained a small percentage of cells expressing either CD25 or CD69, typical for tissue-resident naive CD4 T cells (data not shown).

These results are consistent with those of Kinter *et al.* (216) who showed that isolated tissue-derived CD4 T cells are permissive upon return to HLAC but not when cultured alone. Our work extends these studies by showing that permissivity of tissue-derived naive CD4 T cells can be conferred by HLAC-conditioned medium suggesting a role for soluble factors.

IL-2 and IL-15 are necessary but not sufficient components of the permissivity activity in conditioned medium

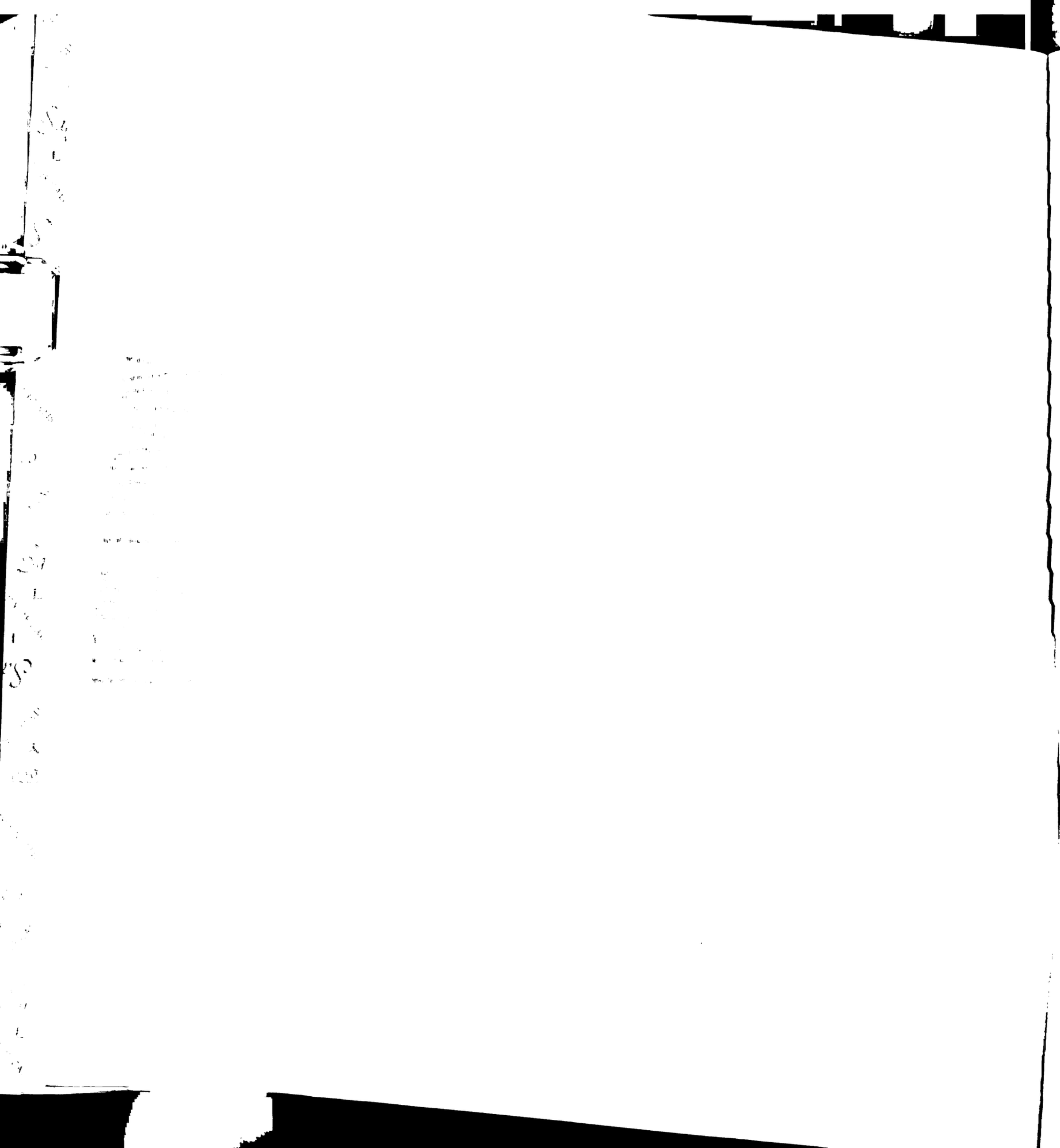
To identify the factors present in conditioned medium necessary to render tissue-derived naive CD4 T cells permissive, we considered the report by Unutmaz *et al.* (186) who showed that 10–20 ng/ml of IL-2, IL-4, IL-7, or IL-15 rendered otherwise unstimulated blood-derived CD4 T cells susceptible to HIV infection. Consistent with this and other studies (185, 217), we found that addition 20 ng/ml of IL-2, IL-4, IL-7, or IL-15 was sufficient for tissue-derived naive CD4 T cells to sustain a spreading infection (data not shown).

We next examined whether depletion of select cytokines from the conditioned medium altered rescue of HIV permissivity in tissue-derived naive CD4 T cells. IL-2 and IL-15, present at 5–10 pg/ml and 2–5 pg/ml, respectively, were depleted from the conditioned medium by treatment with capture antibodies bound to insoluble bead supports. This procedure effectively reduced these cytokines to undetectable levels (data not shown). To control for non-specific inhibition by the depletion procedure, beads bound with antibodies recognizing IL-7 were also employed as IL-7 was undetectable in the conditioned medium. Depletion of either IL-2 or IL-15 markedly reduced the ability of the conditioned medium to rescue viral replication while, as expected, depletion with

antibodies recognizing IL-7 had no effect (Fig. 5.2A). Further supporting a necessary role for IL-2, addition of a blocking antibody specific for the alpha chain of the high affinity IL-2 receptor also inhibited viral replication while a control antibody against the IL-4R did not (data not shown).

Next we tested whether the addition of recombinant IL-2 or IL-15 at levels recapitulating those found in conditioned medium was sufficient to render tissue-derived naive CD4 T cells permissive. By comparison, these experiments were performed with doses three orders of magnitude lower than those previously tested by Unutmaz *et al.* (186). At 5 pg/ml, IL-2 and IL-15 alone or in combination were not sufficient to promote infection of tissue-derived naive CD4 T cells (Fig. 5.2B). Similarly, the combination of IL-2 and IL-15 at 20 pg/ml was ineffective (data not shown). At 500 pg/ml, IL-2 and IL-15 each conferred permissivity (Fig. 5.2B). Thus, IL-2 and IL-15 are necessary but probably not sufficient for the permissivity activity found in conditioned medium.

Other examples of paracrine signaling conferring permissivity to HIV infection have been described. Using cultures from peripheral blood, Swingler *et al.* (218) found that HIV-infected or activated macrophages produce soluble CD23 and soluble ICAM, which act on B cells to render T cells permissive by cell-cell contacts. Since IL-2 and IL-15 act directly on purified naive CD4 T cells, effects on intermediate cells do not appear involved. Others have highlighted a role for paracrine signaling by certain pro-inflammatory cytokines. Endogenous IL-1β, IL-6, and TNF-α are necessary for HIV infection in suboptimally stimulated PBMCs (219, 220). In *ex vivo* lymphoid cultures, neutralization of endogenous IL-1β or IL-6 decreases HIV replication when the tissue is cultured on collagen rafts but not when dispersed in HLAC (207). Since HIV replication



in HLAC is unaffected by neutralization of these cytokines, it is unclear what role, if any, they might play in regulating the permissivity of tissue-derived naive CD4 T cells.

Soluble factors render tissue-derived memory CD4 T cells permissive to HIV infection

Next, we investigated the role of IL-2, IL-15, and other soluble factors in HIV infection of memory CD4 T cells. Since tissue-resident memory CD4 T cells are a major target for HIV infection *in vivo* (153, 154, 157), understanding the molecular basis for permissivity in these cells could advance our understanding of HIV pathogenesis. Like tissue-derived naive CD4 T cells, tissue-derived memory CD4 T cells were refractory to HIV infection unless cultured in the presence of conditioned medium (Fig. 5.3). Surprisingly, conditioned medium depleted with antibodies specific for IL-2, IL-4, IL-7, and IL-15 retained the ability to render the memory CD4 T cells permissive to infection (Fig. 5.3). As expected, the depleted conditioned medium did not confer permissivity on tissue-derived naive CD4 T cell (data not shown). Thus, memory CD4 T cells in lymphoid tissue appear to acquire permissivity for HIV infection through soluble factors different from those that confer permissivity on naive CD4 T cells.

Naive CD4 T cells in the absence of conditioned medium exhibit an early, post-entry block to HIV infection

To determine if conditioned medium alters the ability of tissue-derived naive CD4 T cells to support HIV Env-mediated entry, we used a virion-based fusion assay (221). Equivalent levels of fusion were observed in the presence and absence of conditioned medium (Fig. 5.4A), consistent with an effect occurring after virion fusion.

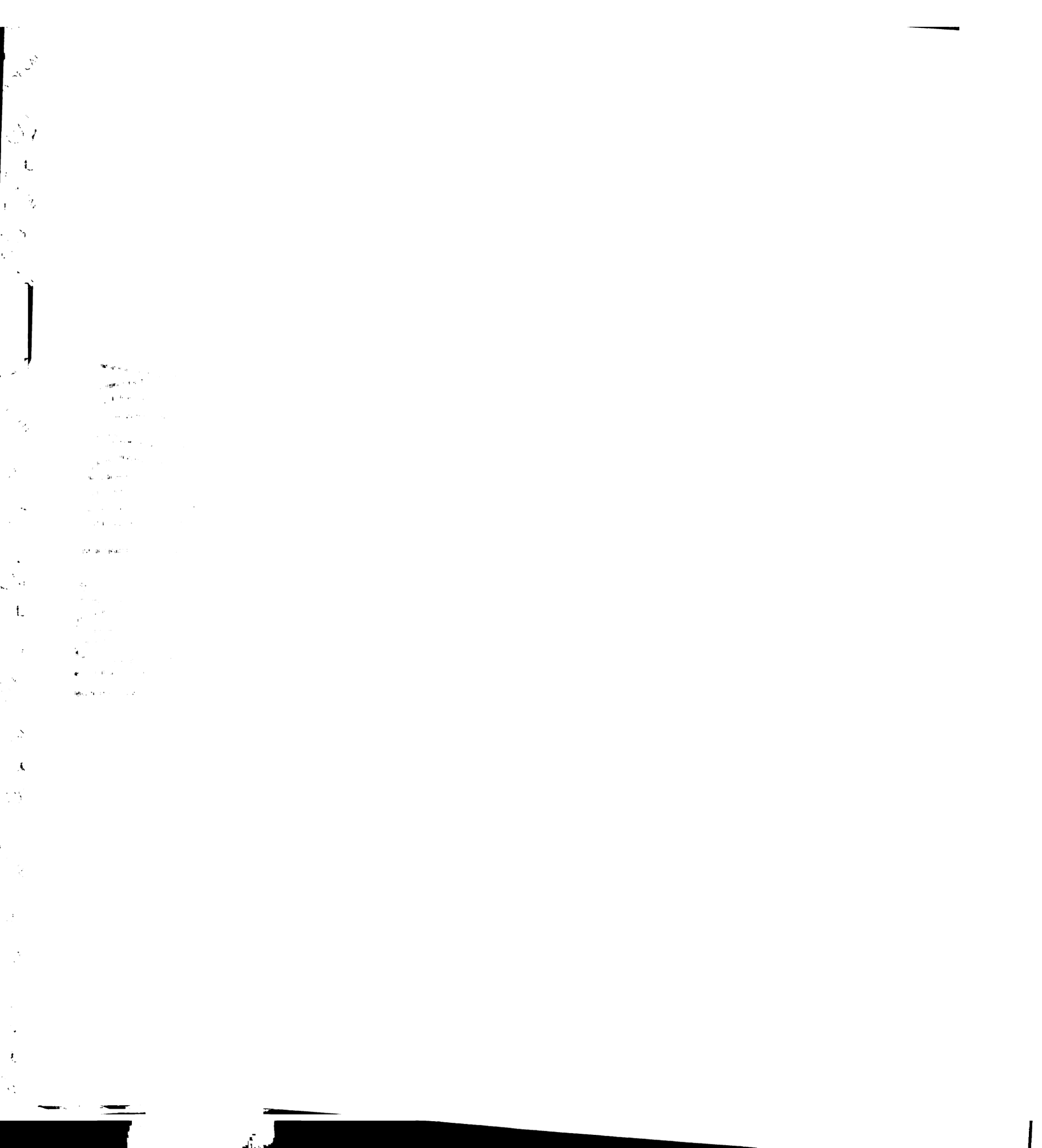
If conditioned medium affects early post-entry steps in the viral life cycle, cells cultured in the absence of conditioned medium would be refractory to infection with single-cycle reporter viruses. However, if the block occurs after integration and gene expression, cells should be permissive in the presence or absence of conditioned medium. In tissue-derived naive CD4 T cells exposed to a single-cycle reporter virus, VSV-G pseudotyped NL4-3 HSA, reporter gene expression was detected only in the presence of conditioned medium (Fig. 5.4B). Thus, conditioned medium appears to overcome an early, post-entry block to HIV infection in tissue-derived naive CD4 T cells.

Naive CD4 T cells contain HMM A3G when isolated from tonsil or when cultured in conditioned medium

The early block to HIV infection in the absence of conditioned medium is reminiscent of the block observed in unstimulated CD4 T cells in peripheral blood (209, 210), which we have shown are protected from HIV infection by expression of LMM A3G (47). If LMM A3G is eliminated by siRNA treatment or is converted to a HMM complex by mitogenic stimulation, these cells are rendered permissive. To determine if HMM A3G is present in tissue-resident, HIV-permissive CD4 T cells, we used velocity sedimentation analysis to compare A3G complexes in purified naive CD4 T cells from peripheral blood or tonsil (Fig. 5.5A). In these gradients, HMM A3G accumulated near the bottom whereas LMM A3G sedimented near the middle, similar in size to tubulin (data not shown). In blood-derived naive CD4 T cells, A3G was in the LMM form, consistent with results in unfractionated CD4 T cells (47). In naive CD4 T cells from tonsil tissue, A3G was detected principally in the HMM form, consistent with the permissivity of these cells to HIV infection (135).

These finding suggest that conditioned medium might render cells permissive to HIV infection by promoting assembly of HMM A3G complexes. As it was difficult to purify enough naive CD4 T cells from tissue for the needed biochemical analysis, experiments were instead performed using cells isolated from the peripheral blood. When cultured in conditioned medium but not medium alone, 1-3% of cells became permissive to infection by HSA-reporter viruses (data not shown). This finding is consistent with the small number of cells expressing CD25 in the conditioned medium-treated cultures (data not shown), indicating low levels of activation. As permissive cells are rare, a highly sensitive biochemical technique must be used to analyze the A3G complexes present in these cultures. In this regard, size-exclusion chromatography is preferable to the velocity sedimentation gradients used earlier. When naive CD4 T cells from the peripheral blood were cultured in medium alone, A3G remained in LMM complexes (Fig. 5.5B). In contrast, when cultured in conditioned medium, both the levels of A3G increased (K. Stopak, manuscript submitted) and importantly a fraction of the LMM A3G was recruited into HMM complexes (Fig. 5.5B). It is likely that the small percentage of A3G present in these HMM complexes reflects the small percentage of permissive cells in these cultures. Given the clear but modest ability of soluble factors alone to drive HMM complex formation in light of the high levels of HMM A3G present in cells isolated directly from tissue, it is possible that the assembly of A3G complexes *in vivo* is regulated by both soluble factors and cell-cell contacts.

In summary, our findings show that endogenously produced IL-2 and IL-15 play a key role in rendering tissue-resident resting, naive CD4 T cells permissive to HIV infection. Investigation is currently underway to identify other soluble factors that



importantly contribute to infection of both naive and memory CD4 T cells. In terms of the molecular basis underlying permissivity of tissue-resident naive CD4 T cells to HIV infection, these cells displayed enzymatically inactive HMM A3G complexes, while nonpermissive naive CD4 T cells from peripheral blood contained LMM A3G, a potent post-entry restriction factor. Further, the addition of HLAC-conditioned medium to resting naive CD4 T cells from the peripheral blood was sufficient to induce recruitment of LMM A3G into HMM complexes. Since the vast majority of CD4 T cells reside in tissues (212), our findings help explain how massive numbers of resting CD4 T cells can be infected and depleted during acute lentiviral infections (153, 154) while circulating resting CD4 T cells remain entirely refractory to HIV infection (209, 210).

Materials and Methods

HLAC and conditioned medium

Human tonsil tissue from routine tonsillectomies were obtained from the National Disease Research Interchange (Philadelphia, PA) and the Cooperative Human Tissue Network (Nashville, TN) and processed for HLAC as described (135, 207). Briefly, tonsils were minced into small pieces, passed through a 40-μm cell strainer, and cultured in 96-well U-bottom polystyrene plates (2×10^6 cells/well) in medium (200 μl/well) consisting of RPMI 1640 supplemented with 15% FCS, 100 μg/ml gentamicin, 200 μg/ml ampicillin, 1 mM sodium pyruvate, 1% nonessential amino acids (Mediatech), 2 mM L-glutamine, and 1% fungizone (Gibco). After 2–4 days of culture, conditioned medium was collected and passed through a 0.2-μm nylon filter. Before use, conditioned medium was mixed with an equal volume of fresh medium to ensure an adequate supply of nutrients and neutral pH.

Cytokine depletion, receptor blocking, and quantitation of conditioned medium

Cytokines were depleted from conditioned medium with capture antibodies specific for IL-2, IL-4, IL-7, and IL-15 (R&D Systems; MAB602, MAB604, MAB207, and MAB647). The antibodies were bound to insoluble bead supports (Pierce; Seize X Protein G Immunoprecipitation Kit) and incubated with the conditioned medium for 4 h at room temperature with constant agitation. The cytokine-depleted conditioned medium was collected after sedimentation of the antibody-containing beads. Blocking antibodies specific for IL-2Rα (2 μg/ml AF-223-NA; R&D Systems) or IL-4R (5 μg/ml MAB230; R&D Systems) were used to inhibit receptor binding. Cytokine levels in conditioned

medium were quantitated by ELISA (sensitivity: IL-2, 1.6 pg/ml; IL-7, 0.156 pg/ml; IL-15, 0.3 pg/ml) (R&D Systems).

Purification of naive and memory CD4 T cells

Naive and memory CD4 T cells in buffy coats (Stanford Blood Bank) or HLAC were first enriched by Ficoll-Hypaque density gradient separation of mononuclear cells. These cells were then sorted for either $CD4^+$ $CD45RA^+$ $CD62L^+$ (naive) or $CD4^+$ $CD45RA^-$ (memory) using a FACS DiVa (BD Biosciences). Alternatively, naive cells were isolated from mononuclear cells by magnetic negative depletion (Miltenyi Biotec; Naive CD4 T Cell Isolation Kit) supplemented with microbeads against CD8, CD14, CD19, and CD45RO. Purity was routinely >95% by flow cytometry and >90% using microbeads. After isolation, naive or memory CD4 T cells from tissue ($1–2 \times 10^5$ in 96-well U-bottom polystyrene plates) or from peripheral blood (2×10^6/ml in 6 well polystyrene plates) were cultured with medium alone, medium supplemented with recombinant IL-2 or IL-15 (R&D Systems), or conditioned medium. For experiments requiring CFSE labeling, cells (5×10^6/ml) were washed once with PBS and incubated with 1 μM CFSE (Molecular Probes) in PBS for 5 min at room temperature. To quench the labeling reaction, cells were washed in fresh medium.

HIV viral stocks

Replication-competent NL4-3 (X4-tropic) and VSV-G-pseudotyped reporter virus (NL4-3 HSA R-E-) encoding heat-stable antigen (HSA, murine CD24) were prepared by calcium phosphate transfection of 293T cells. Virus-containing supernatants were collected 48 h later, clarified by sedimentation, filtered, and concentrated by

ultracentrifugation. For fusion studies, NL4-3 virions containing BlaM-Vpr were prepared as described (221). All viral stocks were quantitated by measuring p24 Gag levels by ELISA (PerkinElmer) or FLAQ (208).

Viral infections

For spreading infections, cells were cultured with or without a reverse transcriptase inhibitor (AZT, 50 μM) for 24 h, incubated with NL4-3 (150 ng of p24 Gag) for 24 h, washed extensively, and cultured; the medium was replaced every 3 days. For single-cycle infections, cells were cultured with or without a reverse transcriptase inhibitor (3TC, 10 μM) for 48 h, inoculated with VSV-G-pseudotyped NL4-3 HSA R-E (200–1000 ng of p24 Gag) in the presence of 5 μg/ml polybrene for 3 h, washed extensively, and returned to culture. For virion-based fusion assays, cells were incubated with NL4-3 virions containing BlaM-Vpr (250 ng of p24 Gag) for 2 h, and assays was performed as described (221). Negative controls were pretreated with a CXCR4-specific entry inhibitor (AMD3100, 250 nM).

FACS analysis of HIV-infected cultures

For intracellular anti-p24 Gag immunostaining, cells were fixed in 1% paraformaldehyde and stained with an anti-p24 Gag antibody (Coulter; KC57) in PBS containing 0.1% saponin (Sigma) and 2% FBS. To measure surface expression of HSA, cells were stained with anti-HSA antibody (BD Biosciences; M1/69) in PBS containing 2% FBS. FACS data were analyzed with FlowJo software (Treestar). For the fusion assay, the levels of virion fusion are shown as a ratio of the blue to green fluorescence

reflecting the presence of cleaved versus uncleaved CCF2 substrate, the former reflecting fusion (221).

Characterization of APOBEC3G complexes

Linear, continuous 15–30% glycerol gradients were prepared in buffer containing 50 mM HEPES, pH 7.4, 125 mM NaCl, and 0.1% NP-40. Naive CD4 T cells were lysed in ice-cold lysis buffer containing 50 mM HEPES, pH 7.4, 125 mM NaCl, 0.2% NP-40, and 1 × EDTA-free protease inhibitor cocktail (CalBiochem). The clarified cell lysates were loaded on the gradients and centrifuged at 130,000 *g* for 16 h at 4°C in an SW55Ti rotor (Beckman). After sedimentation, 22 fractions (each ~210 μl) were collected from the bottom to the top of the gradient. FPLC analysis was performed as described (47) except that due to the limited amount of material, FPLC fractions were concentrated 10-fold (Microcon YM-30, Millipore) prior to detection of A3G by immunoblot analysis. To prevent non-specific loss of sample during concentration, 50–100 μg of glutathione reductase (Sigma) were added to each fraction.

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3 from Dr. Malcolm Martin and pNL4-3 HSA R-E- from Dr. Nathaniel Landau. We thank Dr. J. Burns (University of California, San Diego) for the gift of expression plasmid pVSV-G; Dr. D. Schols (Rega Institute for Medical Research, Leuven, Belgium) for the gift of AMD3100.

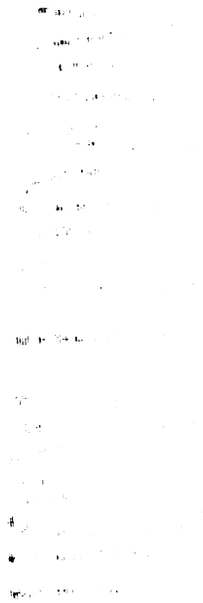


Figure Legends

Figure 5.1. Conditioned medium from HLAC renders tissue-derived naive CD4 T cells permissive to HIV infection. Cells were cultured in medium alone (left panels), labeled with CFSE, and mixed back with autologous HLAC (middle panels) or were cultured in HLAC-conditioned medium (right panels). Intracellular anti-p24 Gag staining was performed 7 days after infection with NL4-3 (150 ng of p24 Gag). AZT (50 μM) was included in some samples to ensure identification of productively infected cells (bottom panels). Data are representative of three experiments in which typically 5–10% of cells cultured in conditioned medium stained positively for intracellular Gag at the peak of infection.

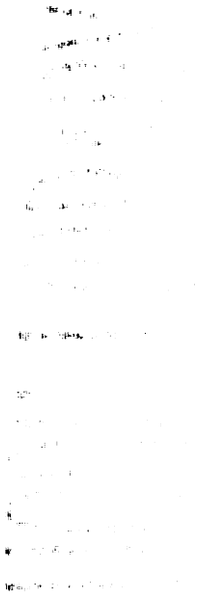
Figure 5.2. IL-2 and IL-15 are necessary but not sufficient components in conditioned medium for HIV infection of tissue-derived naive CD4 T cells. (A) Cells were cultured in medium alone, conditioned medium, or conditioned medium previously incubated with anti-IL-2, anti-IL-7, or anti-IL-15 capture antibodies and inoculated with NL4-3 (150 ng of p24 Gag). After extensive washing, virus-containing supernatants were monitored for p24 Gag content after 4, 7, 10, or 13 days of culture. Select cultures were treated with AZT (50 μM) as a control. (B) Cells were cultured in medium with or without IL-2 or IL-15 (5, 50, or 500 pg/ml). One sample was cultured with IL-2 and IL-15 (5 pg/ml). Cells were infected as described in (A). p24 Gag was measured by FLAQ assay. Data are representative of three independent experiments.

Figure 5.3. Infection of tissue-derived memory CD4 T cells also requires soluble factors. Tissue-derived memory CD4 T cells were cultured in medium alone, conditioned

medium, or conditioned medium previously incubated with anti-IL-2, anti-IL-4, anti-IL-7, and anti-IL-15 capture antibodies. Cultures were inoculated with NL4-3 (150 ng of p24 Gag); after extensive washing, virus-containing supernatants were monitored for p24 Gag content after 4, 7, 10, or 13 days of culture. Select cultures were treated with AZT (50 μM) as a control. p24 Gag was measured by FLAQ assay. The depleted conditioned medium failed to support HIV replication in tissue-derived naive CD4 T cells from the same donor. Data are representative of three independent experiments.

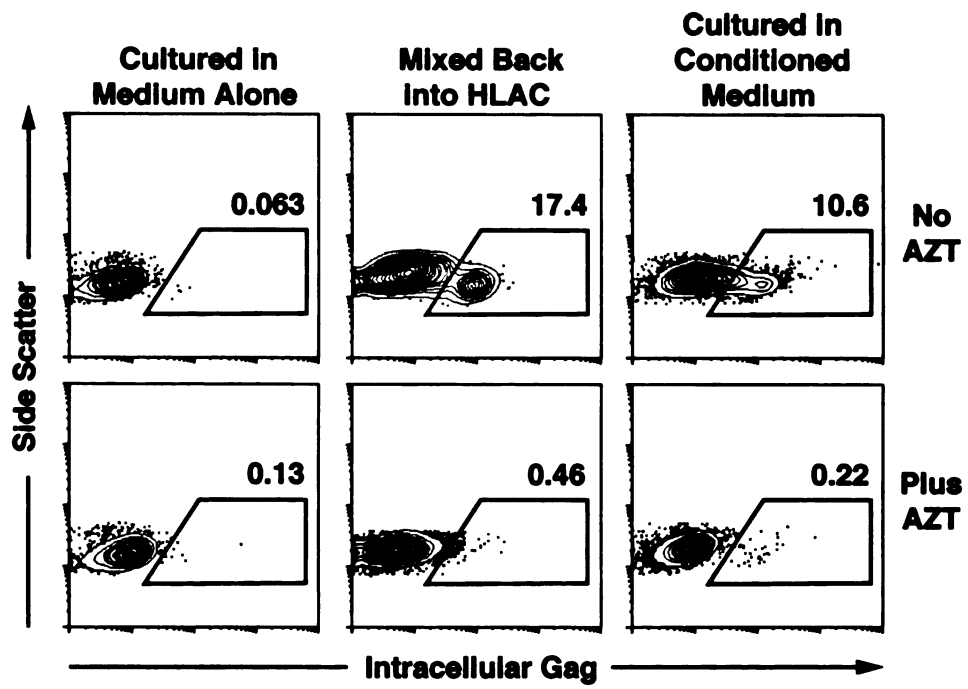
Figure 5.4. Conditioned medium relieves an early post-entry block of HIV infection in tissue-derived naive CD4 T cells. (A) Cells cultured in medium alone or conditioned medium were exposed to NL4-3 virions (250 ng of p24 Gag) containing BlaM-Vpr for 2 h before performing the virion-based fusion assay (221). As a negative control, AMD3100 (250 nM), an inhibitor of CXCR4-mediated entry, was included. Data are representative of three experiments during which levels of fusion ranged from 5–12% in the cell population. (B) Cells were exposed to VSV-G pseudotyped NL4-3 HSA R-E- (700 ng of p24 Gag) for 3 h, washed extensively, and stained for surface expression of HSA 4 days later. Select cultures were treated with 3TC (10 μM) as a control. Similar results were obtained in four additional experiments with levels of HSA infection ranging from 0.7–4.0% in the cell population.

Figure 5.5. Naive CD4 T cells contain HMM A3G when isolated from tonsil or when blood-derived cells are cultured in conditioned medium. (A) Lysates of naive CD4 T cells from peripheral blood or tonsil were subjected to velocity sedimentation analysis. Equal volumes of collected fractions were subjected to SDS-PAGE and immunoblotting for



endogenous A3G using a peptide-specific rabbit anti-hA3G antibody (46). Comparable data were obtained in two additional experiments. (B) FPLC analysis was performed on lysates of blood-derived naive CD4 T cells that were cultured for 6 days in medium alone or conditioned medium. Fractions were concentrated 10-fold prior to immunoblot analysis. These results are representative of three independent experiments.

Figure 5.1


Cultured in Medium Alone
Mixed Back into HLAC
Cultured in Conditioned Medium
0.063
17.4
10.6
No AZT
0.13
0.46
0.22
Plus AZT
Side Scatter
Intracellular Gag

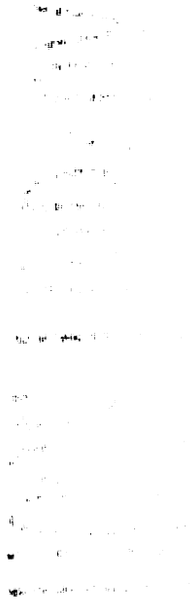


Figure 5.2

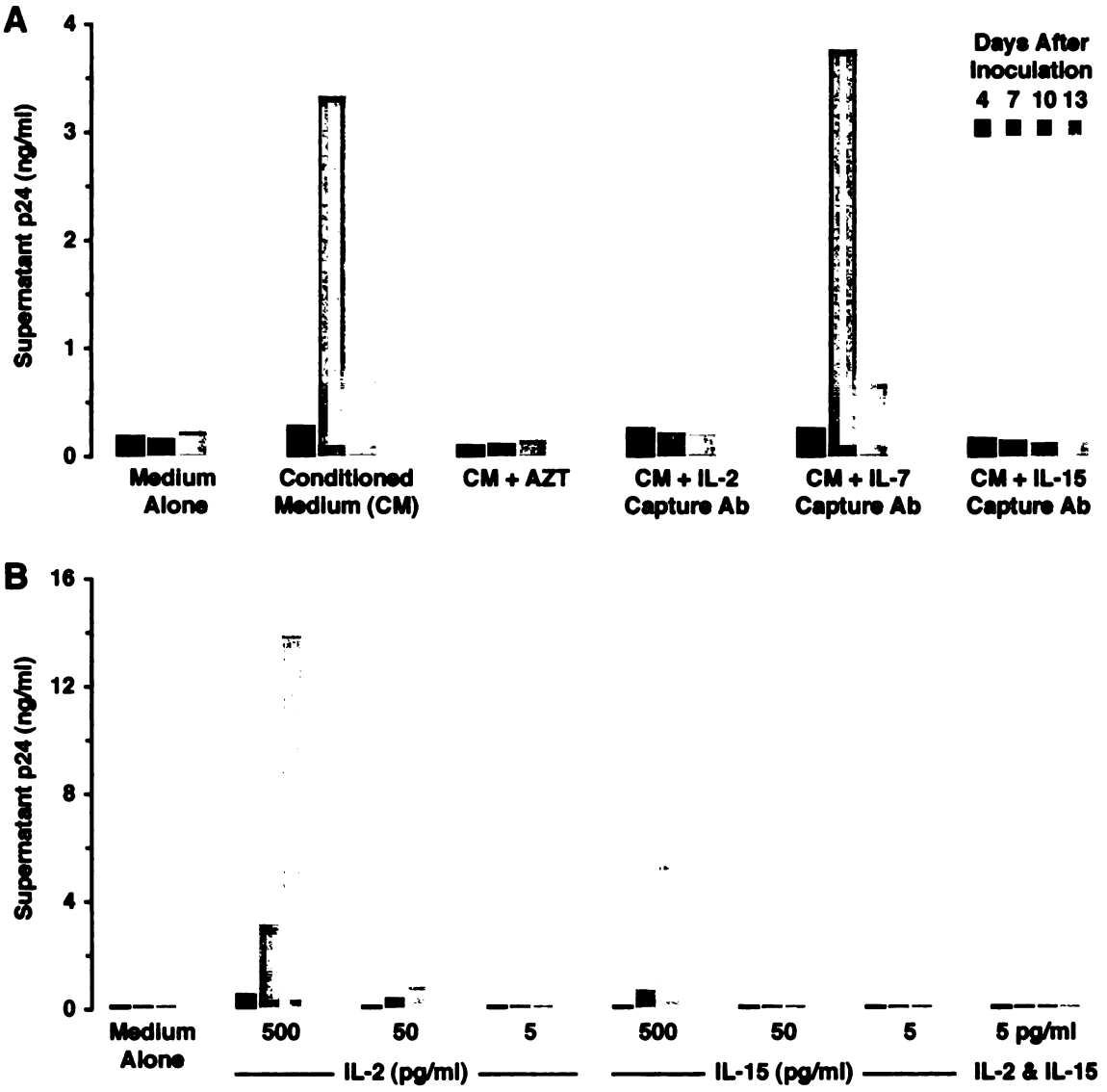

A
Supernatant p24 (ng/ml)
0
1
2
3
4
Days After Inoculation
4 7 10 13
Medium Alone
Conditioned Medium (CM)
CM + AZT
CM + IL-2 Capture Ab
CM + IL-7 Capture Ab
CM + IL-15 Capture Ab
B
Supernatant p24 (ng/ml)
0
4
8
12
16
Medium Alone
500
50
5
500
50
5
5 pg/ml
IL-2 (pg/ml)
IL-15 (pg/ml)
IL-2 & IL-15

Figure 5.3

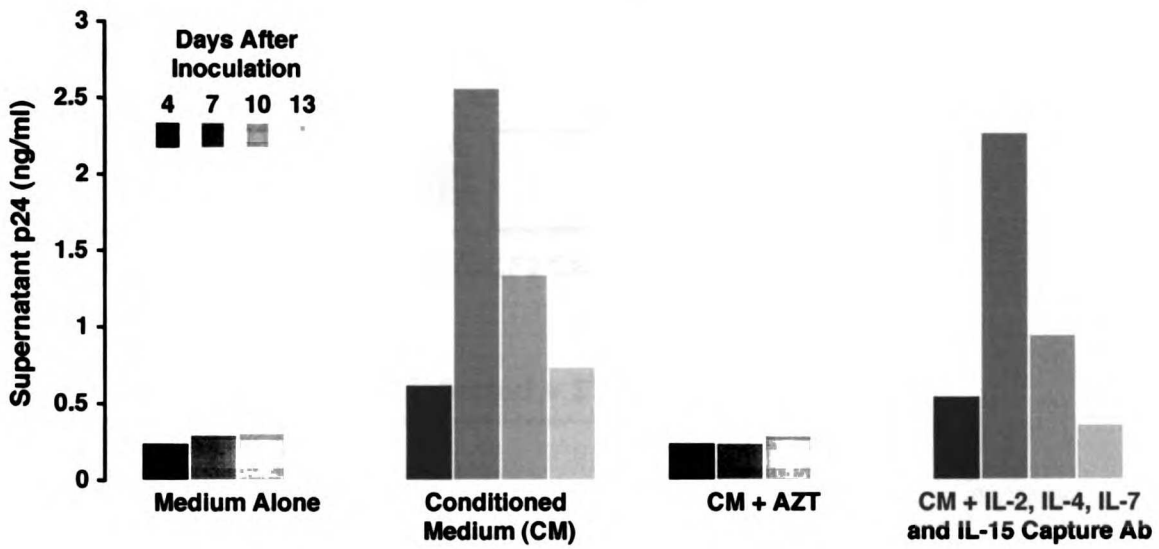

Supernatant p24 (ng/ml)
3
2.5
2
1.5
1
0.5
0
Days After Inoculation
4 7 10 13
Medium Alone
Conditioned Medium (CM)
CM + AZT
CM + IL-2, IL-4, IL-7 and IL-15 Capture Ab

Figure 5.4

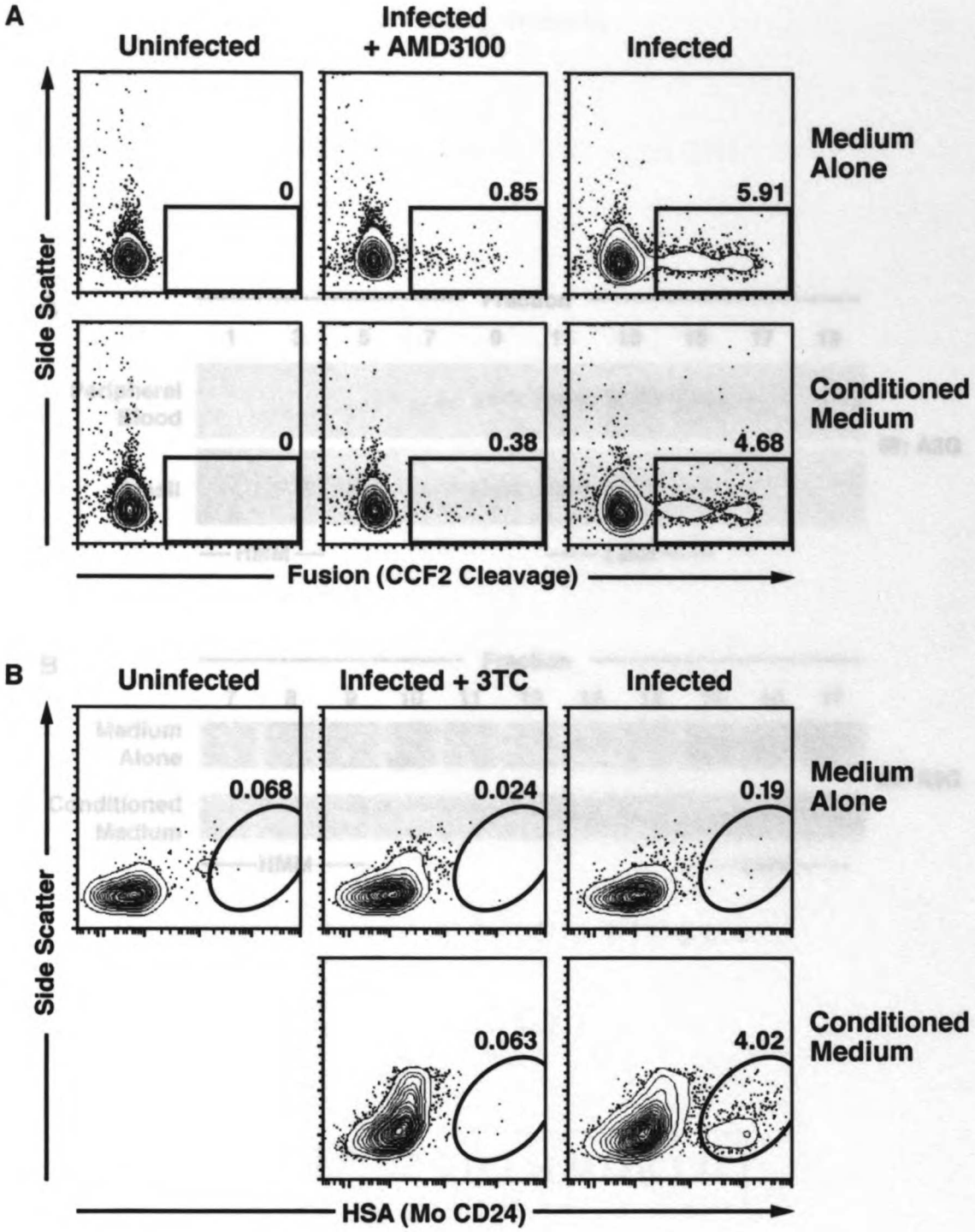
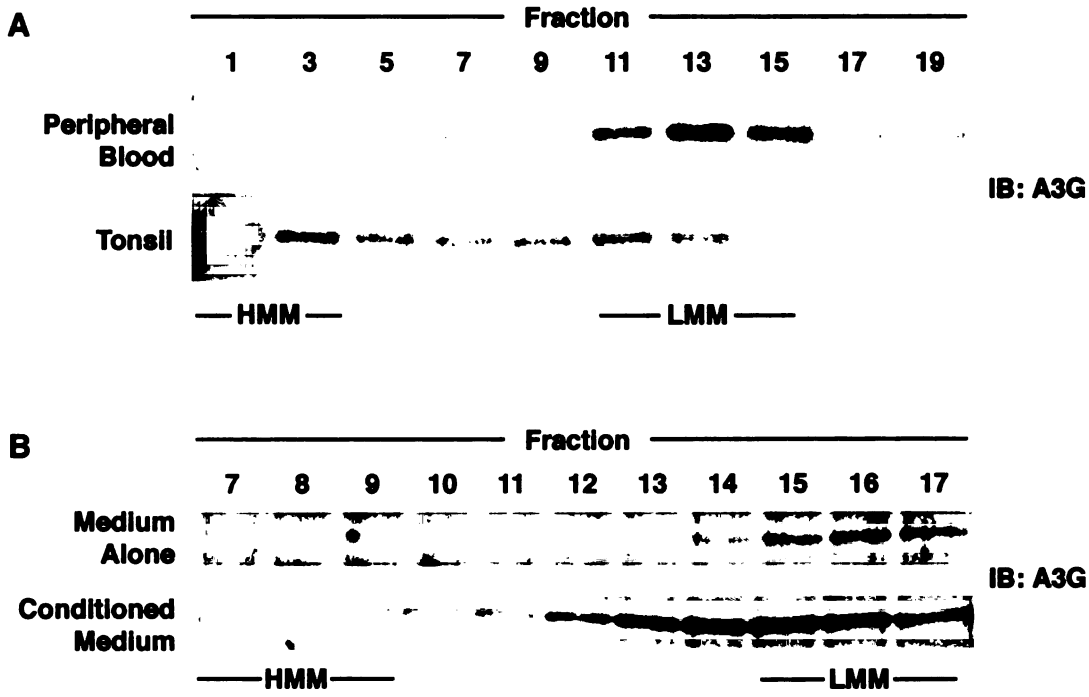

A
Uninfected
Infected + AMD3100
Infected
Medium Alone
Conditioned Medium
0
0.85
5.91
0
0.38
4.68
Side Scatter
Fusion (CCF2 Cleavage)
B
Uninfected
Infected + 3TC
Infected
Medium Alone
Conditioned Medium
0.068
0.024
0.19
0.063
4.02
Side Scatter
HSA (Mo CD24)

Figure 5.5


A
Fraction
1 3 5 7 9 11 13 15 17 19
Peripheral Blood
Tonsil
IB: A3G
HMM
LMM
B
Fraction
7 8 9 10 11 12 13 14 15 16 17
Medium Alone
Conditioned Medium
IB: A3G
HMM
LMM

Chapter 6

Concluding Remarks

To further our understanding of HIV pathogenesis, a thorough appreciation of both the host and viral determinants is required. Although the projects described in this thesis were broad in scope, they all sought to elucidate the role of various host and viral factors in HIV pathogenesis. When we initiated our screen for positive-acting host factors acting early in the viral life cycle, there was scant evidence that host factors played any role in this process. The only well-established factor was Fv1, whose action was and still remain mysterious (30). Although we were unable to identify a required host factor during the early steps of the viral life cycle, recent work from other labs has demonstrated that both positive and negative host factors do play important roles. Through its ability to interact with HIV integrase, LEDGF appears to play a key role in determining integration site selection (12-14), although the biological significance of this preference is not clear. Unlike the preference of HIV to integrate within genes, another retrovirus, MLV, appears to have preferred sites of integration near the start of genes (222). This preference too likely results from the actions of yet unidentified murine host factors. In addition, two recently identified anti-viral factors, A3G (37) and TRIM-5α (31), show that events early in the viral life cycle are subject to host anti-viral intervention.

The factors underlying viral evolution in HIV-infected patients are unclear. Of particular interest are the factors that select for R5-tropic strains early in infection and that later drive the evolution towards X4-tropic strains. A recent study by Ribeiro *et al.* suggests that viral evolution is driven by the distinct properties of naive and memory CD4 T cells (223). They claim that, since naive cells divide less frequently than memory cells, memory cells are more readily infected, thereby favoring strains that can infect memory cells while avoiding infection of naive cells. Although we agree that properties

of naive and memory cells likely influence viral evolution, we do not believe that the key difference is the variable proliferation frequencies. Instead, both ours and previous studies have found that memory cells are a far more permissive cell-type than naive cells even in the absence of cell division. Memory cells are infected more rapidly than naive cells and, upon infection, produce far greater amounts of virus (135). These data suggest that R5-tropic viruses are favored over X4-tropic viruses early in the course of disease not because R5-tropic viruses infect a cell-type that can divide more frequently but because R5-tropic viruses infect a cell more hospitable to the viral life cycle. The molecular differences between these two cell types that regulate susceptibility to HIV infection are unknown.

Although our studies on permissivity factors in lymphoid tissue provide some insights, many questions remain. There are clearly more soluble factors remaining to be identified as we cannot yet reconstitute the permissivity activity found in conditioned medium on tissue-derived naive CD4 T cells with recombinant proteins alone. In addition, we do not yet know the identity of any of the soluble factors important for infection of memory CD4 T cells in tissue. Given the central role of memory CD4 T cells during HIV pathogenesis, understanding these factors could be of great importance. We also know very little about the role of cell-cell contacts in rendering cells permissive. Although tissue-derived naive or memory CD4 T cells are permissive in the absence of other cells types, they are far more permissive in the whole tissue. Clearly then, cell-cell contacts contribute important signals to CD4 T cells, thereby helping them to establish or maintain a permissive state. It will also be important to understand the role of all these

factors not only in HIV infection of lymphoid organs such as tonsil but also in other lymphoid and peripheral sites (154).

Finally, our studies found that in HIV-permissive resting cells *in vivo*, A3G is present in HMM complexes. In addition, conditioned medium containing IL-2 and IL-15 is sufficient both to render resting naive CD4 T cells from the peripheral blood permissive to infection and to promote assembly of HMM A3G complexes. Thus, complex assembly of the host anti-viral factor A3G governs the permissivity of CD4 T cells *in vivo*. This is clearly an exciting opportunity for therapeutic intervention. Two possible drugs can be envisioned: one that prevents HMM complex formation and another that promotes HMM disassembly. A note of caution is warranted though as we do not yet know the role of A3G *in vivo*. Thus, it is unclear what unintended consequences might occur upon prolonged LMM A3G exposure.

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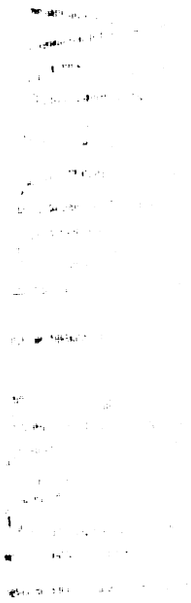

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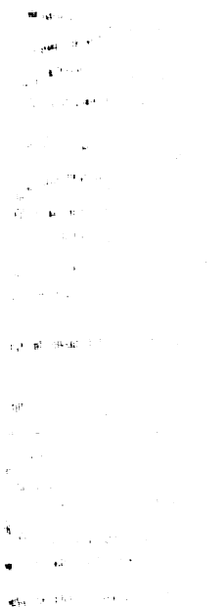

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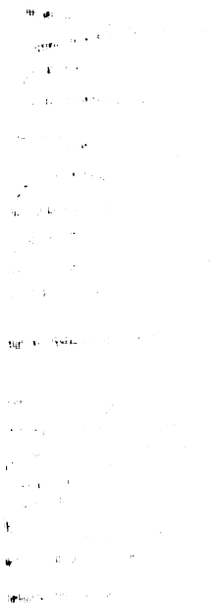

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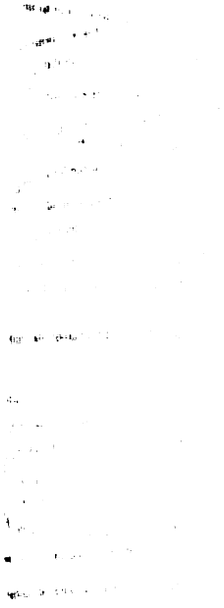

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