RESTRICTION OF MACROPHAGE INFECTION DURING HIV AND SIV INFECTION: THE ROLE OF ANTIBODIES AND CORECEPTOR USE PLASTICITY IN ENFORCING CD4-DEPENDENT ENTRY AND SHAPING TROPISM *IN VIVO*

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ABSTRACT

RESTRICTION OF MACROPHAGE INFECTION DURING HIV AND SIV INFECTION: THE ROLE OF ANTIBODIES AND CORECEPTOR USE PLASTICITY IN ENFORCING CD4-DEPENDENT ENTRY AND SHAPING TROPISM *IN VIVO*

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The pathogenesis of HIV and SIV infection is determined to a large extent by adaptations of the virus that enable it to infect different cell types and either destroy these cells, or establish long-term reservoirs of viral replication. CD4+ T cells are the predominant targets of HIV and SIV infection *in vivo,* although some variants of these viruses have the ability to efficiently infect macrophages, which express exceedingly low levels of CD4. I sought to understand the selective forces that normally favor CD4+ T cell infection while restricting macrophage infection *in vivo*. To investigate these forces, I examined viral envelope glycoprotein (Env) variants that emerged during an *in vivo* SIV infection model in which extensive tissue macrophage infection was observed in experimentally CD4+ T cell-depleted rhesus macaques. I found that plasma-derived viral Envs from these animals had a remarkable ability to mediate entry into cells expressing CCR5 but lacking CD4, a phenotypic hallmark of macrophage-tropic viruses. These CD4-independent Env variants were highly sensitive to neutralization by anti-Env antibodies, as well as to control SIV+ plasma. However, plasma from CD4+ T cell-depleted animals could not neutralize CD4-independent Envs. This suggests that CD4+ T cells enforce CD4-dependent SIV entry by supporting the production of anti-Env antibodies that normally prevent the emergence of macrophage-tropic virus. In further studies, I asked whether other forces might restrict the emergence of CD4-independent Envs *in vivo* by examining entry coreceptor use by these Envs. Despite the ability of CD4-independent Envs to mediate robust entry in the presence of high cell

surface levels of rhesus macaque CCR5, they were impaired in their use of human CCR5, rhesus macaque GPR15, and low levels of rhesus macaque CCR5. These phenotypes indicated that CD4-independent entry is accompanied by reduced plasticity in coreceptor usage, possibly restricting the virus in its range of potential target cells *in vivo*. Future studies will examine whether antibody-mediated enforcement of CD4 tropism and coreceptor usage are selective pressures that also explain the conservation of CD4-dependent entry during HIV-1 infection. It is possible that these forces can be manipulated to reshape viral tropism and eliminate HIV-1 macrophage reservoirs.

TABLE OF CONTENTS

LIST OF TABLES AND ILLUSTRATIONS

CHAPTER I

GENERAL INTRODUCTION

Epidemiology of HIV and challenges to a cure

Human immunodeficiency virus (HIV) is the cause of the acquired immune deficiency syndrome (AIDS) pandemic and remains a major public health concern despite 3 decades of research (1, 2). An estimated 34 million people globally are currently infected and living with HIV, with approximately 2.5 million new infections and 1.7 million AIDS-related deaths each year. While the total number of new infections and the number of AIDS-related deaths both appear to be slowly declining, the total number of people living with HIV continues to rise, especially in sub-Saharan Africa, where as many as one out of every 20 individuals is infected. Anti-retroviral drugs are effective at reducing viral load and immunodeficiency in individuals and slowing the spread of infection, but are not curative, and are available to fewer than one in 3 HIV-infected people worldwide (2).

As large-scale economic and public health initiatives aim to expand access to anti-retroviral drugs and prevent new infections, basic research on HIV molecular virology and pathogenesis reflect at least two longer-term goals: 1) designing an effective HIV vaccine, and; 2) finding ways to fully eradicate the viral reservoirs within infected individuals. Attempts at designing an effective vaccine for the prevention of HIV infection in humans have ranged from partial successes to complete failures (10, 30, 81). However, laboratory studies and non-human primate models of infection (discussed in detail below) continue to provide clues to correlates of protection that inform future vaccine design. Anti-retroviral therapy potently blocks HIV infection at multiple stages in the viral life cycle (discussed in **Chapter II**), but poses severe limitations. It requires lifelong patient adherence for effective suppression viral load, and carries with it the risk of adverse effects and selection of drug-resistant viral variants. Therefore, a major area of basic research involves identification and elimination of the cells harboring the viral reservoir, or alternatively, prevention of the formation of such a reservoir. Underlying both vaccine design and reservoir identification is the need to gain a greater understanding of how HIV infection leads to immunodeficiency.

This thesis examines the regulation of potential target cells for HIV infection *in vivo* using non-human primate simian immunodeficiency virus (SIV) as a model. I will begin by describing SIV as a model system for HIV and providing an overview how viral entry pathways influence cell tropism (**Chapter I**). I will then provide more in-depth background on how HIV/SIV infection can differ between cell types, and how these different cell types impact pathogenesis (**Chapter II**). Next I will present my own body of research, which is an in-depth examination of a model system in which SIV tropism is profoundly altered *in vivo* in response to perturbations in the host immune system and the virus's target cells (**Chapters III, IV, V**). These chapters will discuss the forces that shape this relationship between virus and host *in vivo*. Finally, I will suggest ways in which an understanding of these forces can inform future efforts at HIV eradication (**Chapter VI**).

Primate models of HIV infection and disease

Many insights into HIV disease, including much of the information presented throughout this thesis, have been gained through studying SIV infection of monkeys. It is therefore important to begin with an in-depth examination of SIV models, how they enhance our understanding of HIV-1 infection, and what their limitations are.

The origins of HIV-1 and HIV-2 infection of humans can be traced to zoonotic transfer of genetically- and structurally-related lentiviruses from African non-human primates collectively termed simian immunodeficiency viruses (31, 44). HIV-1 and HIV-2 in fact make up relatively small phylogenic branches within the SIV family, each member of which naturally infects its own unique primate host (17, 37). The M and N groups of HIV-1 in particular, which represent the vast majority of documented cases of human infection and which are responsible for the HIV-1 pandemic, originated from SIVcpz, a virus that infects chimpanzees in the wild (55). SIVcpz appears to have evolved as a recombinant virus, with the 5' half of its genome being closely related to SIVrcm from red-capped mangabeys, and the 3' half being related to SIVgsn from greater spot-nosed monkeys (6, 17). In contrast, HIV-2 infection, which is much less common than HIV-1

and is characterized by a slower rate of disease progression as well as less severe immunodeficiency (20, 54, 66), is most closely related to SIVsmm from sooty mangabeys (44). Interestingly, with extremely rare exceptions, despite chronic high-level replication, SIV causes neither immunodeficiency nor reduced lifespan in its natural hosts ((African non-human primates, (9, 62, 76, 82, 91)). It is generally assumed that millions of years of virus-host co-evolution has selected for this non-pathogenic persistence of SIV, but the specific mechanisms underlying the non-pathogenic nature of SIV in natural hosts are not well-understood, and are an active topic of research (3, 88, 91).

Many strains of HIV/SIV are highly species-specific, in that they have a host range limited to one or a small number of species (62). This includes HIV-1, which productively infects humans, is attenuated in great apes, and cannot infect monkeys. The potential for a monkey SIV model of human HIV infection was first observed when captive Asian macaques in U.S. primate centers developed lymphomas while cohabitating with sooty mangabeys (19, 53). It is now understood that the sooty mangabeys were naturally infected with SIVsmm and that when transmitted to macaques the virus caused an AIDS-like disease. As noted above, this same family of SIV also transmits to humans resulting in HIV-2, but more importantly, the disease observed in the infected macaques closely resembles the AIDS caused by HIV-1 (61), and so experimental SIV infection of Asian macaques is arguably the most important animal model of HIV infection (43, 71).

The SIV macaque model was optimized by passaging SIVsmm in macaques *in vivo* and in cells *ex vivo* to generate the now commonly used SIVmac239 and SIVmac251 isolates (**Figure 1.1A**), highly pathogenic viruses that consistently recapitulate an AIDS-like disease in macaques (18, 19, 61, 80). SIVmac239 and SIVmac251 are closely-related, with the principal difference being that 239 is a clonal virus with a single discrete genotype maintained as a plasmid, while 251 is a heterogeneous "swarm" that is maintained by continuous passage in macaque peripheral blood cell culture *ex vivo* (18, 80). One obvious consequence of this difference is that when referring to the use of

"SIVmac251" we are in fact referring to a collection of related viruses that, while more genetically similar to each other than they are to SIVmac239, have the potential to be highly variable phenotypically (21, 92). At least seven phylogenetically unique stocks of SIVmac251 exist in the U.S., and the overall maximum diversity within each of the various stocks is as high as 2% (21, 63). Indeed, a range of *in vitro* phenotypes has been observed for various clones of SIVmac251 envelope glycoproteins, including variability in neutralization sensitivity and receptor tropism (21, 63, 92). However, despite the diversity of the SIVmac251 swarms and their phylogenic uniqueness from SIVmac239, *in vivo* pathogenesis and natural history in infected macaques are largely considered indistinguishable between SIVmac239 and any of the SIVmac251 swarms, and all are commonly used for pathogenesis and vaccine studies (21, 43). This concept is important in considering the work that will be presented in **Chapters III**, **IV**, and **V** of this thesis, because we operate under the assumption that under normal circumstances selective pressures cause the same types of viral variants to dominate during infection *in vivo*, even when a diverse swarm of variants is presumably available at the start of infection (21).

How well does SIVmac infection of macaques recapitulate and effectively model HIV-1 infection of humans? The similarities and differences between HIV-1 infection of humans and SIVmac infection of rhesus macaques (3, 41, 71) are outlined in **Table 1.1**. Indian rhesus macaques (*Macaca mulatta*) are the most frequently used non-human primate models for HIV/SIV pathogenesis studies and are the basis of the work described in **Chapters III**, **IV** and **V** of this thesis. Importantly, although the timing differs between HIV infection of humans and SIV infection of rhesus macaques, both infections begin with an initial peak in plasma viral load and contemporaneous destruction of the majority of mucosal CD4+ T cells, followed by a decline in viral load to a "setpoint" as an adaptive immune response sets in. The virus continues to adapt to the host immune response, replicating with largely sub-clinical manifestations as CD4+ T cell counts in the blood gradually decline and the adaptive immune system becomes exhausted from continuous activation. Towards the end of the disease course, opportunistic infections and frequently neurologic symptoms (discussed in **Chapter II**) take hold as the host

succumbs to AIDS. In the case of SIV infection, the very late stages of disease are rarely observed because the animals are euthanized for ethical reasons.

Another monkey species, the pigtailed macaque, also succumbs to an AIDS-like disease when infected with SIVmac, although this model is generally thought to be less accurate at recapitulating HIV infection of humans, since progression to AIDS is very rapid in the animals and they are less likely to mount an adaptive immune response. Furthermore, simian-human immunodeficiency virus (SHIV) models have been developed in order to assess the efficacy of potential anti-HIV drugs and vaccines and have also enhanced our understanding of HIV/SIV pathogenesis. One of the original SHIV constructs was generated by replacing the *rev*, *tat*, and *env* genes of SIVmac239 with the *rev*, *tat*, *vpu*, and *env* genes of HIV-$1_{89.6}$ (**Figure 1.1B**). Although $SHIV_{89.6}$ and subsequent modifications thereof provided controlled animal models of viruses genetically similar to HIV-1, *in vivo* infection had some unusual features, such as rapid and sustained depletion of peripheral CD4+ T cells and the frequent failure of infected animals to generate antibodies against the virus. This may be related to the ability of the 89.6 parental virus to use both CCR5 and CXCR4 as entry coreceptors (discussed below), since SIV rarely uses CXCR4. In contrast, some SHIVs (developed from other HIV-1 isolates) are rapidly suppressed by the host, and most SHIVs paradoxically tend to over-estimate the efficacy of potential HIV vaccines (41).

SIVmac infection of rhesus macaques on the other hand provides a consistent and tractable method of observing lentiviral transmission, replication and *in vivo* evolution, as well as innate and adaptive immune responses and progressive immunodeficiency in a small primate host. Thus, while no non-human primate system perfectly recapitulates HIV-1 infection of humans, SIVmac infection of rhesus macaques remains the best method of modeling HIV-1 pathogenesis. For this reason, most of the topics covered in this thesis draw from studies of both HIV-1 and SIV, using concepts studied in the context of one virus to inform the understanding of the other unless there is specific evidence suggesting that it would be inaccurate to do so.

Table 1.1. Characteristics of SIVmac and SHIV89.6 monkey models of AIDS to HIV-1 infection of humans. Information is adapted from various reviews (3, 41, 71).

		HIV-1 infection of humans	**SIVmac infection of rhesus macaques**	**$SHIV_{89.6}$ infection of rhesus macaques**
Virus	**Structure**	See **Figure 1.1**	See **Figure 1.1**	Mac239 with *rev, tat, env* replaced with HIV-1 *rev, tat, vpu, env* (**Figure 1.1**)
	Sequence diversity	10-30% between HIV-1 subtypes	2% between SIVmac variants	N/A (clonal chimera)
	Accessory genes	*vif, vpr, vpu, tat, rev, nef*	*vif, vpr, vpx, tat, rev, nef*	*vif, vpr, vpu, tat, rev, nef*
	Co-receptor usage	CCR5, CXCR4	CCR5, GPR15, CXCR6, others	CCR5, CXCR4
	Macrophage infection	Entry and post-entry restriction	Entry restriction	Post-entry restriction
Host cell	**TRIM5**	Does not restrict HIV-1 or SIVmac replication	Restricts HIV-1 replication, not SIVmac	Does not restrict SHIV replication
	APOBEC3	HIV-1 Vif counteracts human APOBEC3	SIV Vif counteracts APOBEC3 from human and NHPs	SIV Vif is necessary for SHIV infection
	Tetherin	HIV-1 Vpu counteracts tetherin	SIV Nef counteracts tetherin	
	SAMHD1	No counteraction of SAMHD1	Vpx counteracts SAMHD1	Vpx counteracts SAMHD1
	MHC	3 HLA genes	2 Mamu genes	2 Mamu genes
Disease (un-treated)	**Transmission**	Sexual or blood; usually a single founder variant	Mucosal or blood (experimental); single or multiple founder variants depend on dose	Mucosal or blood (experimental); single or multiple founder variants depend on dose
	Time to AIDS	8-10 years	1-2 years	Less than 1 year
	Peak viral load (log copies/mL)	5.0-6.0	6.5-8.4	6.4-8.7
	Set point viral load (log copies/mL)	4.0-5.0	4.4-7.7	3.9-6.5
	CD4+ T cell and lymphocyte turnover	Rapid loss of mucosal CD4+ T cells; gradual decline in blood	Rapid loss of mucosal CD4+ T cells; gradual decline in blood	Rapid loss of mucosal and blood CD4+ T cells
	Time to sero-conversion (est.)	2 months	1 month	Usually fail to sero-convert
	CNS infection	CNS inflammation; infected microglia and perivascular macrophages	CNS inflammation; infected perivascular macrophages	Similar to HIV-1
Other notes	**Vaccine and immunogen testing**	N/A	Immunogens do not predict HIV-1 immunogenicity	Env immunogen identical to HIV-1
	Drug susceptibility	Sensitive to multi-drug regimen	Many HIV-1 drugs not effective	Drug activity depends on target gene

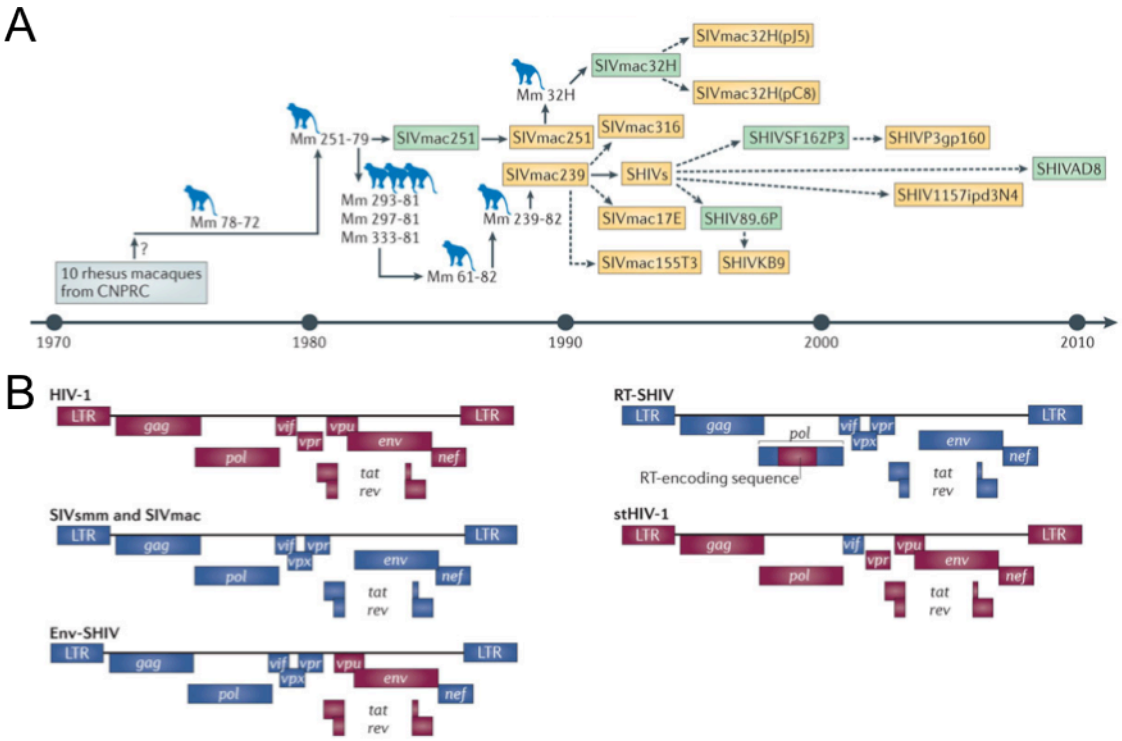

Figure 1.1. Origin and structure of commonly used SIV strains. **A.** Progression from left to right across the figure represents the time at which each of the original isolates were derived beginning in the early 1970s with animals carrying SIVsmm at the California National Primate Research Center. **Green** boxes represent swarm isolates whereas **gold** boxes represent clonal isolates. **Blue** monkey cartoons indicate *in vivo* passage (Mm = Macaca mulatta); solid arrows indicate direct transmission and/or viral isolation; dotted arrows indicate cloning and ex vivo passaging in tissue culture. B. Viral RNA sequences are indicated with known protein-coding genes highlighted along with the 5' and 3' long terminal repeat (LTR) regions. HIV-1 genes are represented in **dark red** and SIV genes are represented in **purple**. Figure was adapted from *Hatziioannou and Evans, 2012* (41).

HIV/SIV entry pathways: overview

HIV and SIV infection lead to the destruction and progressive decline of CD4+ T cells in their hosts. Before the identification of HIV as the causative agent of AIDS, a key observation in the earliest AIDS patients was the profound loss of CD4+ T cells (known at the time as Leu-3 helper cells; (36)). It was later observed that many HIV-1 isolates had a cytopathic effect and replicated in CD4+ T cell culture and T cell lines in vitro, and indeed CD4 was later identified as a specific receptor required for viral entry (64, 67). While much of the loss of CD4+ T cells *in vivo* is the result of direct infection and killing of these cells, a substantial amount of immune dysfunction also arises from indirect effects of the virus such as bystander cell killing, immune exhaustion and chronic activation, all of which can lead to the loss of uninfected cells. Still, infection of CD4+ T cells is the central driver of AIDS pathogenesis and the principal source of viral replication *in vivo,* and latently infected CD4+ T cells are the major source of long-term viral persistence (29, 45, 79, 96). What is less clear is the role of non-CD4+ T cells in supporting viral replication. Infection of myeloid cells (monocytes, macrophages, microglia, astrocytes, and dendritic cells) is known to drive HIV-associated neurologic disorders and is thought to contribute to the formation and maintenance of a long-term viral reservoir (5, 40, 58, 97). However, other consequences of myeloid cell infection, if any, are not clear. Furthermore, it is apparent that evolutionary pressures drive a host-pathogen relationship in HIV and SIV infection that generally favors viral replication in CD4+ T cells over other cell types, a relationship which needs to be understood in order to advance efforts to eradicate infection.

HIV/SIV entry pathways: structural and molecular interactions

HIV and SIV envelope glycoproteins, known as Env, exist as trimers on the surface of virions and infected cells (**Figure 1.2**). Each virion-associated Env trimer consists of non-covalently linked heterodimers of glycoproteins including a gp120 surface subunit that mediates attachment to target cells, and a gp41 transmembrane subunit that mediates fusion between the viral and cellular membranes (65, 73). The Env gp120 and gp41 subunits are coded by the viral *env* gene, which produces a single gp160 protein that is

cleaved by cellular furin during viral assembly (39). During entry into a target cell, viral gp120 engages cellular CD4, triggering conformational changes in Env that expose the V3 and bridging sheet regions of gp120 (46, 56, 84). These regions interact with a cellular 7-trans-membrane (7TM) co-receptor – principally CCR5 or CXCR4 for HIV-1, and CCR5, GPR15, CXCR6, or others for SIV – initiating further conformational changes that expose the fusion peptide of gp41 (24, 68). The helical repeats (HR1 and HR2) of each gp41 molecule within the trimer rearrange to form a six-helix bundle, which together with the fusion peptides allows membrane fusion to occur. This process implicates two crucial elements in the initial targeting of the virus to a cell: 1) gp120 engagement of CD4; and 2) V3 and bridging sheet engagement of co-receptor. The diversity of primate lentiviruses and their tendency to rapidly mutate during replication lead among other things to diversity in entry pathways. With only a small number of genetic changes, viruses can vary in their efficient use of either CD4, the various 7TM receptors, or both.

CCR5 is generally considered the principal 7TM co-receptor for HIV-1. The most important evidence for this stems from two observations: 1) Individuals homozygous for the CCR5-null Δ32 allele are nearly entirely resistant to infection (32, 69, 87); and 2) newly-transmitted variants that establish productive infection exclusively use CCR5 as a co-receptor (51, 77, 78, 86). Switch to CXCR4 or dual CCR5/CXCR4 use occurs in up to half of untreated subtype B HIV-1 infected individuals, and this change is generally associated with more rapid disease progression (8, 14, 42). However, co-receptor switch is not a requisite to acquisition of AIDS, so it possible that the emergence of CXCR4-using variants is a result, not a cause of progression to AIDS (52). The V3 loop of HIV provides the principal basis of co-receptor specificity, possibly bearing a structural homology with natural chemokine ligands (11). Structural and charge alterations in the V3 loop and to a secondary extent the V2 loop and other regions result in changes in receptor preference from CCR5 to CXCR4 (11, 15, 47, 60).

The situation is less well understood with regards to CCR5 use in SIV infection. Most SIV variants tested do in fact use CCR5 as a co-receptor for entry, but unlike HIV-1, many SIV also can use other 7TM receptors such as CCR2b, CXCR6, GPR15, GPR1, APJ, and others (23, 24, 68). There are in fact at least two examples of natural SIV host species that can become infected despite carrying CCR5-null alleles that do not support viral entry, suggesting that at least in some non-human primates, viral entry through cellular CCR5 is not an absolute requirement for viral replication *in vivo* (13, 83). Furthermore, use of CXCR4 is rare among SIV variants in both pathogenic and non-pathogenic infection, adding evidence that co-receptor pathways may be an important divergent characteristic between HIV-1 and SIV infection (23, 70).

For both HIV and SIV, co-receptor engagement generally encompasses at least two key interactions between gp120 and the 7TM co-receptor: 1) an interaction between the second extracellular loop of the 7TM receptor (ECL2) and the V3 loop of gp120; and 2) an interaction between the n-terminus of the 7TM receptor and the gp120 quaternary structure known as the bridging sheet formed from β-sheets at the base of V1/V2 and β20/β21 upon CD4 binding (15, 25-28). Whereas the V3-ECL2 interaction is considered important for co-receptor engagement and specificity (34), this requirement can be relaxed in Envs that are more dependent on the 7TM n-terminus for entry (59, 72). In contrast, the interaction between the 7TM n-terminus and the gp120 bridging sheet is thought to be extremely important in initiating the conformational changes that lead to exposure of the gp41 fusion peptide, with several sulfated tyrosines on the cellular 7TM receptor being indispensible for the interaction (16, 25-28).

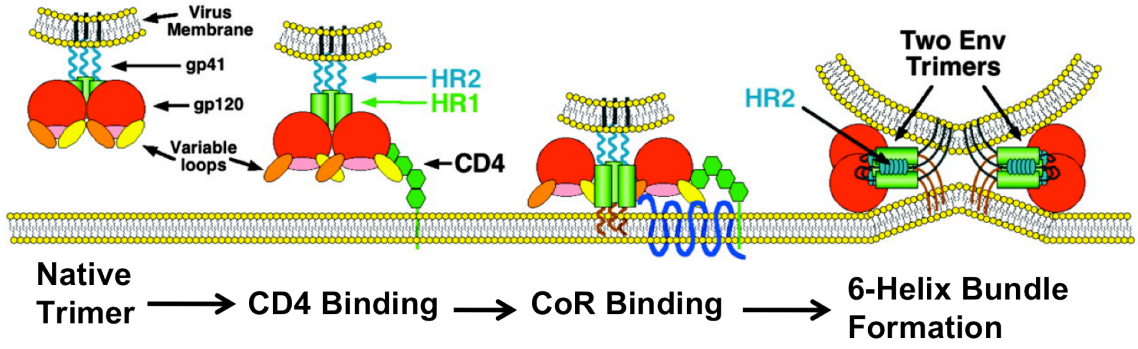

Figure 1.2. HIV entry. The viral Env spike is composed of gp120 and gp41 subunits form a trimer of heterodimers on the surface of the viral membrane (**top**). When the virus encounters a target cell membrane (**bottom**), gp120 engages CD4, initiating conformational changes that form an exposed co-receptor binding site. Interactions between gp120 V3 (**yellow**) and the 7TM ECL2 (**blue**) as well as between the gp120 bridging sheet (**pink**) and the 7TM n-terminus initiate a further cascade of conformational changes that form gp41 HR1 and HR2 into a six-helix bundle that drives membrane fusion. Figure was adapted from *Moore and Doms, 2003* (73).

HIV/SIV entry pathways: tropism *Portions of this section have been adapted from Gorry, Francella, and Collman, 2013* (33).

Viral entry dependence on CD4 together with a 7TM co-receptor is a principal determinant of HIV and SIV cellular host range. Historically prior to the full elucidation of the viral entry pathways, newly-derived HIV isolates were characterized as either T-tropic (capable of infecting immortalized T cell lines and primary CD4+ T cells) or M-tropic (capable of infecting primary macrophages and CD4+ T cells). When CXCR4 and CCR5 were later identified as the co-receptors, it happened that most M-tropic viruses used CD4 in conjunction with CCR5 for entry, while most T-cell tropic viruses used CD4 in conjunction with CXCR4. However, equating T- and M- tropism classification with CXCR4- and CCR5- use is now known to be inaccurate. Whereas most HIV variants that infect macrophages do use CCR5 for entry, not all CCR5-using variants infect macrophages. Furthermore, HIV and SIV variants involved in transmission and destruction of mucosal CD4+ T cells are CCR5-using and non-M-tropic (78, 86). There are also a handful of HIV-1 variants that use CXCR4 to enter macrophages, again arguing against assumptions regarding "T-tropic" and "M-tropic" in relation to co-receptor use (12, 98).

At least one unifying similarity connecting most M-tropic HIV and SIV variants is the ability of viral Env to use low to absent levels of CD4 on the cell surface to mediate entry via a 7TM co-receptor (4). Relaxation of strict CD4-dependence, either through increased affinity of gp120 for low levels of CD4 (22, 93) or through enhanced Env intrinsic triggerability and exposure of the Env co-receptor binding site in the absence of CD4 (38, 46, 56, 84), leads to increased infection of macrophages and other CD4-low and CD4-negative cell types (7, 74, 89). Further evidence suggests that in addition to decreased dependence on CD4, some macrophage-tropic variants may exhibit an altered and more efficient mechanism of CCR5 engagement (33, 35, 94). Importantly, changing as little as one or a select few amino acid residues in Env can impart the altered interactions with CD4 and CCR5 that allow enhanced macrophage infection (4, 86). Conceivably, if widespread macrophage infection *in vivo* was a preferred pathway, the

mutation rate of the virus would allow this to occur. Yet, as stated before, CD4+ T cells, not macrophages, are the preferred target of the virus *in vivo*, and it is their infection and destruction that propagates the virus and leads to disease progression (29, 45). Macrophage infection, when it occurs, is highly compartmentalized, and infection is generally limited to the central nervous system (CNS) and possibly peripheral organs, suggesting that the host actively prevents macrophage infection and the virus adapts to avoid host resistance mechanisms (57, 90).

SIV models of infection have provided important insights into the mechanisms regulating macrophage infection. In both humans infected with HIV-1 and rhesus macaques infected with SIV, high level plasma viremia persists even during late stage disease when virtually all circulating and tissue CD4+ T-cells have been lost. Using a highly-pathogenic SHIV strain, Igarashi et. al. showed by immunohistochemistry that in infected macaques with end-stage disease, greater than 95% of infected cells were tissue macrophages (49). More recently, a CD4+ T-cell depleted model of SIV infection revealed the potential for extensive macrophage infection *in vivo*. Ortiz et. al. artificially depleted CD4+ T-cells in macaques prior to SIVmac infection (75), and while peak viral loads during acute infection were similar in CD4+ T-cell depleted and control animals, there was no post-peak viral load decline in CD4+ T-cell depleted animals as is normally observed. This phase of high level viremia was characterized by extensive tissue macrophage infection. Thus, it appears that tissue macrophages outside the CNS have the capacity to serve as sites of high level virus production, at least under particular circumstances. Analyses of these models suggest that macrophage infection develops in settings where there is both a paucity of CD4+ T-cells that requires the virus to seek alternative cellular targets, and a permissive immune environment that allows the emergence of M-tropic variants. In both the highly-pathogenic SHIV infection and the antibody-mediated CD4+ T-cell depletion model, macrophage infection does not occur until after CD4+ T-cells are depleted (48, 49, 75). An additional factor contributing to immune control of macrophage infection may be a direct antiviral activity of CD4+ T-cells that is uniquely active in suppressing virus in macrophages (85, 95). Of note,

macaque infection with highly M-tropic SHIV in the absence of immune suppression generally does not cause disease, and results instead in rapid disappearance of plasma viremia and induction of neutralizing antibodies (50).

Post-entry restriction and cellular tropism

Multiple host cellular factors restrict HIV/SIV infection at the various stages of the viral life cycle, and the virus has evolved a range of mechanisms of counteracting such restriction. While the bulk of this thesis specifically focuses on restriction of viral infection and determinants of cellular tropism at the level of entry, post-entry mechanisms of restriction are extremely important in determining the range of host cells (and species) that the virus can infect. Mounting evidence demonstrates that, at least for a subset of host restriction factors, expression and anti-viral activity varies by cell type and therefore can direct the rapidly evolving virus to more favorable target cells. This concept, along with a description of the viral life cycle, is covered in **Chapter II**.

Goals of this thesis

HIV and SIV have the ability to infect macrophages, yet selective forces appear to strongly favor preferential infection of CD4+ T cells in the systemic compartment *in vivo*. Multiple mechanisms underlying this preference for CD4-tropism have been proposed, but there is no clear evidence that these forces shape cellular tropism *in vivo*.

The goal of this thesis is to examine how CD4-tropism is enforced during HIV/SIV infection *in vivo*, and why extensive infection of macrophages is rare. I studied an *in vivo* SIV infection model in which macrophages were abundantly infected following experimental depletion of CD4+ T cells. This system enabled examination of the features of the virus and the host immune response that allowed macrophage infection *in vivo*. I found that the lack of an effective antibody response to Env in these animals was associated with the emergence of CD4-independent, neutralization-sensitive viral variants thought to infect macrophages *in vivo*. I conclude from this evidence that the antibody

response normally restrains viral tropism and prevents macrophage tropism (**Chapter III**).

I went on to examine what other features of these CD4-independent Env variants might normally restrain their emergence during infection, and found that that CD4-independent viral entry is associated with a striking lack of coreceptor use plasticity in Env (**Chapter IV**). Preliminary attempts to understand what specific primary cell types can become infected by CD4-independent SIV revealed that these viruses were highly impaired in infection of primary cells and cell lines *in vitro* (**Chapter V**). These studies reveal that at least two forces normally favor CD4-tropic infection *in vivo*: host antibody responses restricting the emergence of CD4-independent virus, and coreceptor plasticity allowing a potentially expanded range of target cells for CD4-dependent viruses.

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CHAPTER II

MACROPHAGE-SPECIFIC ASPECTS OF HIV/SIV INFECTION

This section was adapted from a book chapter in press:

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Abstract

Macrophages are a heterogeneous population of myeloid leukocytes that perform diverse innate and adaptive functions of the vertebrate immune system. They are principally defined as being differentiated, tissue-resident forms of monocytes, with a phagocytic capacity, relatively large size, and expression of various functional markers on their cell surface. Notably, macrophages express the HIV/SIV entry receptors CD4 and CCR5/CXCR4, and have long been known as *in vivo* and *in vitro* targets of infection. However, HIV/SIV that infect CD4+ T-cells vary in their capacity infect macrophages (macrophage-tropism). This variability can be attributed to macrophage-specific characteristics and differences among virus variant, principally in the ability to enter macrophages, as well various levels of restriction outlined in this chapter and adaptations that the virus uses to overcome these restrictions. A central goal of this thesis is to understand the forces that normally restrain macrophage infection in the systemic compartment while enhancing it in the CNS, and this chapter provides background on our current knowledge of these forces. A summary of this chapter is illustrated in **Figure 2.1**.

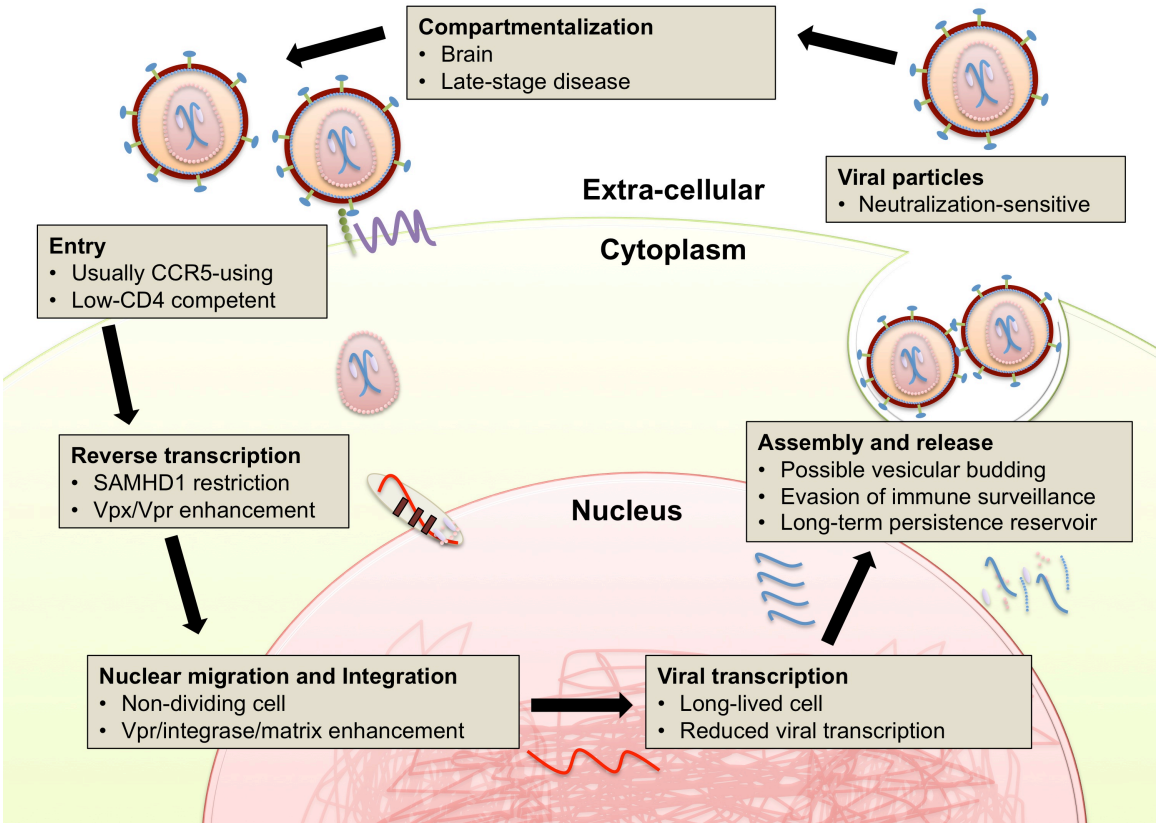

Figure 2.1. The HIV/SIV life cycle in infected macrophages. Unique aspects of infection in macrophages compared to CD4+ T cells are indicated in the boxes.

Introduction

While CD4+ T-cells constitute the majority of infected cells during *in vivo* HIV infection, other important targets of infection are myeloid cells, predominantly macrophages. T-cells and macrophages are vastly different in morphology, proliferative capacity, gene expression profile and interaction with HIV, yet both can become infected and support viral replication.

In the central nervous system (CNS), the majority of infected cells are myeloid cells (macrophages and microglia), and these are responsible for neurological aspects of disease. Outside the CNS, macrophages are involved in events at mucosal surfaces during transmission, can serve as long-lived reservoirs, and may contribute substantially to virus production especially in late stage disease when CD4+ T cells are largely depleted (3). Thus, comparative studies of HIV infection of macrophages versus T-cells continue to be of great interest. Much of the current understanding of HIV molecular pathogenesis comes from studies using tissue culture-adapted viruses and transformed cell lines that resemble neither T-cells nor macrophages. It is therefore essential to consider the HIV life cycle in the context of the very different primary cell types these viruses can infect. While the primary focus of this chapter is HIV-1, critical insights have been gained through studies of SIV and so we will indicate where data is extrapolated from SIV to the context of HIV-1.

Macrophage models used to study HIV/SIV

Much of the understanding of HIV biology has been generated through studies in transformed cell lines, but while useful they do not always reflect unique features of HIV/primary cell interactions. Monocyte/macrophage-like cell lines exist that are sometimes used for HIV infection studies (U937, THP-1, MonoMac, HL60), but since they may not faithfully recapitulate primary cell interactions with the virus, primary cells are often used. As cell-specific experimental targets for HIV, monocyte-derived macrophages (MDMs) are most often generated by *ex vivo* culture and differentiation of blood-derived monocytes. In addition, more physiologic and phenotypically diverse

tissue-associated macrophages can be isolated directly from human and non-human primate mucosal regions such as gut, lung, and vaginal tract, as well as from secondary lymphoid organs, liver, kidneys and central nervous system (5).

Phenotypically, macrophages are distinguished from other leukocytes by their propensity to adhere to culture surfaces, their large irregular morphology, and surface expression of MHC class II as well as myeloid markers such as CD14, CD16, CD68 and CD156. Functionally, macrophages are highly diverse, with important roles ranging from innate immune control, inflammation, phagocytosis and antigen presentation to anti-parasitic and wound healing.

Traditionally, HIV-1 isolates have been phenotypically classified as macrophage-tropic (capable of infecting primary macrophages and primary CD4+ T cells but not transformed T cell lines), T cell line tropic (capable of infecting primary CD4+ T cells and transformed T cell lines but not primary macrophages), or dual-tropic (capable of infecting all three target cell types in vitro). The term "macrophage-tropic" was formerly used interchangeably with "CCR5-tropic" in reference to HIV entry receptors, a correlation that is now recognized to be highly imperfect, as will be discussed in detail below (11). Today, "macrophage tropism" in the context of lentiviral infection generally refers to the ability of a virus to infect and replicate in *ex vivo* cultured macrophages, and/or the ability of viral components (such as the envelope glycoprotein) to mediate infection of macrophages in a single-round assay (9).

Interestingly, monocytes are much more resistant to HIV-1 infection *in vitro* than differentiated macrophages. *In vivo*, HIV DNA can be found in circulating CD14+ monocytes, although typically at levels considerably lower than CD4+ T cells. Monocytes impact HIV pathogenesis partially because they are directly infected by the virus and may be involved in trafficking virus into tissues such as the brain, and partially through bystander and indirect effects associated immune activation (14).

Macrophage-Specific HIV/SIV Entry

The viral envelope (Env) glycoprotein of HIV-1 (as well as HIV-2 and SIV) is comprised of the surface subunit gp120 and transmembrane subunit gp41, which are organized as trimeric units (trimer of heterodimers) on the virus surface. Entry is mediated by gp120 engagement of cellular CD4, which results in structural changes to gp120 that enable interactions with one of two principal cellular co-receptors, CCR5 or CXCR4. Further structural changes in gp41 then lead to fusion of the viral and cellular membranes and viral entry (17).

Entry receptor use as determinant of macrophage tropism. Early studies on HIV-1 suggested that macrophage-tropic viral isolates used CCR5 as a co-receptor, whereas viruses that could not infect macrophages used CXCR4. However, it is now recognized that CCR5 is the principal co-receptor for most HIV-1 strains, and a gradation of macrophage tropism exists among them that is determined by the ability to utilize CCR5/CD4 in the context of their expression levels on the surface of primary cells. Conversely, macrophages also express low levels of CXCR4, and while T-cell line tropic HIV-1 strains that use CXCR4 for entry are usually not able to enter macrophages efficiently, some primary isolates that use CXCR4 for entry can use macrophage CXCR4. Dual-tropic HIV-1 variants, which use both CCR5 and CXCR4, may enter macrophages through either or both pathways. Despite this plasticity in macrophage coreceptor use, however, it is true that most macrophage-tropic HIV variants studied use CCR5. In contrast, non-macrophage-tropic variants may use CCR5, CXCR4, or both (11).

Among CCR5-using viral variants, efficiency of CD4 use is a major entry determinant of macrophage tropism. Macrophages express less CD4 on their cell surface than CD4+ T-cells, and most macrophage-tropic viral variants are capable of infecting cells expressing little CD4. The viral Env determinants of coreceptor usage (CCR5 versus CXCR4) map in large part although not exclusively to the third hypervariable (V3) domain (7) . Among R5 variants, the viral Env determinates that regulate the efficiency of CD4 use and thus macrophage tropism are more varied, and include changes in the first and

second variable loops (V1/V2) as well as loss of glycosylation in the CD4-binding site and second constant domain (C2) (9).

Of note, different tissue macrophages vary in their expression of CCR5, and thus permissiveness to HIV-1 infection. For example, vaginal mucosal macrophages are susceptible to infection in vitro and in the rhesus macaque/SIV model in vivo, whereas small intestinal mucosal macrophages are reported to lack entry coreceptors and resist infection (12).

Endocytosis in macrophage entry. HIV entry occurs predominantly at the plasma membrane surface, in contrast to viruses that enter via endocytic pathways. However, some models suggest that HIV entry into macrophages (and some cell lines) may involve endocytic pathways, although its relative importance remains to be fully defined (17).

Immunological correlates of macrophage tropism. Macrophage-tropic HIV-1 strains are generally more sensitive to neutralization by HIV+ serum and monoclonal antibodies than non-macrophage tropic variants. At least one explanation for this is that these variants exhibit increased constitutive exposure of regions within Env, such as the co-receptor interacting domains, that enable viral entry in the presence of reduced CD4 levels. These regions are highly vulnerable targets of neutralizing antibodies. In contrast, variants that are not macrophage-tropic normally shield such epitopes until entry is triggered by CD4 (2). Macrophage-tropic variants are able to tolerate this enhanced sensitivity to neutralization because they typically reside in immune-privileged compartments like the CNS, emerge in late-stage disease, or possibly avoid antibodies by infecting new target cells via cell-to-cell spread.

Anatomic compartmentalization of macrophage infection and macrophage tropism. The neutralization-sensitivity of macrophage-tropic viral variants has implications reflected in observations of HIV infection *in vivo*. While infected macrophages can be found in the gut, vaginal tract and lungs, infected macrophages and highly macrophage-tropic viral

variants are most abundant in the CNS. In patients with AIDS-associated neurologic disorders, microglia and perivascular macrophages are the principal infected cells and viral reservoir in the CNS, rather than T-cells. The propensity of macrophage-tropic HIV-1 to preferentially emerge in the CNS is likely due to both the abundance of myeloid cells and paucity of T cells in this compartment, and the relative shelter from peripheral immune pressure that allows the low-CD4-using, neutralization-sensitive variants to exist (2).

Viral RNA can be detected in the cerebrospinal fluid in most HIV-infected individuals who are not on ART treatment (6), and as many as 40-70% of untreated individuals experience some form of HIV-associated neurologic disorder (18). However, productive infection of macrophages in the CNS may be less common, as infected CNS-resident cells have been detected in approximately 20% of patients when examined during autopsy (6). Based on decay rate following ART initiation, CSF virus appears to be derived from a combination of short-lived and long-lived cells, with the former likely reflecting virus originating from blood T cells, and the latter likely originating from brain macrophages and more dominant in individuals with more severe neurologial disease.

While non-CNS HIV-1 variants are typically poorly macrophage tropic, macrophage-tropic variants may be more often isolated from the blood and lymph nodes of late-stage and highly-immunodeficient AIDS patients. Like the immune-privileged CNS, emergence of macrophage-tropic viral variants in late stage disease with advanced T-cell loss may also reflect a setting in which there are limiting numbers of T cell targets combined with diminished immune pressure. Consistent with this notion, studies in the rhesus macaque model infected with SIV or SHIV (SIV/HIV chimeras) show that macrophages may be an important, or even principal, reservoir in very late stage disease when nearly all detectable CD4+ T cells have been depleted, or following experimental CD4+ T cell depletion (3).

Post-Entry Early Events in Replication Cycle

Following fusion and entry, the viral genome is reverse-transcribed from RNA into DNA and transported into the nucleus within a structure known as the pre-integration complex, comprised largely of viral capsid proteins.

Cellular restriction factors. Mammalian cells have a variety of intrinsic proteins that have as major (if not exclusive) functions, the ability to restrict incoming retroviruses, including among them lentiviruses such as HIV and SIV. In cases where virus successfully establishes infection in target cells, it either has evolved to avoid these restriction factors or has acquired specific genes to impede their function. These restriction factors are typically interferon-stimulated, contributing to innate immunity augmenting intrinsic cellular resistance to infection.

A cellular restriction factor that has a unique role in macrophages is SAMHD1, which impedes HIV-1 reverse transcription by hydrolyzing deoxynucleosides in the cytoplasm. As non-dividing cells, macrophages have lower pools compared to T cells of deoxynucleoside precursors required for reverse transcription of viral RNA into DNA (10). HIV-2 and most SIVs encode a *vpx* gene, whose gene product interferes with SAMHD1 and enhances viral permissiveness in macrophages. In some SIVs lacking *vpx*, a similar function is ascribed to Vpr, but HIV-1 lacks both the *vpx* gene and any as-yet identified gene with equivalent function. Nevertheless, HIV-1 does infect macrophages *in vitro* and *in vivo*, and macrophage-tropic Env allows productive infection of macrophages *in vitro*, despite the absence of Vpx, relatively high levels of SAMHD1 and low levels of nucleotides in the cytoplasm. This suggests that SAMHD1 restriction of HIV-1 modulates but is not sufficient to fully block infection of macrophages.

The APOBEC3 family of proteins restricts virus replication by multiple mechanisms, the most well-defined of which is cytosine deamination during reverse transcription that leads to G-to-A hypermutation, unless countered in the virus-producer cell by the virally-encoded Vif protein. While APOBEC3 proteins are not known to differentially restrict

HIV infection in T-cells versus macrophages, it has been suggested that APOBEC3 expression in immature blood monocytes contributes to their resistance to infection relative to differentiated macrophages, but this line of evidence is not yet conclusive. TRIM5α and related members of the TRIM family are intrinsic cellular resistance factors that target the capsid of incoming viruses for degradation. While simian TRIM proteins block efficient HIV-1 infection, HIV-1 has evolved to avoid targeting by human TRIM proteins. TRIM proteins are expressed in both macrophages and T-cells, and some evidence suggest that different members of the large TRIM are differentially expressed in macrophages compared to T cells, although they likely function similarly in the different cell types (1).

Antiviral restriction factors place HIV and SIV under continuous evolutionary pressure to avoid triggering innate host immune signaling while maximizing replication and target cell tropism. For example, the cellular DNase TREX1 degrades cytoplasmic viral DNA, but rather than acting as an antiviral mechanism, this process actually enhances HIV infection because the virus hijacks the TREX1 system in order to prevent self-integration and reduce the risk of triggering an interferon response, particularly in T cells. In contrast, viral genomes persist for a longer time in myeloid cells, and thus are more prone to detection by innate immune sensors. One current hypothesis is that the inability of HIV-1 to antagonize myeloid-specific restriction factors leads to reduced infection of macrophages and dendritic cells and, consequently, only weak triggering of the interferon response in these cells. In contrast, HIV-2 and various SIVs, which possess Vpx and Vpr proteins that overcome restriction in these cells, may then trigger a robust interferon response that in turn reduces viral replication across multiple cell types (19). However, despite this intriguing and plausible hypothesis, there is as yet no evidence that *in vivo* macrophage infection is greater in SIV compared to HIV or that the viruses elicit distinct interferon responses *in vivo* (16).

Nuclear migration and integration. Macrophages differ from activated CD4+ T cell targets of infection in that they are terminally differentiated non-dividing cells. In

contrast to other retroviruses, which are unable to enter the nucleus of nondividing cells, lentiviruses including HIV have the ability to efficiently enter the nucleus of nondividing cells and establish integration. The viral Vpr protein plays a major role in enabling nuclear migration in non-dividing cells, interacting with cellular proteins to allow nuclear entry without disruption of the nuclear envelope, and thus is an important factor for efficient macrophage infection. In addition to Vpr, the viral matrix, capsid, and integrase proteins also contribute to efficient nuclear migration in non-dividing cells. On the other hand, Vpr plays an important role during infection of proliferating activated CD4+ T cells by inducing cell cycle arrest, a function that is not operative in non-dividing macrophages (13).

HIV-1 has a distinct predilection for integration into genes, and especially into transcriptionally active genes. This is a feature common to both macrophages and CD4+ T cells. One difference between cell types, however, is that while integration is considered essential for efficient viral gene expression in CD4+ T cells, some models suggest that low levels of transcription from unintegrated viral DNA may occur in macrophages, driven by Vpr and enabled by the non-proliferating nature of macrophages that allows persistence of unintegrated genomes (15).

Viral expression: transcription, RNA processing and translation

Key factors that determine whether an integrated virus is productively expressed or latent include the complement of cellular transcription factors present and state of cellular activation. In macrophages in particular, the relatively quiescent activation state compared to activated CD4+ T-cells and distinct transcription factor patterns are associated with generally lower levels of viral gene expression. Compared to CD4+ T-cells, macrophages possess higher ratios of repressive forms of transcription factors such as C/EBPβ, Sp3 and OKT18. In addition, macrophage-specific chromatin conformations and recruitment of histone deacetylases to the HIV-1 promoter may also contribute to relative transcriptional quiescence. Together, these factors contribute to overall lower viral expression in macrophages compared to T cells, and may also contribute to the

potential for long-term survival of infected cells via decreased direct cytopathogenicity or immune recognition *in vivo* (15).

Viral assembly and release

Following structural gene expression, the virus is assembled and is released from the cell, either as cell-free virus at the plasma membrane or, perhaps especially in the case of macrophages, via cell-to-cell transfer through viral synapses. Differences in viral assembly and release between macrophages and T cells are a dynamic area of research.

Budding versus exocytosis. In macrophages, mature HIV-1 virions have been observed in large vesicular structures within cells. There is longstanding debate as to whether this reflects budding into intracellular multivesicular body-like structures, or into deep invaginated pockets of the cell membrane. Thus, it is also unclear whether HIV-1 is released from macrophages following fusion of these multivesicular bodies with the plasma membrane (exocytosis), or if the virus buds from the cell surface as it does other target cells, only into these invaginated pockets (4). In either model, the nature of the budding site is believed to provide a mechanism shielding virions from immune surveillance.

Tetherin The host cell restriction factor tetherin inhibits release from the cell surface of HIV-1 virions (as well as many other enveloped viruses). The HIV-1 viral protein Vpu counteracts tetherin by promoting its degradation, enabling release of virus from the cell surface. Macrophages express high levels of tetherin relative to primary T cells. Release of virus carrying mutant Vpu is more attenuated in macrophages *in vitro* than in CD4 lymphocytes, which have lower levels of tetherin. This implies that tetherin may play a particularly significant role in viral restriction in macrophages relative to other target cell types (8).

Cytopathicity and reservoir function

Macrophages are relatively resistant to the cytopathic effects of HIV-1 infection *in vitro* and are able to produce virus for prolonged periods of time, in contrast to productively infected CD4+ T cells that are rapidly killed following infection. The *in vivo* half-life of tissue macrophages is estimated to be approximately 2 weeks in healthy individuals. *In vitro*, the lifespan of macrophages is not reduced by HIV infection, and current dogma that the same is true *in vivo* (3), although this remains an active area of research (5, 14).

In vivo reservoir. Infected individuals receiving antiretroviral therapy, even those with viral loads below the limit of detection for many years, experience viral rebound after termination of therapy. In addition, even when antiretroviral therapy reduces plasma viremia below the threshold of clinical assays, most patients maintain very low levels of plasma virus that can be detected with ultra-sensitive methods. The source of both persistent low level plasma virus on treatment and rebound virus when therapy is stopped is a key area of current investigation. While productively infected activated CD4+ T cells survive for only a short time *in vitro* and *in vivo*, resting CD4+ T cells that are latently infected are one important reservoir for long-term persistence. In addition, because of their resistance to viral cytopathicity and long natural lifespan, macrophages have the potential to serve as another long-lived HIV-1 reservoir in patients treated with antiretroviral therapy (15). Because macrophages penetrate and reside deep within tissues, they may be particularly shielded from antiretroviral drugs due to their anatomic localization.

Brain macrophages are an especially important current focus of interest as a long-term reservoir, given the low tissue-specific bioavailability of many antiretroviral drugs in the CNS. Although infected macrophages have been found in many tissues, they are the predominant infected cell type in the brain. In addition to their role in viral persistence, they are the source of viral expression and inflammation contributing to HIV-1 associated neurological disorders, even in infected individuals with undetectable peripheral viral loads on long-term antiretroviral therapy (18). Furthermore, CNS viral load exhibits

slower decay kinetics than does plasma viral load following anti-retroviral therapy, although viral RNA is generally undetectable in the CNS in long-term treated patients (3).

Consequences of HIV-1 infection for macrophage function

In contrast to the clear evidence that HIV-induced CD4+ T cell loss and dysfunction lead to immune dysfunction and AIDS, it is not clear whether there is substantial macrophage immune dysfunction induced by HIV infection that directly contributes to immunodeficiency. On the other hand, monocyte-macrophage activation induced by HIV-1 binding and/or infection clearly contribute to disease pathogenesis. Generalized immune activation is a central feature of HIV pathogenesis, and levels of the monocyte activation marker soluble CD14 are strongly linked to disease. In HIV-infected people blood monocytes show changes in transcriptomic profiles, and in SIV infection monocyte turnover is linked to rapid disease progression (14).

Unlike most neurotropic or neurovirulent viruses, HIV does not directly infect neurons, and HIV-associated neurological disorders result from indirect effects mediated by glial cells, primarily infected or activated brain macrophages and microglia. Viral Env binding to the CD4/chemokine co-receptor complex triggers intracellular signaling cascades, which lead to release of cytokines, chemokines and other mediators that augment inflammation. In addition, productive macrophage infection leads to release of neurotoxic cytokines and small molecule mediators that injure neurons. Thus, HIV-induced activation and infection of macrophages are responsible for the indirect neuronal injury seen in HIV-associated neurological disorders, which persist albeit at lesser degrees of severity even in people on antiretroviral therapy (18).

Conclusion

HIV-1 infects macrophages both *in vitro* and *in vivo*. Several aspects of HIV-1 infection in macrophages distinguish infection of these cells from other HIV-1 target cells. HIV-1 viruses that infect macrophages are primarily CCR5-tropic and generally neutralization-sensitive given their adaptation to low levels of CD4 expressed on these cells. A number of host restriction factors and other cellular proteins dictate unique aspects of HIV-1 entry, reverse transcription, nuclear migration, and egress in macrophages relative to HIV-1 infection of CD4 T cells. HIV-1 infection of macrophages in the CNS has a well-defined role in AIDS-associated neurological disorders, and an apparent but less clear role in other tissues. Importantly, macrophages may be a reservoir of persistent infection in patients treated with antiretroviral therapy. Defining the specific role of macrophages in disease progression, understanding mechanisms of macrophage HIV-1 infection, controlling HIV-1 triggered macrophage immune activation, and finding ways to successfully eliminate persistent macrophage reservoirs will be central to developing successful therapies to treat and perhaps block HIV-1 infection. Importantly, this thesis helps understand the forces that restrain widespread macophage infection in the systemic compartment while enabling macrophage infection in the CNS.

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CHAPTER III

CD4+ T CELLS SUPPORT PRODUCTION OF SIV ENV ANTIBODIES THAT ENFORCE CD4-DEPENDENT ENTRY AND SHAPE TROPISM *IN VIVO*

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Abstract

CD4+ T cells rather than macrophages are the principal cells infected by HIV-1/SIV *in vivo*. Macrophage-tropism has been linked to the ability to enter cells through CCR5 in conjunction with limiting CD4 levels, which are much lower on macrophages than on T cells. We recently reported that rhesus macaques (RM) experimentally depleted of CD4+ T-cells before SIV infection exhibit extensive macrophage infection, as well as high chronic viral loads and rapid progression to AIDS. Here we show that early time point and control Envs were strictly CD4-dependent, but by day 42 post-infection, plasma virus of CD4+ T cell-depleted RM were dominated by Envs that mediate efficient infection using RM CCR5 independently of CD4. Early time point and control RM Envs were resistant to neutralization by SIV+ plasma but became sensitive if pre-incubated with sCD4. In contrast, CD4-independent Envs were highly sensitive to SIV+ plasma neutralization. However, plasma from SIV-infected CD4+ T cell-depleted animals lacked this CD4-inducible neutralizing activity, and failed to neutralize any Envs regardless of sCD4 pre-exposure. Enhanced sensitivity of CD4-independent Envs from d42 CD4+ T cell-depleted RM was also seen with monoclonal antibodies that target both known CD4-inducible and other Env epitopes. CD4-independence and neutralization sensitivity were both conferred by Env amino acid changes E84K and D470N that arose independently in multiple animals, with the latter introducing a potential N-linked glycosylation site within a predicted CD4-binding pocket of gp120. Thus, the absence of CD4 T cells results in failure to produce antibodies that neutralize CD4-independent Envs and CD4 pre-triggered control Envs. In the absence of this constraint and with a relative paucity of CD4+ target cells, widespread macrophage infection occurs *in vivo* accompanied by emergence of variants carrying structural changes that enable entry independently of CD4.

Introduction

CD4+ T cells rather than macrophages are the primary target cell infected by HIV-1 and SIV in vivo (62). CD4+ T cells and macrophages both express the principal entry co-receptor CCR5, but macrophages express much lower levels of CD4, and macrophage-tropism is associated with the ability of Env to enter using CCR5 in conjunction with very low-to-absent CD4 (6, 11, 22, 65, 77). The two-step entry process that requires CD4 binding prior to co-receptor engagement is known to render virus more resistant to antibody neutralization (7, 25, 28, 39) suggesting that immune forces may limit emergence of macrophage-tropic variants *in vivo*. Indeed, infection of macaques using cloned CD4-independent or macrophage-tropic SIV results in either transient, non-progressive infection or dissemination to the central nervous system (CNS), without immunodeficiency (49, 50). In the CNS, unlike in the systemic compartment, myeloid lineage cells are the dominant targets of infection and macrophage-tropic viruses are common, where a paucity of CD4+ T cell targets combined with the immune-privileged nature of the CNS is thought to contribute to the unique tropism pattern in the brain (20, 38, 60, 62, 72, 79). Macrophage-tropic variants have also been reported to arise during very late-stage disease in some studies (47) or following rapid profound virus-induced CD4+ T cell depletion (31, 32). However, the forces that maintain strict CD4 dependence and regulate tropism in the systemic compartment have not been explicitly demonstrated *in vivo*, nor have mechanisms that might enable widespread systemic macrophage infection.

Recently we showed that extensive macrophage infection developed in rhesus macaques (RM) experimentally depleted of CD4+ T-cells from the blood and lymph nodes prior to infection with SIVmac251, and variants capable of using human CCR5 in the absence of CD4 emerged in plasma during chronic infection (58). Depleted animals also experienced high chronic viral load and progressed rapidly to AIDS. Thus, CD4+ T cell depletion prior to infection resulted in adaptation of SIV to decreased CD4-dependence and macrophage tropism *in vivo,* and provides a model in which the forces that regulate tropism during infection can be elucidated.

In this study we show that very efficient CD4-independent use of rhesus macaque CCR5 arose in CD4+ T cell-depleted macaques during the post-peak phase of infection and is associated with sensitivity to neutralization by control SIV+ plasma, but not by autologous plasma. A key distinguishing feature was the presence of antibody activity in control RM plasma, but not CD4+ T cell depleted RM plasma, that neutralized control Envs if pre-incubated with sCD4, but not without sCD4 exposure. In the absence of this CD4-inducible neutralization activity, and with a paucity of CD4+ T cell targets in CD4+ T cell depleted animals, circulating SIV Envs acquired 2 amino acid changes in gp120 that impart CD4-independent entry through CCR5. Thus, CD4+ T cells contribute to the production of antibodies targeted to conserved Env conformations that normally are only induced by CD4 engagement. These antibodies were associated with strict CD4 dependence of Env, maintenance of CD4+ T cell targeting, and restrained tropism for CD4-low macrophages *in vivo*.

Methods

Ethics statement

All animal experimentation was conducted following guidelines established by the Animal Welfare Act and the NIH for housing and care of laboratory animals and performed in accordance with Institutional regulations after review and approval by the Institutional Animal Care and Use Committees (IACUC) at the Yerkes National Primate Research Center (YNPRC) or the Tulane National Primate Research Center (TNPRC). Studies were also reviewed and approved by the University of Pennsylvania IACUC.

SIV_{Mac} envelope clones, mutagenesis, and pseudotyped virus

The *env* genes from d11 and d42 SIV-infected rhesus macaque plasma were PCR amplified using a procedure for endpoint diluted single genomes as previously described (58). Mutations were introduced into SIV envelopes using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Inc., Santa Clara CA) and verified by sequencing. SIVmac239 and SIVmac251.6 Env clones were used as reference controls. Luciferase expressing pseudotyped viruses carrying SIV Envs on an HIV-1 backbone were generated as previously described (18), and were treated with DNAse prior to use in infection.

Virus infection and receptor function assays

Human 293T cells were maintained in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum (D10 media). Entry of pseudotyped viruses was assayed in 293T target cells expressing CD4 and CCR5 or CCR5 alone. Target cells were transfected with plasmids encoding rhesus macaque CCR5 with or without rhesus macaque CD4, using pcDNA 3.1 as "filler" plasmid (66). Cells transfected with pcDNA 3.1 only were used as a negative control. Target cells ($2x10^4$ per well in 96-well plates) were infected with pseudotyped viruses (20 ng p24 antigen) by spinoculation at 1200xg for 2 hrs. Cells were then incubated for 72 hrs at 37°C and infection quantified by measuring luciferase content in cell lysates as previously described (66). All data represent a minimum of 3 independent replicate experiments.

Env structural mapping

The SIV Env core structure (3) was visualized with Jmol (35), and predicted CD4-binding residues were highlighted based on homology with HIV as previously described (3, 45). Residues highlighted were as follows: HIV-CD4 direct contact model (more stringent) included residues 107, 293-295, 297, 381, 384, 386, 387, 438-443, 468-472, 479, 482-484; HIV-CD4 loss of solvent accessibility (less stringent) in addition to the above included residues 105, 106, 108, 272, 292, 296, 380, 481, 485-487. Residue 84 was additionally highlighted.

Neutralization assays

Monoclonal antibodies 7D3, 36D5, 17A11, 171C2, and 35C11 have been previously described (14). Plasma from d11 and d56 animals in this study or pooled plasma from two chronically SIVmac251-infected macaques (kindly provided by P. Marx) were heat-inactivated at 56°C for 1 hour. Soluble CD4-183 (sCD4; Pharmacia, Inc.) was obtained from the NIH AIDS Reference Reagents Program. Neutralization and sCD4 exposure assays were performed as previously described (55), (7) with modifications. Pseudotyped virus was mixed with sCD4 in D10 medium to achieve a concentrations of virus of 0.8 ng/µl of viral p24 antigen and 50 ng/µl sCD4, and incubated at 37°C for 1 hr. Aliquots (25 µL) of the virus/sCD4 mixture were then transferred to wells of a 96-well v-bottom plate, and mAb or plasma was added to final concentrations as noted. The mixture was incubated at 37°C for an additional 1 hr, after which 25 µL was added to target cells (293T cells expressing rmCD4 and rmCCR5) plated in 96-well flat bottom plates (2x104 cells in 100 µL medium). Cells were subject to spinoculation as above, incubated for 72 hours, and lysed for measurement of luciferase activity as an indication of infection. Neutralization was determined based on the percentage of infection in the absence of antibody (using virus incubated with or without sCD4, as appropriate, for the no-antibody treatment). Neutralization data represent a minimum of at least three independent replicate experiments.

Plasma binding antibody.

Binding antibodies to SIV Env protein were assessed by ELISA as described previously (83). Briefly, 96-well plates were coated with 1 μg/ml SIVmac239 gp140 protein (Immune Tech, New York, NY) by overnight incubation at 4°C, then incubated with with serial dilutions of plasma for 2 h, followed by detection using HRP-conjugated goat anti-monkey IgG (1:10,000 dilution, AlphaDiagnostic, Owings Mills, MD) and TMB substrate. A dilution series of SIVmac239 gp140-specific IgG standard with known concentration (generously provided by P. Kozlowski, Louisiana State University) was included in parallel.

Nucleotide accession numbers

The sequences of the SIV *env* clones described in this study have been submitted to GenBank under the following accession numbers: KF276976 to KF277047.

Results

CD4-independent SIV Env emerges in CD4+ T cell-depleted rhesus macaques

SIVmac251 infection of animals following antibody-mediated depletion of CD4+ T cells from peripheral blood, lymph node and bone marrow, but not mucosal sites, resulted in extensive *in vivo* macrophage infection, high post-peak viremia and rapid disease progression (58). We previously showed that Env clones derived from post-peak (d42) plasma of these CD4-depleted SIV-infected rhesus macaques were able to enter target cells expressing human CCR5 in the absence of CD4, reaching levels approximately 50% of that in cells expressing CCR5 together with CD4 (58). Here we asked whether CD4-independent variants were present in plasma at an earlier time point, during peak viremia (d11), and if CD4-independence was maintained using species-matched rhesus macaque CCR5 and CD4 (65). SGA-derived Env clones were generated from 4 depleted and 2 control animals, and 6 Envs from each animal at each time point (72 total Envs) and were used to generate a panel of Env pseudotyped reporter viruses. Pseudotyped viruses were tested for their ability to enter 293T cells transfected with CCR5 in the presence or absence of CD4 (**Figure 3.1**).

Envs at d11 from both the CD4-depleted and control animals required CD4 for efficient entry, both with rhesus CCR5 (**Figure 3.1A**) and with human CCR5 (data not shown). A few Envs mediated low level entry in the absence of CD4, and while this appeared to be slightly greater among CD4-depleted than control d11 Envs, it was variable and did not reach statistical significance. In contrast, nearly all d42 Envs from the CD4-depleted group mediated robust CD4-independent entry (**Figure 3.1B**). In fact, using these rhesus-derived receptors, most clones were essentially indifferent to the presence of CD4 (mean CD4-independent entry was 94±7% compared with CD4-dependent entry). In contrast, d42 Envs from the control animals all required CD4 for entry through rhesus CCR5. These data indicate that in animals depleted of CD4+ T cells, CD4-independent variants emerged *in vivo* during the post-peak phase of SIV infection and dominated in plasma during chronic infection.

Emergence of SIV Env D470N and E84K confers CD4-independence

We next asked what molecular determinants conferred the CD4-independent phenotype that arose *in vivo* in multiple CD4-depleted animals. We aligned the amino acid sequences of the 72 d11 and d42 SGA Envs, and found that 3 amino acid changes in gp120, G62N, E84K, and D470N (based on SIVmac239 numbering (3)) were enriched in CD4-depleted RM Envs compared to controls (**Figure 3.1**). E84K and D470N were found exclusively in d42 CD4-depleted RM Envs, and were both significantly associated with CD4-independent entry ($p<0.001$; McNemar test for associations). In contrast, G62N was found in CD4-depleted RM Envs at both d11 and d42 time points, as well as in a few control Envs, and was not significantly associated with CD4-independent entry ($p=0.14$). One partially CD4-independent Env clone, RUf6.d42.6.1, carried neither E84K nor D470N, but did contain D385N, G383R, and D180N, which had each been previously described in CD4-independent SIV Envs (68). Only one d42 Env from a CD4-depleted animal did not mediate appreciable CD4-independent entry (RZj5.D42.9.2), and did not carry D470N, E84K, or any previously-described polymorphisms associated with CD4-independence. G62N, E84K, and D470N have not been explicitly characterized in previous studies, but an analysis of SIVmac sequences revealed that G62N has ~5% frequency within SIVmac251 viral stocks (9, 37, 74, 75), while D470N and E84K are extremely rare and present mainly in *in vivo*-passaged SIVmac251-related isolates (9, 17, 46, 67, 76). Of note, all instances of G62N, E84K, and D470N that arose in these animals were the result of G-to-A nucleotide changes (**Figure 3.1C**).

To determine whether G62N, E84K, or D470N regulate CD4-independent entry, we introduced each of these mutations, as well as E84K+D470N, into a representative control Env, RZu4.d42.7.1 (**Figure 3.2A**). G62N had negligible effects on RZu4.d42.7.1 entry and did not enable CD4-independence. E84K and D470N each severely attenuated total entry, although the signal remained significantly above assay background and represented bona-fide Env-mediated entry. Despite the overall low entry levels, however,

D470N entry was similar whether or not CD4 was present, while E84K remained CD4-dependent. When E84K and D470N were simultaneously introduced into RZu4.d42.7.1, robust entry was restored and was equivalent in the presence and absence of CD4. Thus, D470N appears to play a central role in CD4 independence but results in attenuation, while E84K restores overall efficiency of entry in this Env background.

We also introduced the reciprocal mutations, N62G, K84E, N470D, and K84E+N470D into a representative CD4-independent Env, RPe6.d42.7.2 (**Figure 3.2B**). Similarly, N62G had little effect. K84E severely attenuated overall entry, and also modestly reduced relative entry independent of CD4. N470D also reduced CD4-independent entry, although again only modestly (76% reduction). However, the combination of mutations in K84E+N470D restored overall entry to that of the parental Env, and dramatically reduced CD4-independent entry (95% reduction). Thus, in the CD4-independent background, both N470D and E84K contribute to CD4-independence and interact to maintain overall efficiency of entry.

To see if the effects of G62N, E84K and D470N in these 2 representative Envs were generalizable to other viruses from additional animals, we introduced D470N and E84K+D470N into a larger panel of CD4-dependent Envs from the animals in this study, including Envs from d11 control, d11 CD4-depleted and d42 control animals (n=7). We also tested the reciprocal mutations in an expanded panel of CD4-independent Envs from d42 CD4+ T cell-depleted animals (n=4). Similar to the representative Envs, D470N, and more potently D470N+E84K, regulated CD4-independent entry of multiple Envs, including CD4-independent variants that arose independently in different animals (**Figure 3.2C, D, S3.1**).

Structural significance of CD4-independence mutations that arise in vivo

Using Jmol (35), we mapped predicted CD4-binding residues onto the previously-described core structure of SIVmac32H gp120 (3) based on homology with known HIV gp120 binding residues (3, 45), and highlighted residues E84 and D470 (**Figure 3.2E**)

(the published core structure of SIV gp120 is N-terminally truncated and excludes G62). This analysis indicates that D470 is a predicted CD4 contact residue residing in a cavity flanked by other CD4 contact residues, while E84 is proximal to the predicted gp120 CD4-binding surface, situated on the first alpha helix and at a distance of 13.3Å to 19.2Å from D470. In addition, owing to its linear position upstream from a threonine residue in SIVmac251 gp120, D470N introduces a potential N-linked glycosylation site (PNLG) within a predicted CD4-binding pocket (**Figure 3.2F**).

To address whether the PNLG introduced into SIV Env by D470N was important in CD4-independent entry (**Figure 3.2F**), we introduced D470Q and N470Q changes into our representative CD4-dependent and CD4-independent Envs (**Figure 3.2A, B**), as well as the extended panel of Envs (**Figure 3.2C, D, S3.1**). Q was chosen as a conservative change compared to N that would maintain the charge but abrogate glycosylation, as described previously (63). D470Q in control Envs did not substantially increase CD4-independent entry (**Figure 3.2A, C, S3.1**), and N470Q in CD4-independent Envs reduced CD4-independence similarly to N470D (**Figure 3.2B, D, S3.1**). These findings suggest that the PNLG in this deep pocket on the CD4-binding surface is an important component of CD4-independent entry.

Constitutive sensitivity of CD4-independent Envs to mAb neutralization

Since Envs from CD4+ T cell-depleted animals were capable of utilizing CCR5 for entry in the absence of CD4, we performed a neutralization assay using the Env monoclonal antibody (mAb) 7D3 (**Figure 3.3, S3.2**), which targets the predicted CD4-induced (CD4-i) binding-binding site of SIV Env (14, 81, 82). As shown in Figure 3.3A, control Env RZu4d11.2.1 was resistant to neutralization by 7D3, but became sensitive if pre-incubated with sCD4. This confirmed that 7D3 targets a CD4-inducible (CD4-i) neutralizing epitope. In contrast to the control Env, the CD4-independent Env RUt5d42.4.3 was potently neutralized by 7D3 regardless of sCD4 pre-incubation, suggesting that this Env constitutively exposes the 7D3 CD4-i neutralization epitope. We then tested the extended panel of d42 CD4-independent Envs that arose independently in

multiple CD4-depleted animals and confirmed 7D3 neutralizing sensitivity for all, as well as the other CD4-dependent Envs from d11 or control animals, which were all resistant but became sensitive following sCD4 exposure (**Figure S3.2** and data not shown).

We then asked whether the same molecular determinants that regulate CD4-independent entry also determine sensitivity to neutralization by 7D3. As shown in **Figure 3.3B**, introduction of D470N and E84K into control Env RZu4d11.2.1 rendered the virus sensitive to 7D3 neutralization in the absence of sCD4 triggering. When the reciprocal mutations, N470D and K84E, were introduced into the CD4-independent Env RUt5d42.4.3, it rendered this Env resistant to 7D3 neutralization unless first exposed to sCD4 (**Figure 3.3C**). Thus, E84K+D470N are principally responsible for sensitivity to neutralization by 7D3.

To determine whether these amino acids were determinants of CD4-inducible antibody neutralization among other Envs from this panel, and the role of each residue specifically, we introduced single mutations at positions 84 and 470 into our extended panel of CD4-dependent and independent Envs. As shown in **Figure 3.4**, introduction of D470N into CD4-dependent Envs conferred sensitivity to 7D3, while E84K alone did not. Furthermore, introduction of D470Q into the CD4-dependent Envs did not confer 7D3 sensitivity, implicating glycosylation at the N470 PNLG site. In contrast, neither N470D nor K84E alone made CD4-independent Envs resistant to 7D3 (with the exception of Ruf6.d42.2.5, which already carries E84 and was rendered 7D3-resistant by N470D alone; **Figure 3.1B** and **S3.2**). Introduction of both N470D and K84E together, however, led to 7D3 resistance for all Envs tested. Thus, both D470N and E84K contribute to CD4-i antibody neutralization sensitivity.

We then tested a panel of additional SIV Env monoclonal antibodies with differing target epitopes (14) for the ability to neutralize control macaque CD4-dependent Envs without and with sCD4 pre-exposure, and neutralization of these Envs carrying E84K, D470N and D470Q mutations (**Figure 3.5**). Both a V3-specific mAb (**Figure 3.5A, B**) and an

anti-gp120 conformational epitope mAb (**Figure 3.5C, D**) showed patterns similar to 7D3, in which Env was neutralized only if pre-exposed to sCD4, or if mutated to carry D470N (+/- E84K) but not D470Q. In contrast, a V1/V2-specific mAb was poorly neutralizing regardless of sCD4 pre-treatment, but did inefficiently neutralize the mutant Envs (**Figure 3.5E, F**), whereas a gp41 mAb failed to neutralize either the sCD4-treated or mutant Envs (**Figure 3.5G, H**). Thus both sCD4 pre-triggering and the D470N mutation that emerged in CD4-depleted RM render SIV Envs sensitive to antibodies with several distinct, but not all, epitope targets.

CD4-independent Envs are sensitive to neutralization by CD4-i activity in SIV+ plasma

Previous studies have shown that commonly used SIV strains, such as mac239, mac251, and SM-E660 are highly resistant to neutralization by most SIV+ plasma (2, 5, 26, 58). However, it has also been shown that HIV+ plasma contains antibodies that target broadly conserved CD4-i binding-binding structures and can neutralize viruses if pre-triggered with sCD4 (7). Therefore, we asked if SIV+ plasma contain antibodies with similar activity, and whether these SIV Envs could be neutralized by pooled plasma from chronically SIVmac251-infected macaques (**Figure 3.6**).

As expected, CD4-dependent control Env RZu4d11.2.1was resistant to plasma neutralization (**Figure 3.6A**). However, pre-incubation with sCD4 rendered this Env sensitive to neutralization, confirming that SIV+ plasma contains CD4-i neutralizing activity. In contrast, CD4-independent Env RUt5d42.4.3 from a d42 CD4-depleted animal was sensitive to neutralization regardless of CD4 pre-triggering. The D470N mutation, either alone or in combination with E84K, rendered the control CD4-dependent Env sensitive to neutralization by SIV+ plasma without sCD4 pre-exposure (Figure 3.6B and data not shown). Similarly, K84E/N470D rendered the CD4-independent Env resistant to plasma neutralization unless pre-exposed to sCD4 (**Figure 3.6C**), while, like mAb 7D3, neither N470D nor K84E alone rendered CD4-independent Envs plasma neutralization-resistant (data not shown).

To determine whether this sensitivity to SIV+ plasma neutralization was common among the viruses that arose in CD4-depleted animals, we tested a larger panel of Envs from our study, along with SIVmac239 and SIVmac251.6 reference Envs for comparison. As shown in **Figure 3.7A**, d11 Envs from both control RM (solid lines) and CD4+ T cell-depleted RM (dotted lines) were resistant to pooled plasma neutralization, but became sensitive if first exposed to sCD4. SIVmac239 and SIVmac251.6 showed a similar pattern of CD4-induced neutralization sensitivity (**Figure 3.7A**). At d42, all Envs from CD4+ T cell-depleted animals were sensitive to plasma neutralization regardless of sCD4 pre-treatment (**Figure 3.7B**). Control animal d42 Envs were resistant to plasma neutralization, but became more sensitive with sCD4 pre-treatment (**Figure 3.7B**). Some Envs exhibited a drop in infectivity at the highest concentrations of plasma, but this was also observed with uninfected control plasma (data not shown) and likely due to nonspecific effects of high concentration plasma as has been previously described (55).

Thus, the CD4-independent Envs that arose in CD4-depleted macaques are constitutively sensitive to CD4-inducible neutralization by SIV+ plasma, which targets Env conformations regulated by D470N and E84K. These findings also suggested that this antibody activity generated during normal SIV infection could restrict the emergence of CD4-independent variants *in vivo*.

CD4+ T cell-depleted macaques lack robust plasma CD4-inducible neutralization activity

Both the CD4+ T cell-depleted and control animals in this study seroconverted by western blot and ELISA (58). Having observed the marked sensitivity to neutralization by SIV+ plasma of CD4-independent Envs that emerged in CD4+ T cell-depleted animals, we hypothesized that these animals might lack CD4-I neutralization activity. To address this, we used chronic infection plasma samples (d56) from the CD4+ T cell-depleted and control animals, and tested their ability to neutralize autologous and heterologous Envs (**Figure 3.8**). As expected, plasma from control animals did not

neutralize autologous d11 or d42 Envs in the absence of sCD4, but did neutralize Envs pre-exposed to sCD4, confirming the presence of CD4-i activity (**Figure 3.8A-D**; black lines). In addition, plasma from control animals also neutralized heterologous CD4-independent Envs from d42 CD4-depleted animals even in the absence of sCD4 (**Figure 3.8E-H**; black lines). In sharp contrast, plasma from CD4-depleted animals showed markedly reduced ability to neutralize heterologous CD4-dependent Envs (**Figure 3.8A-D**; red lines), autologous d11 CD4-dependent Envs (Figures 8E, G; red lines) or autologous d42 CD4-independent Envs (**Figures 8F, H**; red lines), regardless of sCD4 treatment (although very weak neutralization at high plasma concentrations cannot be completely excluded). Thus, plasma from CD4+ T cell-depleted animals in which CD4-independent viruses emerged lacks robust CD4-i neutralizing activity. Plasma from earlier time points, d14 and d25, had no detectable CD4-i neutralizing activity (**Figure S3.3**), indicating that CD4-i neutralizing antibody activity emerges between days 25 and 56 after infection in normal SIV infection.

Finally, we asked if total anti-Env IgG was reduced in CD4 T cell-depleted animals' plasma, using an ELISA against SIVmac230 gp140. As shown in **Figure 3.9**, Env-binding antibodies were present in all macaques, but titers were significantly lower in the CD4 T cell-depleted animals. Thus, CD4 T cell depletion results in global reduction of antigen-specific antibody production, consistent with the poor CD4-inducible neutralization capacity of CD4 T cell-depleted plasma.

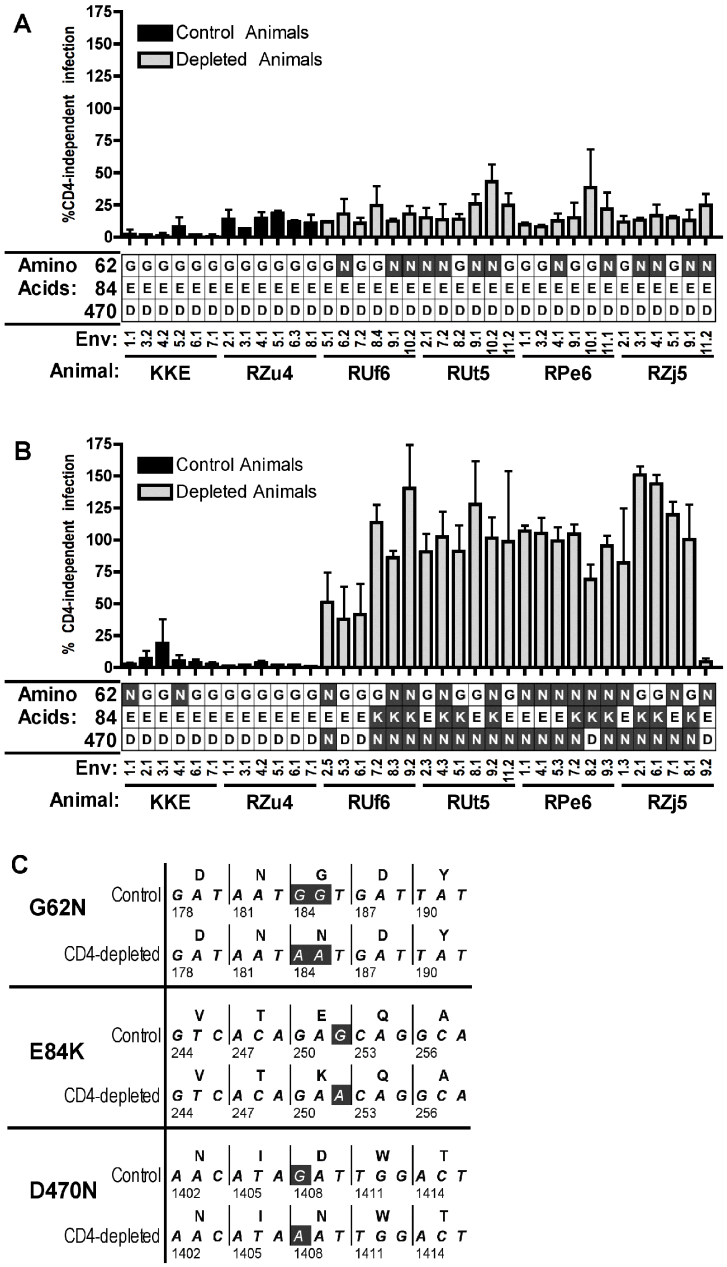

Figure 3.1. Envs from CD4+ T cell-depleted macaques mediate CD4-independent entry. Env clones obtained by single genome amplification (SGA) from plasma at d11 **(A)** and d42 **(B)** after infection from 2 control animals (solid bars) and 4 CD4-depleted animals (open bars) were used to generate HIV virions pseudotyped with SIV Env and containing a luciferase reporter. These virions were used to infect 293T cells transfected with rmCCR5, with and without rmCD4. Values indicated are expressed as percent entry in the absence versus presence of CD4 on target cells (means ± SEM of triplicate experiments). Predicted amino acids at sites 62, 84 and 470 (based on SIVmac239 numbering) are indicated below the clone number, and are also shown in the context of amino acid and nucleotide sequences **(C)** for a representative Env from a control animal (RZu4.d42.7.1) and from a CD4-depleted animal (RPe6.d42.7.2).

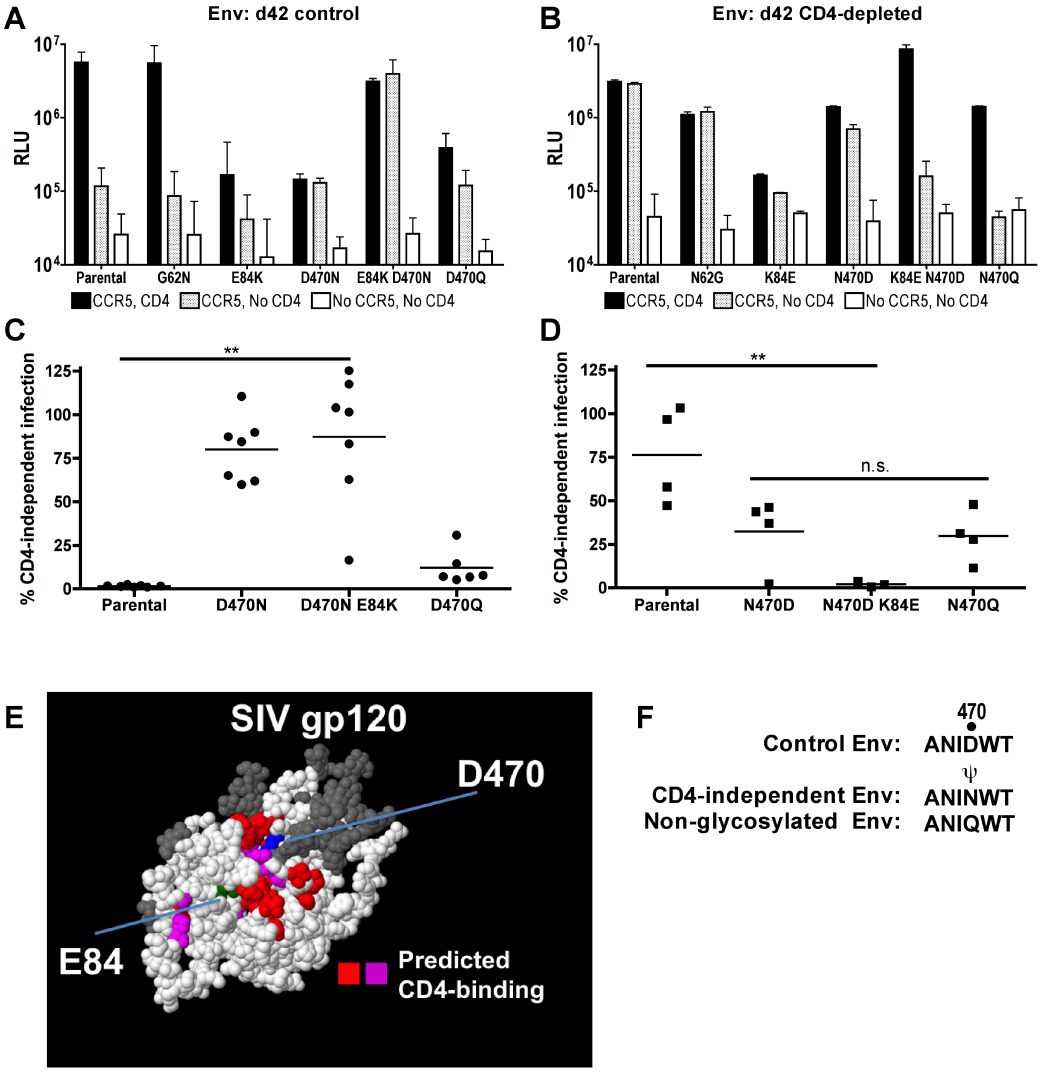

Figure 3.2. D470N and E84K regulate CD4-independent entry. (A, B) Mutations G62N, E84K, D470N, and D470Q were introduced into a representative CD4-dependent Env (RZu4.d42.7.1; panel **A**), while N62G, K84E, N470D, and N470Q were introduced into a representative CD4-independent Env (RUt5.d42.4.3; panel **B**). Columns and error bars indicate the mean and standard error, respectively, from 3 independent experiments. The mutant Envs were tested for their ability to mediate infection of 293T cells transfected with rmCCR5, with and without rmCD4, indicated by relative light units (RLU). **(C, D)** The same mutations were introduced into an extended panel of CD4-dependent (**C**) and CD4-independent (**D**) Envs, and CD4-independent entry is shown as a percentage of entry in the presence of CD4, with each individual Env clone indicated as a point and each horizontal line indicating the mean percent entry among the various clones. Mean percent CD4-independent entry was compared using a pair-wise T-test. Note that in one CD4-independent Env (RUf6 d42 2.5), E84 was present in the parental form so only N470D was introduced. **(E)** The locations of E84 (green) and D470 (blue) are shown based on a core structure of SIVmac32H (3), with the predicted Env-CD4 binding site indicated based on homology with HIV-HxB Env-CD4 direct contact (red) and loss of solvent accessibility (magenta) residues. Peptide residues are colored white and carbohydrates are colored grey. **(F)** The potential N-linked glycosylation site introduced by D470N and non-glycosylated mutant D470Q are shown.

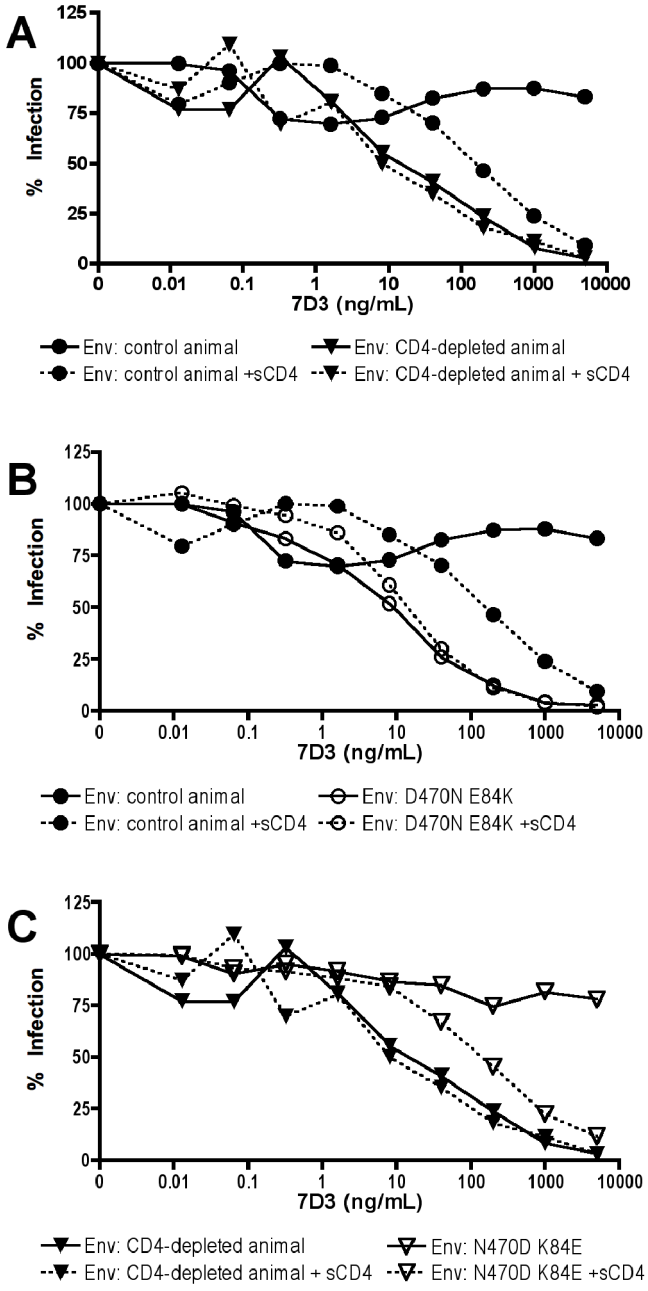

Figure 3.3. A CD4-inducible epitope monoclonal antibody neutralizes CD4 independent Envs. Pseudotype viruses carrying a representative CD4-dependent Env from a control animal (RZu4.d11.2.1) and CD4-independent Env from a CD4-depleted animal (RPe6.42.7.2) were incubated with or without sCD4 followed by various concentrations of monoclonal antibody 7D3, prior to infection of 293T cells transfected with rmCD4 plus rmCCR5 (**A**). The parental and E84K/D470N mutant forms of the CD4-dependent Env were similarly tested for neutralization by 7D3 **(B)**. The parental and K84E/N470D mutant forms of the CD4-independent Env were tested for 7D3 neutralization **(C)**.

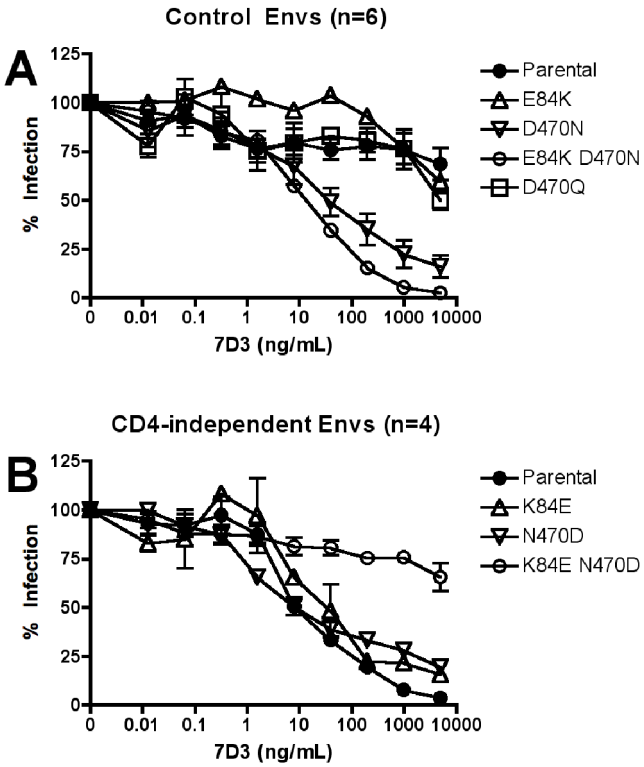

Figure 3.4. Molecular determinants of 7D3 neutralization are consistent across the extended panel of Envs. **(A)** CD4-dependent Envs from either control animals or d11 CD4-depleted animals (n=6) were mutagenized to introduce E84K, D470N, or both, or non-PNLG mutant D470Q, and then tested for neutralization by monoclonal antibody 7D3. Data show the means ± SEM of all Envs analyzed. **(B)** Means ± SEM for 7D3 neutralization of CD4-independent Envs from CD4-depleted d42 animals and Env mutants is shown.

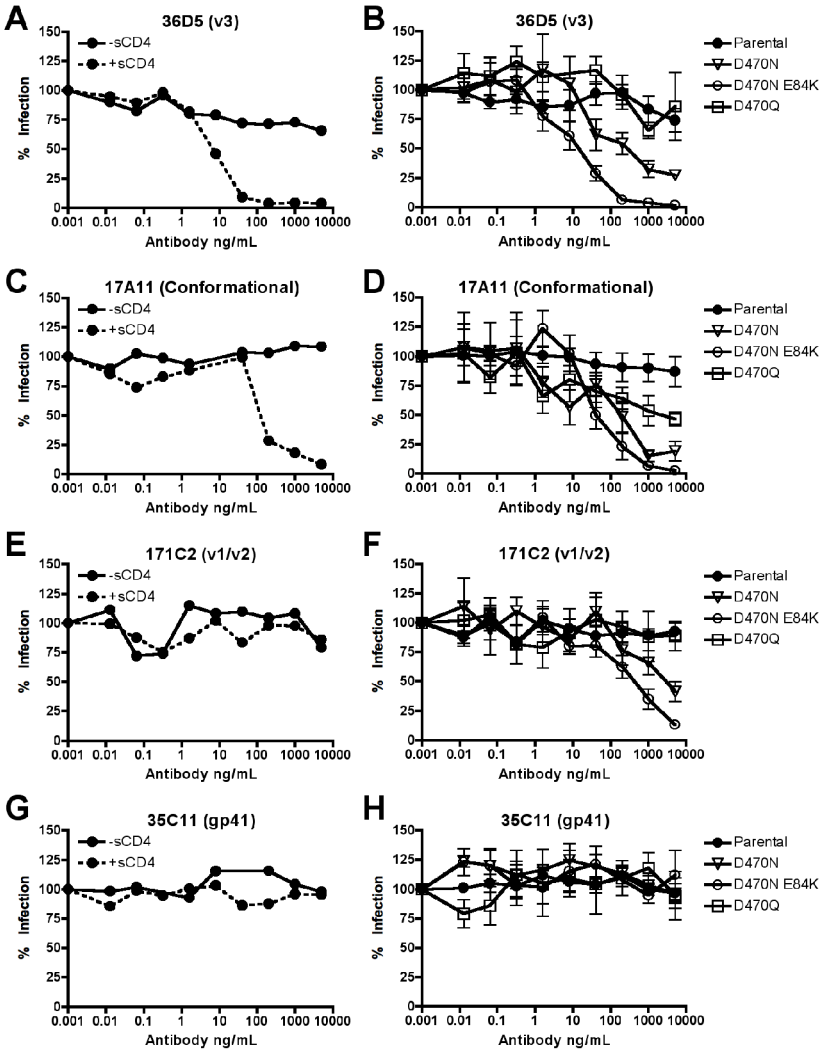

Figure 3.5. CD4-inducible epitope targeted monoclonal antibodies neutralize CD4-independent Envs (A, C, E, G): Representative CD4-dependent (RZu4.d11.2.1) Env pseudotyped virus was incubated with or without sCD4 followed by various concentrations of monoclonal antibodies with indicated specificities prior to infection of 293T cells transfected with rmCD4 plus rmCCR5. **(B, D, F, H):** The parental and E84K/D470N mutant forms of multiple CD4-dependent Envs were similarly tested for neutralization and the result is expressed as the mean value of the various clones tested ± SEM (n=4). 17A11 is believed to target a conformational epitope that overlaps both the CD4- and CCR5-binding site.

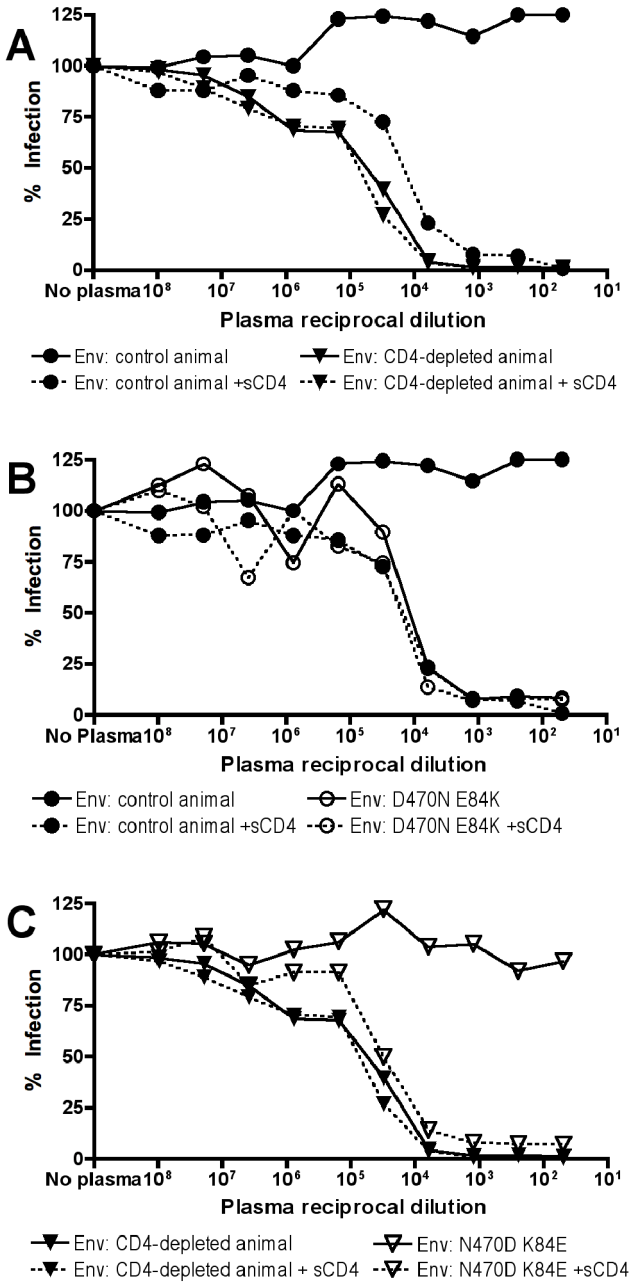

Figure 3.6. Pooled SIV+ plasma neutralizes CD4-independent Envs and CD4-triggered CD4-dependent Env. (A) Representative CD4-dependent (Rzu4.d11.2.1) and CD4-independent (Rpe6.42.7.2) Env pseudotyped viruses were incubated with or without 50 μg/mL sCD4, then incubated with serial dilutions of SIV+ plasma pooled from two chronically SIVmac251-infected macaques (not from this study). Viruses were then used to infect 293T cells transfected with rmCD4 plus rmCCR5. (**B**) The parental and E84K/D470N mutant forms of a CD4-dependent Env were tested for plasma neutralization. **(C)** The parental and K84E/N470D mutant forms of a CD4-independent Env were tested for plasma neutralization.

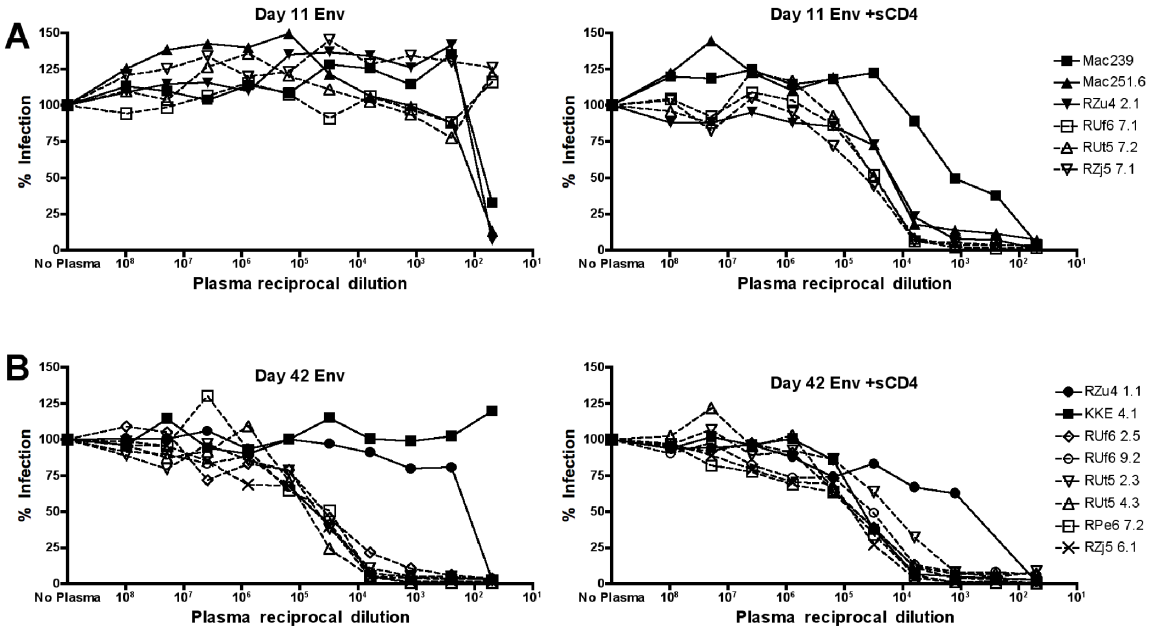

Figure 3.7. SIV+ plasma neutralization of the extended panel of CD4-independent and control Envs and prototype SIV Envs. Pseudotype viruses carrying Envs from d11 animals (**A**) and d42 animals (**B**) were pre-incubated without **(left)** or with **(right**) sCD4, exposed to various dilutions of pooled SIV+ plasma, and were used to infect 293T cells expressing rmCD4 and rmCCR5. Envs from CD4-depleted animals are represented by dotted lines, and Envs from control animals are represented by solid lines. Reference Envs from SIVmac239 and SIVmac251.6 were tested in parallel and are included in panels A.

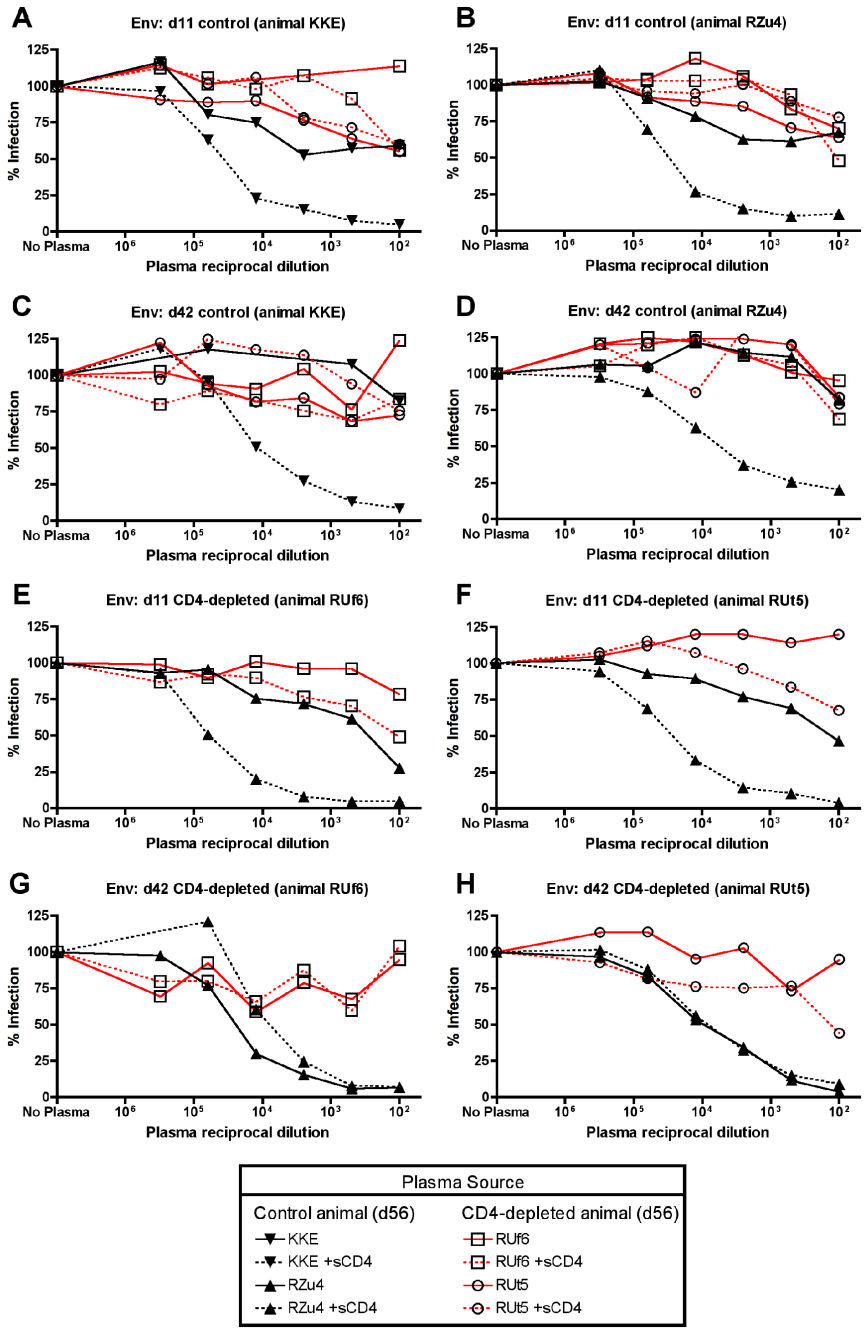

Figure 3.8. Reduced CD4-inducible SIV Env neutralization activity in plasma from CD4+ T cell-depleted animals. Chronic infection plasma (d56) from control animals (KKE, RZu4; black lines) or CD4-depleted animals (RUf6, RUt5; red lines) was used in neutralization studies with pseudotyped viruses carrying Envs from control animals (panels **A-D**) or CD4-depleted animals (**E-H**), with or without pre-incubation with sCD4. Black lines in panels A-D represent neutralization by autologous non-CD4-depleted plasma, while red lines represent neutralization by heterologous plasma from CD4-depleted animals. Red lines in panels E-H represent neutralization by autologous CD4-depleted plasma, while black lines represent neutralization by heterologous non-CD4-depleted plasma.

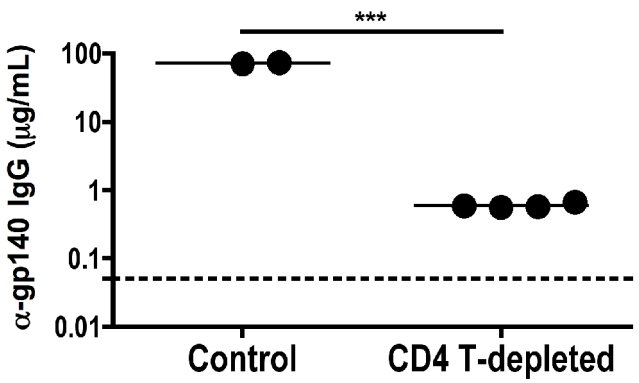

Figure 3.9. Reduced antigen-specific IgG in CD4 T cell-depleted animals. Total anti-gp140 plasma IgG from control and CD4 T cell-depleted macaques at d. 42 post-infection was measured by ELISA. The mean anti-gp140 titer differed significantly between control (n=2) and CD4-depleted (n=4) animals by T-test. The assay threshold of detection is indicated by a dotted horizontal line. *Assay performed by Peng Xiao, Emory University*.

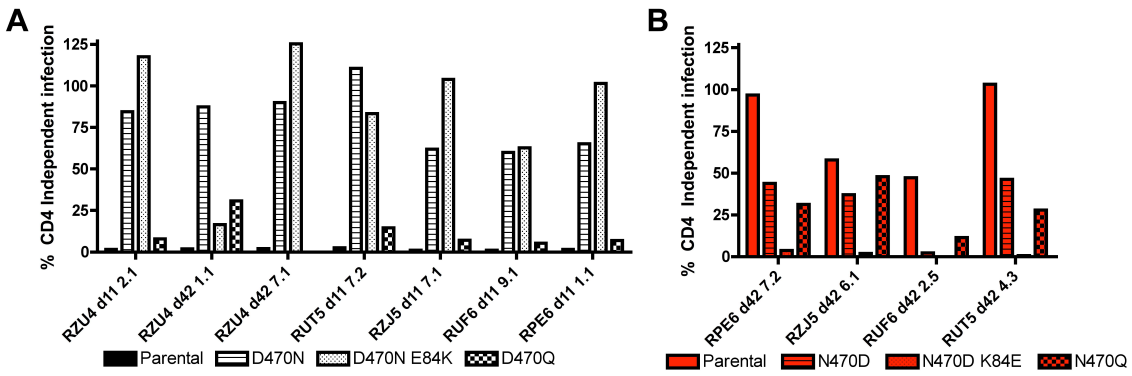

Figure S3.1. E84K and D470N confer CD4-independent entry in multiple parental Envs. Mutations E84K, D470N, and D470Q were introduced into multiple CD4-dependent Envs **(A)**, while K84E, N470D, and N470Q were introduced into multiple CD4-independent Envs **(B)**. The mutant Envs were tested for their ability to mediate infection of 293T cells transfected with rmCCR5, with and without rmCD4, and CD4-independent entry is shown as a percentage of entry in the presence of CD4. Note that in one CD4-independent Env (RUf6 d42 2.5), E84 was present in the parental form so only N470D was introduced.

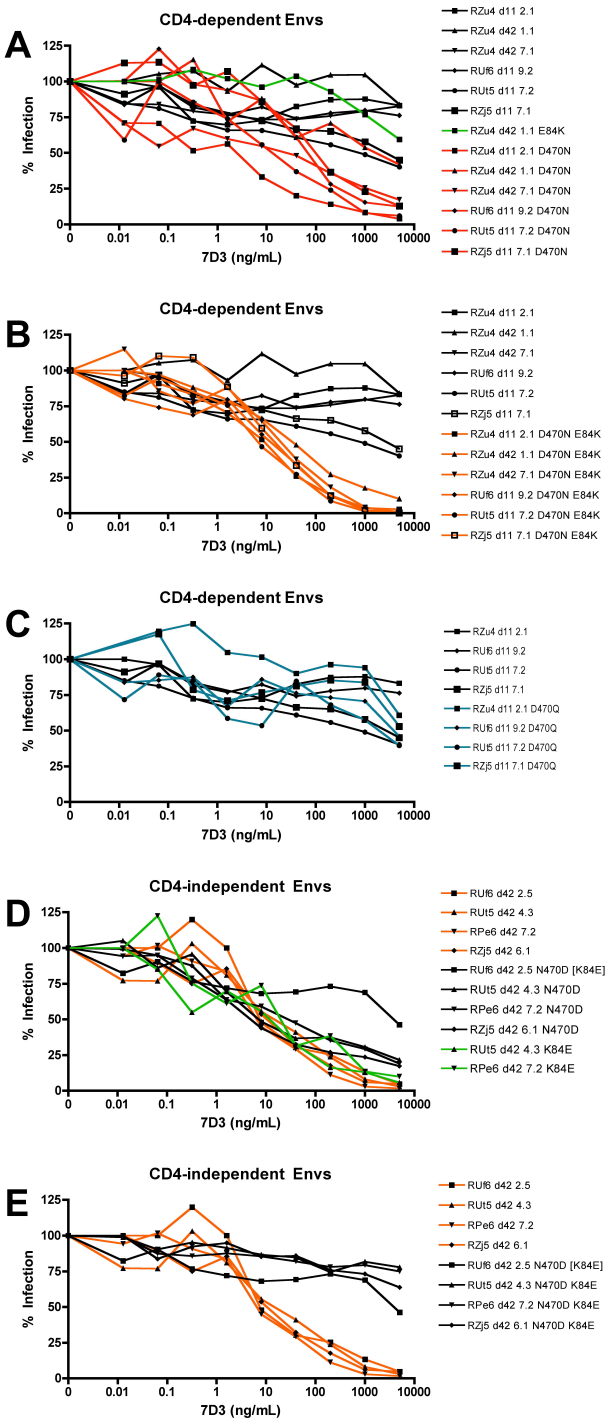

Figure S3.2. Molecular determinants of 7D3 neutralization are consistent across the extended panel of Envs. **(A, B, C)** CD4-dependent Envs from either control animals or d11 CD4-depleted animals were mutagenized to introduce E84K, D470N **(A)**, or both **(B)**, or non-glycosylated mutant D470Q **(C)**, and then tested for neutralization by monoclonal antibody 7D3. (D, E) CD4-independent Envs from day 42 CD4-depleted animals were mutagenized to introduce K84E, N470D **(D)**, or both **(E)**.

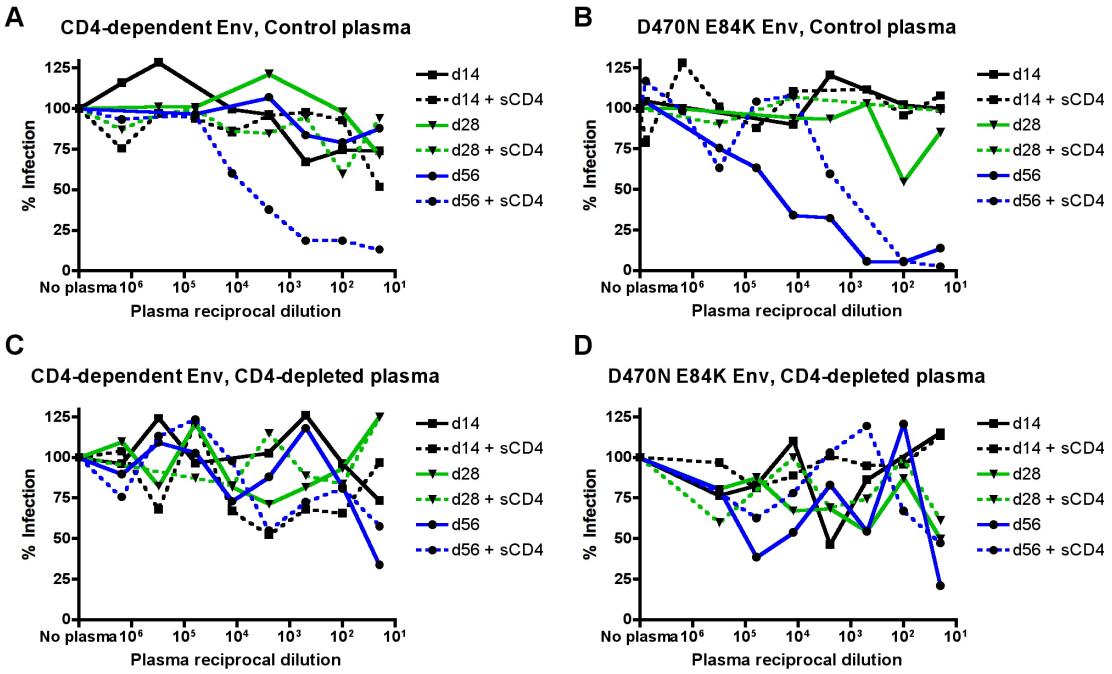

Figure S3.3. Plasma from control infected animals at d14 and d25 post-infection lacks CD4-inducible neutralizing activity. Plasma from control (**A,B**) or CD4 T-depleted (**C,D**) animals was tested for neutralizing activity against a CD4-dependent parental Env (**A,C**) or its D470N E84K mutant (**B,D**). Plasma from d14 (black), d25 (green) and d56 (blue) was tested with or without pre-treatment of the target virus with sCD4.

Discussion

SIV infection of rhesus macaques that were largely depleted of CD4+ T cells, as described here and in (58), reveals that CD4-independent viral variants emerge and consistently dominate circulating virus in this setting. Emergence of CD4-independent Env variants corresponded with extensive *in vivo* infection of macrophages (58), a relationship which has been previously addressed *in vitro* (10, 12, 64, 65) and in the CNS or lung compartments (16, 21, 23, 30, 41, 53, 61). Here we demonstrate for the first time the factors governing CD4 dependence and target cell tropism in the systemic compartment *in vivo*. Animals that were not CD4+ T cell-depleted before infection produced a robust antibody response which likely contributes to maintaining strict CD4-dependence and CD4+ T cell tropism. Such antibodies, which are non-neutralizing against control virus unless pre-triggered with sCD4, are also abundantly present in chronic HIV-1 infection (7). The absence of such antibodies in the CD4+ T cell-depleted animals was associated with amino acid changes in Env gp120 that confer entry through CCR5 independently of CD4, enabling a shift in tropism to macrophages. These findings highlight the critical role that CD4-inducible neutralizing antibody activity has *in vivo* in shaping Env function, regulating CD4 use, and defining cell targeting early after infection.

We mapped the molecular determinants of CD4-independence to two amino acids in Env gp120, D470N and E84K. These changes were strongly associated with CD4-independence among Env variants, arose independently in multiple animals, and conferred the phenotype by mutagenesis studies. Several pathways to CD4 independence in HIV and SIV have previously been described (4, 8, 16, 28, 40, 68), but our observation suggests that this particular pathway is especially favored *in vivo*, at least in the context of this SIVmac251 infection. SIVmac251 is a heterogeneous swarm, so it is conceivable that E84K and D470N were present as polymorphisms in the inoculum used to infect the animals in this study. However, our review of database sequences indicates that these amino acids are exceedingly uncommon, and the fact that D470N and E84K were found neither in Envs from control animals nor from d11 CD4-depleted animals demonstrates

that these changes are not favored until post-peak infection in the relative absence of CD4+ T cells. In contrast to D470N and E84K, G62N was enriched in CD4-depleted animals at both d11 and d42, but was not significantly associated with CD4-independent entry. G62N is present at 4-5% overall frequency among SIVmac sequences and occasionally represents the dominant sequence among some SIVmac251 swarms (9, 75). Thus, additional selective forces in the CD4-depleted animals other than CD4-independence *per se* appear to be responsible for selection at this residue. Of note, all occurrences of G62N, E84K, and D470N resulted from G-to-A nucleotide changes, implicating the APOBEC3 family of proteins (33) and suggesting an important role for this mechanism in achieving diversity rapidly in the context of this selective environment *in vivo*.

While both E84K and D470N contribute to the full CD4-independent phenotype, D470N appears to have a primary role, with E84K reflecting at least in part a compensatory change that reversed the degree of attenuation induced by D470N. Structural mapping of D470N on the unliganded core crystal structure of SIV gp120 (3) places a potential glycosylation site deep within a pocket of CD4-binding residues. In contrast to N at that location, Q470 Envs were not able to efficiently use CCR5 independently of CD4, suggesting that glycosylation may be responsible for allowing CD4-independent entry rather than simply loss of a negative charge. It is possible that placement of a bulky carbohydrate within that pocket could affect gp120 structure in a manner similar to CD4 docking itself, causing Env to adopt a CD4-bound conformation and expose the conserved co-receptor binding site. Whether differences in structure of an intact SIV gp120 compared to the core structure employed here (as has been suggested for HIV-1 gp120 (43, 48, 52, 81, 82)) would affect the position of this residue is unknown. The homologous HIV-1 gp120 amino acid (D457) maps to the CD4 binding region in the HIV-1 gp120 core crystal structure and trimeric cryo-EM models (43, 45, 48), suggesting that the structural significance of D470 modeled here may be generalizable. How E84K might contribute to CD4-independence or to reverse D470N attenuation is unclear. This residue is near the predicted CD4 binding region but not a predicted contact point, and

lies within an alpha-helix thought to move with CD4 engagement (3, 29, 44). This drastic charge change may have indirect effects that affect structure, or perhaps may stabilize inter- or intra-molecular interactions induced by the D470N effects that mimic CD4 engagement.

Although these residues have not been explicitly characterized in previous studies, analysis of database sequences reveal that both E84K and D470N are very rare. E84K is present at low frequency in some stocks of SIVmacBK28 and SIVmac251 (9, 17, 75), and D470N is present in a few brain and plasma sequences in a rapid disease progression model (67). E84K is also present in an *in vitro*-derived CD4-independent variant of SIVmac239 (15, 46), although its specific contribution to CD4-independence was not assessed. In addition, both E84K and D470N were found in Envs from experimentally CD8-depleted, SIVmac251-infected rhesus macaques experiencing brain disease (76). CNS disease is driven largely by macrophage-tropic infection of myeloid cells in the brain, in the overall immune-privileged CNS environment (21). The presence of these mutations in the brain and in highly immunodeficient animals in previous studies is consistent with our observation of macrophage infection *in vivo* in CD4 T cell-depleted animals (58).

Recently, it has been suggested that the generally greater sensitivity CD4-independent viruses to antibody neutralization is due to greater "intrinsic reactivity" of these Env variants (25). This property enables them to spontaneously sample a range of conformations in the absence of CD4-binding, leading to both greater efficiency of co-receptor engagement and also greater sensitivity to structural perturbation (and thus neutralization) by antibodies with a range of epitope specificities, not just those reflecting the co-receptor binding site exposed upon binding to CD4. Our CD4-independent variants were sensitive to antibodies targeting several (although not all) SIV gp120 epitopes (Figs 4, 5), which is consistent with this model. Thus, the mutations that arose in these Envs may not only expose discrete CD4-i co-receptor binding site epitopes on gp120, but may also increase the intrinsic reactivity, rendering them more sensitive to

neutralization by multiple epitope antibodies. This is the first study to directly demonstrate *in vivo* the functional constraints on Env imposed by this mechanism, and consequences for co-receptor use and target cell tropism that are governed by such constraints.

In our model, CD4+ T cells were profoundly depleted from blood and inductive lymphoid compartments, although incompletely from mucosal sites (58). It has been proposed that CD4-i epitope antibodies arise *in vivo* through B cell recognition of Env complexed with CD4, which exposes the conserved binding-binding site to the B cell receptor (19). Our observation that CD4+ T-cell depletion leads to decreased CD4-inducible epitope antibody activity, despite the availability of these epitopes directly on these CD4-independent Envs for potential recognition by B-cells, suggests that it is the loss of CD4+ T helper function in these animals that leads to the dysfunctional antibody response.

Along with the *in vivo* tropism shift towards macrophage target cells, CD4+ T cell-depleted animals exhibited rapid disease progression and an absence of post-peak decline in plasma viremia. While the role of antibody activity in shaping altered tropism in the CD4-depleted animals seems clear, the mechanisms responsible for sustained high-level plasma viremia are less clear. Robust autologous or heterologous neutralizing activity was not detected in the control animals even at the d56 time point ((58), **Figure 3.8**). In addition, studies differ on whether direct B cell depletion prior to infection of rhesus macaques affects post-peak viral setpoint (51, 54, 71). Similarly, although CD8+ T cells clearly play a central role in post-peak viral decline (1, 24, 34, 42, 70), current paradigms are that CD4+ T cell help is dispensable for primary CD8 T cell responses (36, 57, 59) and no differences in CD8+ T cell responses were seen between control and CD4-depleted animals (58). In contrast, a variety of direct HIV or SIV-specific antiviral activity has been ascribed to CD4+ T cells (56), including a role in post-peak decline following acute infection (58, 73). CD4+ T cells have also been reported to have particular antiviral activity against SIV-infected macrophages (69). Thus, loss of direct

CD4+ T cell antiviral mechanisms may be responsible for the sustained viremia. At the same time, loss of strict CD4+ T cell dependence with consequent expansion to macrophages as a second robust target cell population may be an additional factor favoring sustained post-peak viremia, particularly in this setting of limited CD4+ T cells.

Some SIV-infected macaques that progress rapidly to AIDS fail to seroconvert (13, 27), a phenomenon that has been linked to early B cell defects following infection (78). In addition, a few CD4-independent SIV Env variants have previously been reported to arise spontaneously in SIV rapid progressors (68). Brain disease in SIV infection is also far more common in rapid than normal progressor animals (80), and macrophage infection within the CNS was also seen in these CD4 T cell-depleted animals (data not shown). Our results suggest that failure to develop CD4-inducible neutralization activity, whether as a consequence of direct virus-induced B cell defects as in spontaneous rapid progressive disease, or of experimental abrogation of CD4+ T cell help as shown here, may be a common factor that enables emergence of these macrophage-tropic variants with expanded host range and propensity for CNS infection. Furthermore, while most HIV and SIV blood variants exhibit modest macrophage tropism, highly macrophage-tropic variants may emerge in very late stage disease when profound CD4+ T cell loss has developed (31, 32, 47). Infection with X4-tropic SHIV also results in rapid profound CD4 depletion, with emergence of macrophage-tropic variants (31, 32). Furthermore, brain disease developed in all animals in another recent study that CD4-depleted macaques prior to SIV infection using a slightly different approach than ours, although CD4-independence was not assessed (57). Future studies will help elucidate whether variations in antibody activity in different stages of disease may also regulate tropism and cell targeting.

In summary, our findings reveal a critical role for CD4 T cell-dependent production of CD4-inducible neutralizing activity early after infection *in vivo* in shaping Env function and target cell tropism. These data suggest that, normally, there is a balance in which CD4+ T cells support the production by B cells of antibodies that, paradoxically, enforce

strict CD4+ T cell tropism for the virus. In this model, CD4+ T cell depletion results in both a profound reduction of CD4+ T cell targets and impaired production of these antibodies. As a result, strict CD4 dependence is lost and expanded tropism for CD4-low macrophages emerges. In addition to the forces regulating normal infection, these findings have implications as well for infection in the CNS and, possibly, late-stage disease upon severe CD4+ T cell depletion.

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CHAPTER IV

DECREASED PLASTICITY OF CORECEPTOR USE BY CD4-INDEPENDENT SIV ENVS THAT EMERGE *IN VIVO*

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Abstract

HIV and SIV generally require CD4 binding prior to coreceptor engagement, but Env can acquire the ability to use CCR5 independently of CD4 under various circumstances. The ability to use CCR5 coupled with low-to-absent CD4 levels is associated with enhanced macrophage infection and increased neutralization sensitivity, but what additional features of these Envs may affect cell targeting is not known. Here we report that CD4-independent SIV variants that emerged *in vivo* in a CD4+ T cell-depleted rhesus macaque model display markedly decreased plasticity of co-receptor use. While CD4-dependent Envs can use low levels of macaque CCR5 for efficient entry, CD4-independent variants required high levels of CCR5 even in the presence of CD4. CD4-dependent variants also mediated efficient entry using human CCR5, whereas CD4-independent variants had impaired use of human CCR5. Similarly, CD4-independent Envs used the alternative coreceptor GPR15 less efficiently than CD4-dependent variants. Env amino acids D470N and E84K that confer the CD4-independent phenotype also regulated entry through low CCR5 levels and GPR15, indicated a common structural basis. Similarly, treatment of CD4-dependent Envs with soluble CD4 enhanced entry through CCR5 but reduced entry through GPR15, suggesting that induction of CD4-induced conformational changes by non-cell surface-associated CD4 impairs use of this alternative co-receptor. Thus, CD4 independence is associated with more restricted coreceptor interactions. While the ability to enter target cells through CCR5 independently of CD4 may enable infection of CD4 low-to-negative cells such as macrophages, this phenotype may conversely reduce the potential range of targets such as cells expressing low levels of CCR5, conformational variants of CCR5, or possibly even alternative coreceptors.

Introduction

During HIV and SIV entry, gp120 engagement of CD4 is normally required to initiate the conformational changes in Env that form and expose a coreceptor binding site, which then allows CCR5 engagement and subsequent entry steps to occur (14). Although extremely rare in natural infection *in vivo*, several pathways have been described by which HIV and SIV can adapt to using CCR5 in the presence of little-to-no CD4 (9, 13, 17-19, 30, 34). The factors that serve to restrain CD4 independence during normal infection, and their relationship to coreceptor use and entry, have important implications for cell targeting and tropism *in vivo*.

We recently described a unique SIV model in which such CD4-independent Env variants emerged and dominated in the plasma of multiple rhesus macaques experimentally depleted of peripheral (but not mucosal) CD4+ T cells prior to infection (13, 24). These animals displayed widespread infection of tissue macrophages, which express very low levels of CD4 compared to CD4+ T cells, indicating a mechanism by which virus could expand its target cell range in this setting of limited CD4+ T cell targets. The CD4-independent Env variants that arose in CD4+ T cell depleted macaques were exquisitely sensitive to antibody neutralization, a characteristic that is common among previously-described CD4-independent HIV and SIV (17-19, 30). Unlike CD4-independent Envs that were constitutively sensitive to neutralization by normal SIV+ plasma and monoclonal antibodies, CD4-dependent control Envs became sensitive only if first incubated with soluble CD4 (sCD4). CD4+ T cell-depleted animals failed to produce this CD4-inducible neutralization activity, however, enabling emergence of CD4-independent variants in these animals. These findings, along with other studies (17-19, 30), suggest that antibodies shape the cellular tropism of the virus *in vivo* by preventing the emergence of CD4-independent variants during typical infection. Both CD4 independence and neutralization sensitivity were conferred by D470N/E84K mutations in Env that arose in these animals (13), indicating that these are linked phenotypes resulting from a common structural basis.

In this study, we wished to determine whether there were additional biological features of CD4-independent Envs that arise in this model that might impact cell entry and targeting, which could impact emergence of such variants in addition to sensitivity to antibody neutralization. It has previously been reported that some HIV isolates that can enter cells using low levels of CD4 may be limited in their capacity to efficiently engage low levels of CCR5 (1, 27). Since most CD4+ T cells express relatively low levels of CCR5 (12, 21, 23, 25, 26), this property could impact cell targeting by restricting infection *in vivo* to the limited subset of cells expressing high levels of CCR5. In addition, CCR5 is expressed on primary cells in a variety of conformational forms, suggesting that efficient infection may require some degree of plasticity in Env function that allows it to interact with such conformational variants (3, 7, 20). Thus, seeking to understand whether more restricted coreceptor use might be a factor, in addition to immune pressure, that could limit the emergence of CD4-independent Env *in vivo*, we tested our panel of plasma-derived CD4-independent Envs for their ability mediate entry through low-level CCR5, through human CCR5, and through the alternative coreceptor GPR15. Low-level CCR5 clearly reflects a biologically important feature of primary target cells in vivo (12, 21, 26), while use of the human CCR5 molecule and of GPR15 may reflect a degree of "plasticity" by which Env can function using related but structurally distinct molecules.

Here we report that CD4-independent SIV Env variants that arise *in vivo* are impaired in their ability to use low levels of rhesus macaque CCR5 for entry even in the presence of CD4. Furthermore, they are significantly less efficient in use of both human CCR5 and GPR15 than CD4-dependent control Envs. Each of these phenotypes was conferred by the same molecular determinants that regulate CD4-independence, indicating shared structural basis. These findings suggest that CD4-independent variants may have an expanded capacity for infection of CD4-low targets, but may have an otherwise narrower range of potential cellular targets *in vivo* due to more limited ability to infect CCR5-low cells and/or cells expressing CCR5 conformational variants. Thus, in addition to neutralization sensitivity, a more restricted coreceptor utilization capacity may also serve to limit the emergence of CD4-independent variants during natural infection.

Methods

SIV_{Mac} envelope clones and receptor plasmids

The SIV *env* genes were cloned by single genome amplification from d11 and d42 plasma of SIV-infected rhesus macaques, and mutations introduced using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA) and verified by sequencing, as previously described (24). Pseudotyped viruses carrying SIV Envs on the NL4.luc/env^-/vpr^+ HIV-1 backbone that has luciferase in place of *nef* (8) were generated as previously described (10), and were treated with DNAse prior to use.

Preparation of target cells

Human 293T cells were maintained in DMEM containing 10% fetal bovine serum. Coreceptor-transfected target cells were prepared by transfecting 10^6 293T cells with 1 μg plasmid encoding co-receptor with or without 1 μg plasmid encoding CD4, using Fugene transfection reagent (Promega, Madison, WI) as per manufacturer's instructions, and plasmid pcDNA 3.1 as "filler" to maintain a total of 2 μg plasmid per transfection. To prepare target cells expressing variable levels of CD4 and CCR5, 10^6 cells were transfected with 1, 0.1, 0.01, or 0 μg plasmid encoding rmCCR5 along with 1, 0.1, or 0 μg plasmid encoding rmCD4, using pcDNA 3.1 as "filler". One day after transfection, target cells were re-plated into 96-well plates ($2x10^4$ per well), and then infected the following day with pseudotyped viruses. In parallel, cells transfected with varying levels of CD4/CCR5 were subject to FACS analysis for CCR5 and CD4 expression using antibodies CD4 (L200)-FITC and CCR5 (3A9)-APC (BD Pharmingen; Franklin Lakes, NJ).

Primary human PBMCs from healthy volunteers were cultured at $2x10^6$ cells/mL in RPMI containing 10% fetal bovine serum, stimulated with PHA (5 μg/mL) for 3 days, and maintained thereafter with IL-2 (20 U/mL). On d. 4 after isolation, cells were plated at 10^5 cells per well in 96-well plates, incubated with or without the CD4 blocking mAb #19 (5 ug/ml; (11)), then infected with pseudotype viruses.

Infection of target cells

Target cells were infected with Env-pseudotyped viruses (20 ng p24 antigen per virus) by spinoculation at 1200xg for 2 hrs. Cells were then incubated for 72 hrs at 37°C and infection was quantified by measuring luciferase content in cell lysates as previously described (29). All data represent a minimum of 3 independent experiments, each carried out in duplicate. Where appropriate, entry of each virus into cells expressing the various combinations of CD4 and co-receptor was calculated as a percentage of entry of that virus into cells expressing the highest levels of CD4 and CCR5.

Soluble CD4-183 (sCD4; Pharmacia, Inc.) was obtained from the NIH AIDS Reference Reagents Program, and sCD4 exposure assays were performed essentially as previously described (13). Pseudotyped virus was mixed with sCD4 to achieve a concentration of virus of 0.8 ng/μl of viral p24 antigen and 50 ng/μl sCD4, and incubated at 37°C for 1 hr. A volume of this mixture (or a mixture of virus incubated without sCD4) containing 20 ng p24 was used to infect cells as described above.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, San Diego, CA). Paired T-tests on log-transformed data were used to compare data sets where a single panel of Env variants was tested for entry on 2 different target cell samples, while unpaired T-tests were used for all other mean comparisons. Linear regression analysis was performed using default parameters in Prism, without constraints and fit to mean values.

Results

CD4-independent SIV Envs have reduced capacity compared to CD4-dependent Env to use human CCR5

We previously described a model for *in vivo* emergence of CD4-independent SIV in which rhesus macaques were experimentally depleted of CD4+ T-cells before SIVmac251 infection (13). Envs isolated from CD4+ T cell-depleted animals at day 42 (d42) post infection were capable of mediating infection through CCR5 independently of CD4, while Envs from CD4+ T cell-depleted macaques early after infection and control macaques both early and late after infection were strictly CD4-dependent (13).

As shown in **Fig. 4.1A**, the CD4-independent Envs mediated equivalent levels of pseudotype virion entry into 239T cells transfected with rhesus macaque CCR5 (rmCCR5) with and without rmCD4, whereas the control Envs were CD4-dependent. In addition, CD4-dependent and CD4-independent Env variants had equivalent levels of entry overall into cells expressing both rmCD4 and rmCCR5. Thus, the CD4-independent Envs are as efficient as CD4-dependent Envs at mediating entry through rmCCR5 in this transfection system, and are essentially indifferent to the presence or absence of CD4 in the presence of rmCCR5.

In contrast to entry through RM receptors, the CD4-independent Envs were significantly less efficient than CD4-dependent Envs in entering cells expressing human CCR5 and CD4 (**Fig. 4.1B**). The lower overall entry through human receptors was due to less efficient use of human CCR5, as entry through rmCCR5/huCD4 was as efficient as rmCCR5/rmCD4, and entry through huCCR5/rmCD4 was similar to huCCR5/huCD4 (data not shown). The CD4-independent Envs were CD4-independent on human CCR5, although entry was lower in cells expressing huCCR5 alone compared to huCCR5/huCD4. Thus, the CD4-independent Envs are attenuated in their use of huCCR5 compared to CD4-dependent Envs.

We further examined whether the CD4-independent phenotype we observed during infection of cells over-expressing CCR5 also applied to primary cells, and whether the CD4-independent Envs were less efficient than CD4-dependent Envs in huCCR5 use in the context of primary cells (**Fig. 4.1C**). Primary human PBMCs were treated with or without the CD4 monoclonal antibody #19, which blocks CD4-dependent HIV and SIV entry (11). As expected, entry into PBMC by CD4-dependent Envs was reduced to background levels by mAb #19, whereas CD4-independent variants were largely resistant to entry blockade by #19, confirming that the CD4-independent phenotype extends to primary cells as well. Overall entry by CD4 independent viruses (in the absence of #19) was attenuated compared to CD4-dependent Envs, which was in contrast to the equivalent entry into rmCCR5/rmCD4 transfected cells for the CD4-independent and –dependent viruses (**Fig. 4.1A**), but similar to lower entry for the CD4-independent viruses into huCCR5/huCD4 transfected cells (**Fig. 4.1B**). Thus, CD4 independent variants are also less efficient than control Envs at entry into human CCR5/CD4-expressing primary cells. The attenuated entry by CD4-independent viruses likely reflects less efficient use of human CCR5, but since primary cells express CCR5 at lower levels than transfected cells, could also reflect inefficient use of lower levels of coreceptor compared with CD4-dependent Envs.

CD4-independent SIV Envs use low levels of rmCCR5 less efficiently than CD4-dependent Envs

To address the impact of CCR5 expression levels, we tested the ability of CD4-independent SIV Env variants to mediate entry into 293T cells transfected with various amounts of rmCD4 and rmCCR5 (**Fig. 2**). Flow cytometry analysis showed that transfection of 0.0, 0.01, 0.1, or 1.0 μg CCR5 plasmid resulted in mean fluorescence intensity (MFI) levels of approximately 5, 6, 10, and 29, respectively, while transfection with 0.0, 0.1, or 1.0 CD4 plasmid resulted in MFI levels of 4, 9, and 15, respectively (data not shown), confirming that varying plasmid transfection results in correspondingly varying levels of receptor surface expression.

As expected, CD4-dependent control Envs exhibited high infectivity at high levels of CCR5, which gradually dropped at lower levels of CCR5 (**Fig. 4.2A**). In contrast, CD4-independent Envs exhibited a steep decline in infectivity even at slightly reduced levels of CCR5 (**Fig. 4.2B**). We then analyzed a larger panel of Envs with this assay, and determined the relationship between CD4-independence and the ability to use low levels of CCR5 (**Fig. 4.2C**). This analysis revealed a strong inverse correlation between entry into cells expressing low levels of rmCCR5 (in the presence of CD4) and entry into cells expressing high levels of rmCCR5 without CD4 ($p = 0.001$). These results indicated that among SIV Env variants that arise *in vivo* in infected macaques, CD4-independence is strongly associated with inefficient use of low levels of CCR5, even in the presence of CD4.

Decreased efficiency of GPR15 use by CD4-independent and CD4-dependent Envs

In addition to CCR5, several other G protein-coupled receptors can be used as entry co-receptors by SIV *in vitro*, reflecting a substantial degree of plasticity in the molecules with which these Envs can interact. To determine whether the efficiency of alternative coreceptor use differed between CD4-dependent and independent Envs, we assessed their ability to enter cells transfected with the orphan receptor GPR15, which is commonly used by SIVmac variants, in conjunction with CD4 (**Fig. 3**).

CD4-dependent control Envs mediated efficient entry in rmGPR15/rmCD4-expressing cells, reaching a mean of 61% (range 50-80%) of entry compared to rmCCR5/rmCD4, which confirms that GPR15 is used by these SIVmac251 variants. However, CD4-independent Envs were significantly impaired in their use of GPR15 compared to control Envs (mean of 30%; range 2-60%). Regression analysis showed a highly significant inverse correlation between the ability of Env to use CCR5 in the absence of CD4 and the ability to use GPR15 in the presence of CD4 ($p < 0.0001$; **Fig. 4.3B**), indicating that CD4 independence is associated with decreased efficiency of GPR15 use.

D470N and E84K regulate efficiency of low-CCR5 use and GPR15 use.

Since the CD4-independent variants were all derived from plasma of macaques experimentally depleted of CD4+ T cells prior to infection, the decreased efficiency of alternative coreceptor and low-CCR5 use among these variants could conceivably have resulted from selective pressures on these Envs *in vivo* unrelated to their CD4-independent phenotype. Therefore, in order to ask whether CD4 independence and decreased low-CCR5 and GPR15 use were inextricably linked, we asked whether the molecular determinants of CD4-independence also regulated these characteristics. We recently identified two amino acids that emerged in the CD4+ T cell-depleted macaque Envs that conferred CD4 independence when introduced into CD4-dependent Envs (D470N, E84K), and which abrogated CD4 independence when reverted in the CD4-independent Envs (13).

When D470N and E84K were introduced into control Envs, they conferred CD4 independence but markedly reduced the ability to enter into cells expressing low levels of CCR5 even in the presence of CD4 (**Fig. 4.4A**, **B**, **E**). Conversely, when N470D and K84E were introduced into CD4-independent Envs, they reduced the ability to utilize CCR5 independently of CD4, but enhanced the ability to utilize low levels of CCR5 in the presence of CD4 (**Fig. 4.4C**, **D**, **E**). Finally, introduction of D470N and E84K into control Envs reduced use of GPR15, while N470D and K84E enhanced GPR15 use (**Fig. 4.4F**). Thus, the molecular determinants that regulate CD4-independent use of CCR5 are also the molecular determinants of co-receptor use plasticity, indicating that they are linked properties of these Envs, rather than independent but co-selected phenotypes of virus variants that emerged in these animals.

Conformational changes induced by soluble CD4 inhibit GPR15 use

Envs capable of using CCR5 in the absence of CD4 are thought to constitutively exist in a CD4-triggered conformation, and it is known that soluble CD4 (sCD4) is also capable of triggering conformations in SIV Env that enable entry through CCR5 in the absence of CD4 (32). To determine if sCD4 triggering has an effect on use of GPR15 similar to the CD4-independent phenotype, we tested the impact of sCD4 on the ability of CD4-

dependent control Envs to enter through GPR15. As shown in **Fig. 4.5**, sCD4 pre-triggering enabled entry by CD4-dependent control Envs into cells expressing CCR5 in the absence of CD4, as expected, and slightly enhanced entry into cells expressing CCR5/CD4. In contrast, sCD4 treatment resulted in a significant (~50%) reduction in entry through GPR15 (**Fig. 4.5C**), which is similar to the relative deficiency in GPR15 use by CD4-independent compared with control Envs (**Fig. 4.3A**). Treatment of CD4-independent Envs with sCD4 had no impact on entry through CCR5, with or without CD4, nor on entry through GPR15. Thus, even though cell surface CD4 engagement is required for GPR15-mediated entry, CD4-induced conformational changes in Env that occur independent of cell surface CD4 inhibit the use of GPR15, concordant with the reduced ability of CD4-independent Envs to use GPR15 compared with CD4-dependent Envs. Notably, the lack of blocking through CCR5/CD4 indicates that sCD4 did not impact entry via effects on cellular tethering, gp120 shedding, or similar mechanisms, suggesting that sCD4 inhibition of entry through GPR15 is likely due to effects on gp120 interactions with the coreceptor.

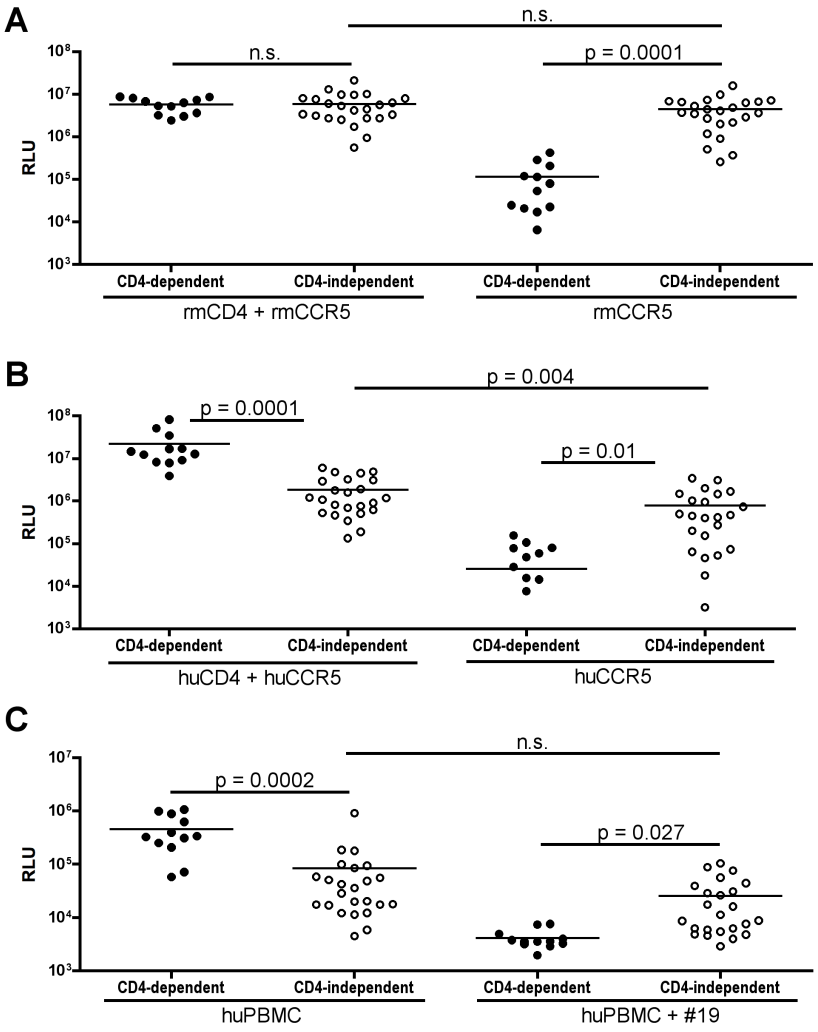

Fig. 4.1. Lower efficiency of human CCR5 use by CD4-independent compared to CD4-dependent SIV Envs

Pseudotyped viruses carrying Envs from d.42 CD4+ T cell depleted macaques (CD4-independent Envs; open circles) or control macaques (CD4-dependent Envs; closed circles) were used infect 293T cells transfected with receptor molecules (**A, B**) or primary human PBMCs (**C**). (**A**) 293T cells were transfected with rhesus macaque (RM) CCR5 with or without rhesus CD4 and infected with pseudotype viruses. Entry was assessed based on luciferase production 48 hours after infection and expressed as relative light units (RLU). (**B**) SIV Env-pseudotyped viruses were used infect either 293T cells transfected with human CCR5 with or without human CD4. (**C**) Envs were assessed for their ability to mediate viral entry into PHA/IL2-stimulated human PMBCs that were pre-treated with or without a CD4 blocking antibody (mAb #19). Unpaired T-tests were used to compare entry between the 2 sets of Envs in a given target cell type, while paired T-tests were used to compare entry of Envs in the presence versus absence of CD4.

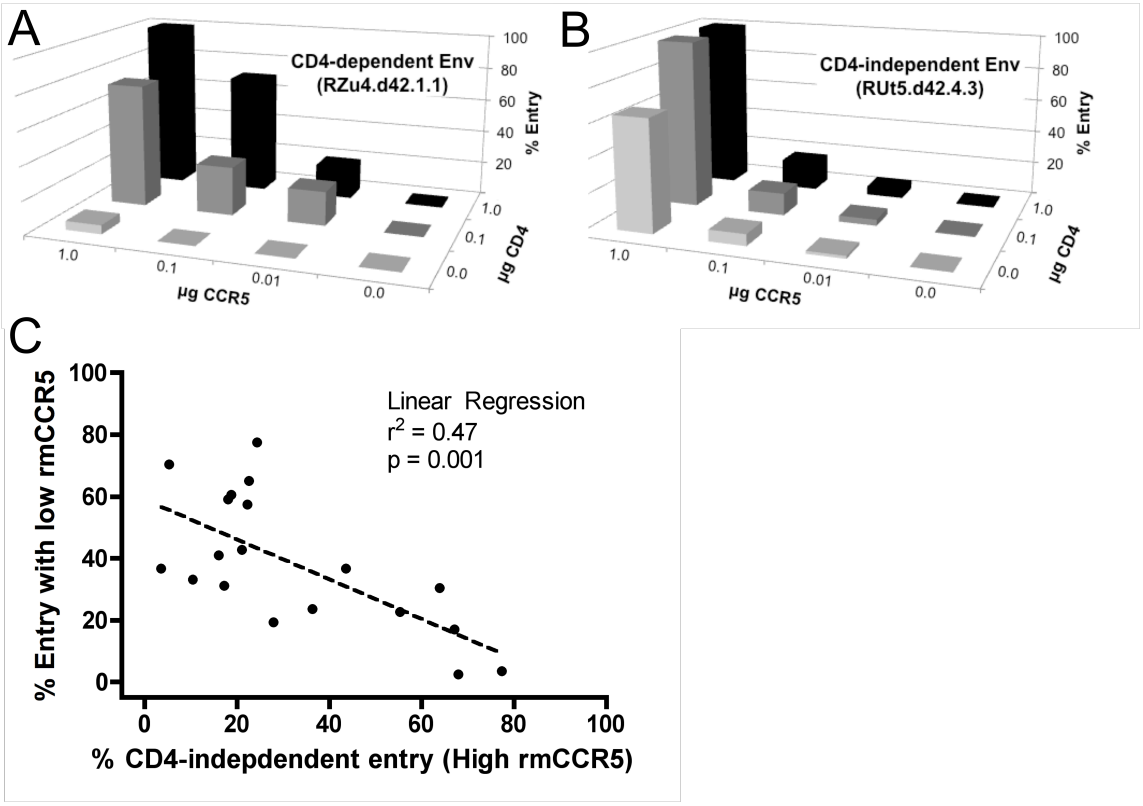

Fig. 4.2. CD4-independence is associated with reduced ability to use low levels of rmCCR5 SIV Env-pseudotyped viruses were tested for their ability to infect 293T cells transfected with different amount of rmCD4 and rmCCR5. (**A, B**) 3D plots showing entry by representative CD4-dependent (A) and CD4-independent (B) Envs. The, horizontal (X) axis indicates the amount of transfected CCR5 decreasing from left to right, the depth (Z) axis indicates the amount of transfected CD4 decreasing from back to front, and the vertical (Y) axis indicates the level entry with 100% set at entry in the presence of maximal rmCD4 and rmCCR5. (C) The panel of Envs were tested as in A and B, with the X axis indicating CD4-independent entry (no CD4, maximal rmCCR5) and Y axis indicating entry via low CCR5 (maximal rmCD4 but only 10% of maximal rmCCR5). Each Env is indicated as a single point on the plot. Linear regression analysis was used to assess the correlation between CD4-independent entry and low-CCR5 entry.

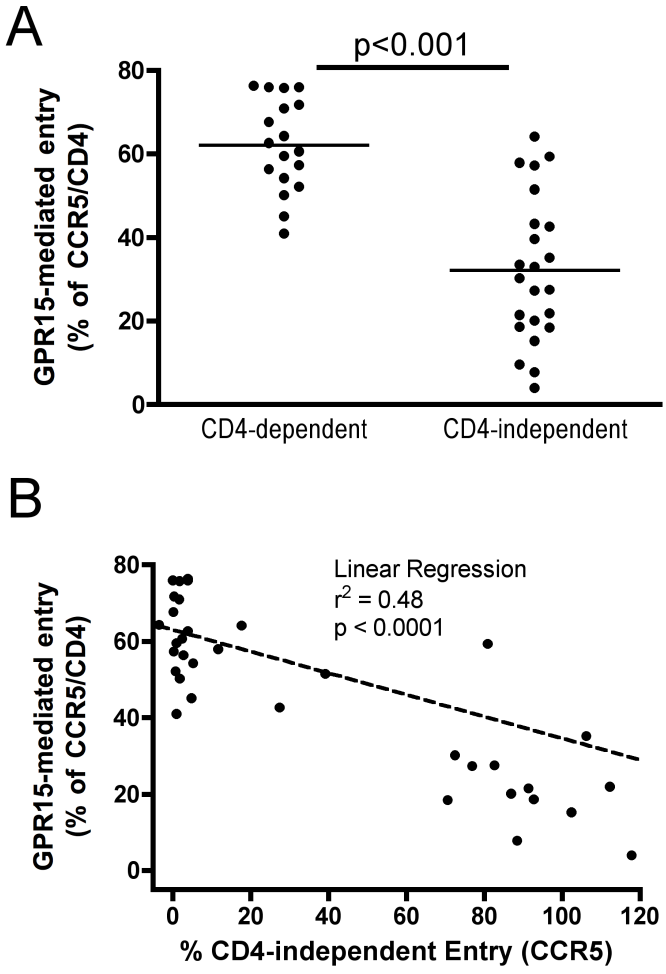

Fig. 4.3. Reduced entry through GPR15 by CD4-independent Envs
Env-pseudotyped viruses were tested for their ability to infect cells transfected with rmGPR15 and rmCD4. (**A**) Entry into 293T cells transfected with rmGPR15 and rmCD4 was measured for each Env, and is indicated as a percentage of entry by that Env using rmCCR5 and rmCD4. (**B**) Envs from (A) were plotted showing entry in cells transfected rmCCR5 without CD4 on the X axis, and entry into cells transfected with rmGPR15 and rmCD4 on the Y axis. Each Env is indicated as a single point on the plot, and linear regression analysis was performed to assess the correlation between CD4-independent entry and GPR15 use.

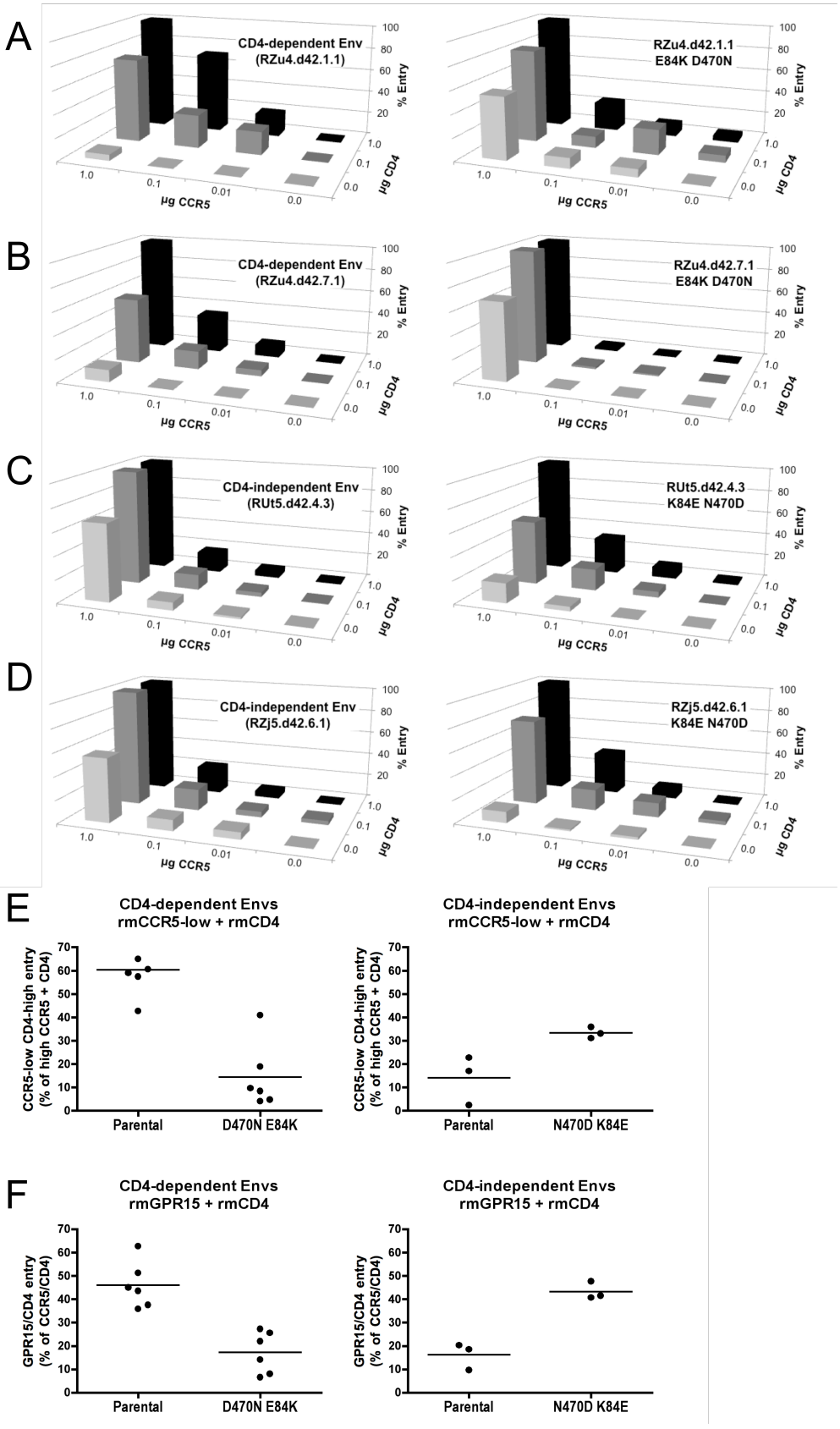
A
CD4-dependent Env
(RZu4.d42.1.1)
RZu4.d42.1.1
E84K D470N
B
CD4-dependent Env
(RZu4.d42.7.1)
RZu4.d42.7.1
E84K D470N
C
CD4-independent Env
(RUt5.d42.4.3)
RUt5.d42.4.3
K84E N470D
D
CD4-independent Env
(RZj5.d42.6.1)
RZj5.d42.6.1
K84E N470D
% Entry
μg CCR5
μg CD4
E
CD4-dependent Envs
rmCCR5-low + rmCD4
CD4-independent Envs
rmCCR5-low + rmCD4
CCR5-low CD4-high entry
(% of high CCR5 + CD4)
Parental
D470N E84K
N470D K84E
F
CD4-dependent Envs
rmGPR15 + rmCD4
CD4-independent Envs
rmGPR15 + rmCD4
GPR15/CD4 entry
(% of CCR5/CD4)

Fig. 4.4. Env D47N and E84K determine efficiency of CCR5 and GPR15 use
Mutations that emerged in vivo which conferred CD4 independence (D470N and E84K) were introduced into CD4-dependent Envs, and mutations that abrogate CD4 independence (N470D and K84E) were introduced into CD4-independent Envs. **(A-D)** Parental (left) and mutant (right) Envs were assessed for their ability to mediate entry into cells transfected with varying amounts of rmCCR5 and rmCD4, as described in **Fig. 4.**2. **(E)** Use of low rmCCR5 levels by all parental and mutant Envs (10% maximal CCR5 in the presence of maximal CD4), with each Env indicated as a dot and entry in the presence of maximal CCR5/CD4 set at 100%. (**F**) Use of GPR15 by parental and mutated Envs were tested based on entry into cells transfected with rmGPR15 and rmCD4, with each Env indicated as a dot and expressed as a percentage of entry in the presence of rmCCR5/rmCD4.

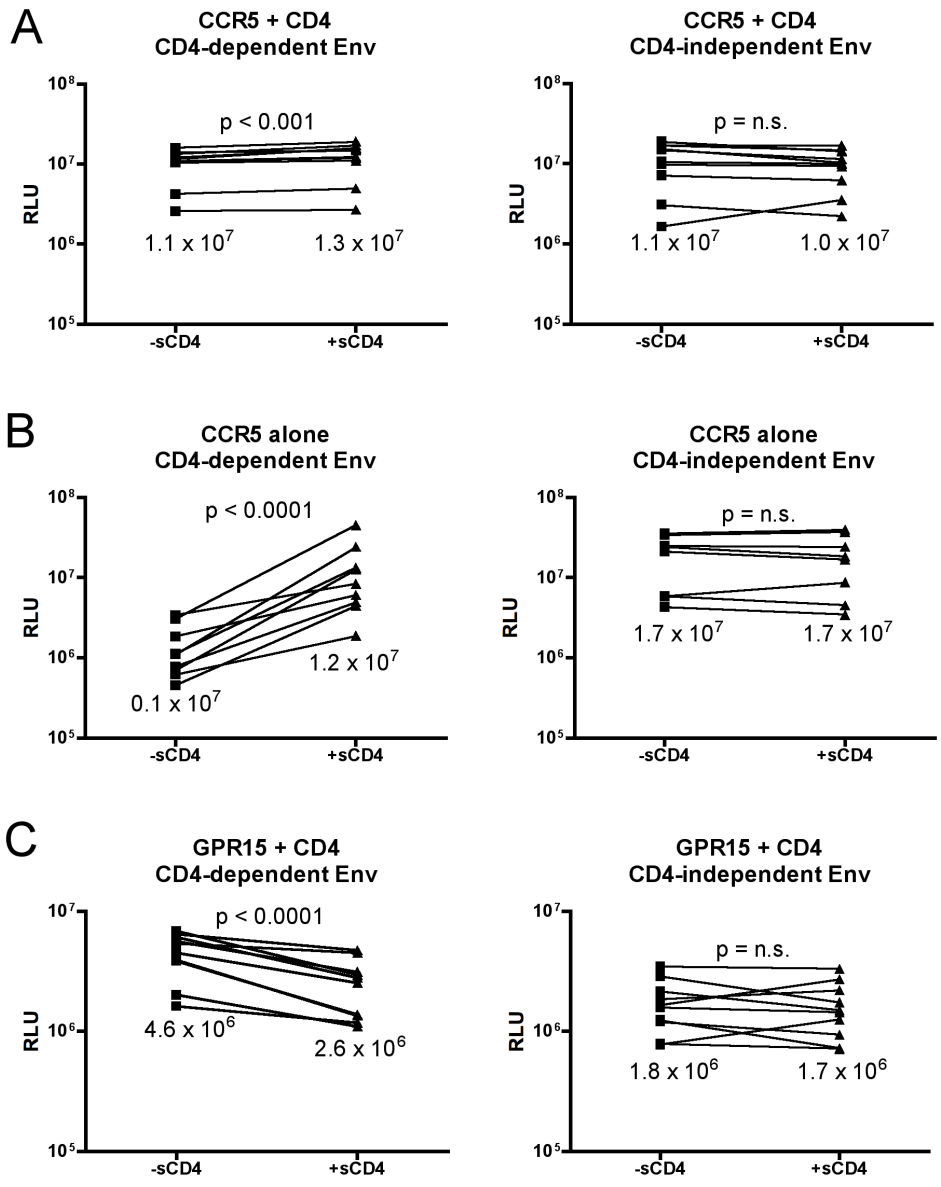

Fig. 4.5. Reduced use of GPR15 by SIV Envs pre-triggered with sCD4
Pseudotype viruses carrying CD4-dependent or CD4-independent Envs were incubated with or without sCD4 (50 ug/ml for 1 hour) and assessed for their ability to infect cells 293T cells expressing rmCCR5 and rmCD4 **(A)**, rmCCR5 without CD4 **(B)**, or rmGPR15 and rmCD4 **(C)**. Each Env is indicated as a box (without sCD4) and a triangle (with sCD4), and the two conditions for each individual Env are connected by a line. Paired T-tests were used to compare entry with and without sCD4 incubation.

Discussion

We focused here on a panel of SIV Envs derived from the plasma of rhesus macaques that had been experimentally depleted of CD4+ T cells prior to infection, in which CD4-independent virus variants arose in multiple animals in association with widespread macrophage infection. The ability to use CCR5 in conjunction with low-to-absent CD4 is a common feature of viruses that efficiently infect macrophages, which express extremely low levels of CD4 (2, 22, 33). However, while entry by these viruses through rmCCR5 in transfected cells is very efficient with or without CD4, the Envs display decreased efficiency of entry in three areas: ability to use low levels of CCR5; ability to use the human homologue of CCR5; and use of the alternative coreceptor GPR15. Because these features are all tightly linked, we suggest that they reflect a common property of overall decreased "plasticity" of Env function, as a consequence of having acquired the ability to use CCR5 without CD4.

While multiple reports have described CD4-independent SIV and HIV generated in various model systems, strict CD4 dependence is a general rule for viruses *in vivo* during normal infection. Understanding the consequences of CD4 independence in settings where it is acquired, therefore, provides insight into critical factors that maintain CD4 dependence in normal infection. We previously reported that the CD4-independent variants in these animals are highly sensitive to neutralization by SIV+ plasma and several monoclonal antibodies, similar to other CD4-independent variants described (13, 17-19, 30), whereas control Envs are resistant to neutralization unless pre-triggered by sCD4. Since the CD4+ T cell depleted animals in which these variants arose failed to produce such CD4-inducible neutralizing activity, one clear factor that appears to contribute to maintaining CD4 dependence is avoidance of plasma neutralization. However, our findings here that CD4-independent Envs exhibit more restricted coreceptor use suggest that additional factors may also impact function of CD4-independent Envs and regulate their relative fitness *in vivo*.

We found that defective use of low levels of CCR5 is linked to CD4 independence both phenotypically (**Fig. 4.2C**) and structurally (**Fig. 4.4A-E**). In contrast to the CD4+ T cell depleted model in which these viruses arose, where a shift towards macrophage targets occurred, CD4+ memory T cells are the principal targets of HIV and SIV in normal infection. In general, CCR5 is expressed on CD4+ T cells at relatively low levels (21, 23). Thus, while CD4 independence may enable the virus to infect an expanded range of CD4-low cell types, particularly macrophages, a consequence of this feature may be a restricted capacity to infect primary CD4+ T cells expressing low or moderate levels of CCR5.

CD4 independence of these Envs was also linked to less efficient use of human CCR5, despite equivalent entry through transfected rmCCR5 (**Fig. 4.1**). In addition to overall lower levels of expression, CCR5 on primary CD4+ T cells exists in a range of structural conformations, which is reflected in conformational antibody and/or Env binding characteristics (20, 23, 25). It has been previously suggested that the ability to engage alternative conformations of CCR5, or even the capacity to engage ligand-bound receptor, enables Env to more efficiently use the heterogeneous conformations that are expressed on CD4+ T cells (3, 7, 12, 20, 21, 26). CCR5 of human and macaque origin share 97.7% amino acid identity (6), and Env use of the two homologues is generally highly concordant. However, our observation that CD4-independent Envs are relatively impaired in use of huCCR5 compared with control Envs suggests that they are more sensitive to subtle structural variations in the molecule. We speculate that the greater use of huCCR5 that we observed in our control CD4-dependent Envs compared to CD4-independent Envs may reflect a greater ability to tolerate structural variations that might allow these Envs to effectively engage CCR5 on a broad range of CD4+ T cells, whereas CD4-independent Envs may be more limited in the structural variation they can tolerate, and thus a more limited range of CD4+ T cell targets.

The CD4-independent Envs in our study also displayed impaired entry into cells expressing rmGPR15 compared to control CD4-dependent Envs, even in the presence of

CD4. SIV Envs typically use a range of alternative coreceptors *in vitro*. However, while alternative entry pathways are used *in vivo* in nonpathogenic infection of sooty mangabey and red-capped mangabey natural hosts (5, 29), it is unclear whether any of these pathways, including GPR15, play a role in SIVmac infection of rhesus macaques, and there appears to be no consequence *in vivo* to loss of GPR15 utilization (28). Thus, while we cannot completely exclude the possibility that less efficient GPR15 use by CD4-independent variants may limit infection of GPR15+ cells *in vivo*, we think it is more likely that this feature reflects simply another aspect of the overall more coreceptor-constrained, less plastic function of the CD4-independent Envs.

Our observation that the ability of SIV to enter independently of CD4 correlates inversely with efficiency of CCR5 use differs from some previous studies that examined variants with the capacity to use exceedingly low levels of CD4 for entry, which were frequently also better able to utilize low levels of CCR5 (4, 15, 16, 35). In those studies, the ability to scavenge very low levels of CD4 on the cell surface was thought to result from increased affinity for CD4, or more stable interactions, triggering the CD4-induced conformational changes that enable coreceptor engagement (31). In contrast to those studies, our CD4-independent variants were not sensitive to inhibition by sCD4 (**Fig. 4.5A**) and could function in the complete absence of CD4 in the context of high CCR5 expression (**Fig. 4.1A**). It appears that our CD4-independent variants exist constitutively in or spontaneously acquire the CD4-bound conformation, even in the absence of CD4 (13). Therefore, loss of coreceptor use plasticity may be specific to Envs that have a pre-formed or spontaneously exposed coreceptor binding site (17-19, 30) rather than Envs that scavenge low levels of CD4 on the cell surface (4, 9, 15, 16, 34, 35). Taken together, these data suggest that diverse mechanisms exist by which virus can expand its host range into CD4-low or negative cells, which may have different consequences for coreceptor interactions. Nevertheless, because these variants arose and came to dominate plasma virus in multiple animals, this mechanism reflects a bona-fide pathway of evolution in this CD4+ T cell depleted model that highlights factors impacting Env function.

In conclusion, we have identified loss of coreceptor use plasticity as a potential consequence of CD4-independent entry capacity. The depletion of CD4+ T cell targets in our model likely forced the virus to adapt to a CD4-independent phenotype to enable macrophage targeting, in the permissive environment lacking antibody activity that would otherwise neutralize these variants. An additional consequence of this adaptation, however, is decreased ability to use low levels of CCR5 and related but distinct coreceptor structures. As a result, even though these variants have broadened their range of targets to include CD4-low/CCR5-high cells, they may have a paradoxically narrower range of potential target cells among CD4+ cells that express lower levels or conformational variants of CCR5 such as primary CD4+ T cells. Under normal circumstances, the targeting of such primary CD4+ T cells may be an additional factor that, along with immune pressure, normally limits the emergence of CD4-independent Env variants *in vivo*.

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CHAPTER V

EXAMINATION OF THE TARGET PRIMARY CELLS FOR CD4-INDEPENDENT SIV

Introduction

In the previous chapters we examined the phenotypic and genetic properties of CD4-independent Env variants that emerged in the plasma of experimentally CD4-depleted animals. In this chapter we look at the ability of these Env variants to mediate entry into monocyte-derived macrophages and examine replication in tissue culture of a full-length SIV carrying a CD4-independent Env. Although this chapter focuses mostly on negative data, the information and reagents will be useful for future studies using our infectious CD4-independent virus for *in vitro* evolution or *in vivo* infections.

The ability of Env to use CCR5 in conjunction with low or undetectable levels of CD4 is generally considered a hallmark of macrophage tropism (1, 11, 15). This chapter presents our observation that our SIV Env variants from CD4-depleted animals at d42 post-infection, which were almost all able to mediate entry independently of CD4 in transfected cells, also mediated entry into human monocyte-derived macrophages (MDM). The infection, as measured by a single-round infection of a pseudotype reporter virus was relatively low overall, but was significantly higher than MDM infection mediated by control CD4-dependent Env variants.

Our observation in **Chapter IV** that our panel of CD4-independent SIV Envs used human CCR5 for entry poorly compared to control Envs led us hypothesize that the using species-matched cells for primary infections may lead to a more robust signal, and so we attempted infection of MDMs from rhesus macaques. Our pseudotype virus assay uses HIV-derived viral particles, but non-human primate cells are known to be largely refractory to HIV infection (8), and myeloid cells in particular can restrict HIV and SIV infection at post-entry steps (6, 9). For these reasons, we employed an assay using a β-lactamase reporter that detects viral infection rapidly after fusion. However, using this assay, although we detected infection of cells by a VSVg-pseudotyped, we were unable to detect SIV Env-mediated infection of either human or rhesus macaque MDMs, suggesting that these particular cells may not accurately reflect the tissue macrophages that harbor CD4-independent macrophage-tropic virus *in vivo*. When we similarly tested

rhesus macaque PBMCs for infection by our panel of CD4-independent and control Env variants, we observed that control Env could mediate viral fusion to these cells but that CD4-independent Env could not. This latter observation is consistent with the possibility, discussed in **Chapter IV**, that CD4-independent variants, while exhibiting an expanded host range for macrophages *in vivo*, may have a more constrained ability to infect CD4+ T cells.

Because Env-pseudotyped single-round infection assays may not fully predict the phenotype of the Env in the context of a live virus, we constructed chimeric SIVmac239 clones containing the Env of a plasma-derived CD4-independent or control Env and tested the ability of virus to replicate in rmCCR5+ T cell lines and primary human CD4+ T cells. We found that the virus carrying the CD4-independent Env was replication-competent even in the absence of CD4, but was highly attenuated in both the presence and absence of CD4. Taken together, the data in this chapter tell us that CD4-independent Env is highly attenuated, but may mediate modest infection of MDMs, and is also functional in the context of an infectious virus.

Methods

Production of pseudotype virus

The *env* genes from d11 and d42 SIV-infected rhesus macaque plasma were previously described (12). Mutations were introduced into SIV envelopes using the QuickChange II XL Site-Directed Mutagenesis Kit and verified by sequencing. Luciferase expressing pseudotyped viruses carrying SIV Envs on an HIV-1 backbone were generated as previously described (4), and were treated with DNAse prior to use in infection. For viruses to be used for β-lactamase (BlaM) readout, BlaM-vpr gene was co-transfected with *env* and backbone at a ratio of 10:6:5 *backbone*:*env*:*vpr* as previously described (5). BlaM-vpr pseudotype viruses were concentrated on a sucrose cushion as described (5).

Primary T cells

Aphaeresis material from healthy human donors or peripheral blood from healthy rhesus macaques was placed in a Ficoll gradient to purify PBMCs (100% Ficoll was used for human blood, 95% Ficoll for RM blood). For some experiments, CD4+ T cells were further purified by negative selection. Blood from 3 human and 3 macaque donors was used, but only data from 1 representative macaque is presented. Cells were cultured at $2x10^6$ cells/mL in RPMI containing 10% fetal bovine serum (R10 media) with 5 μg/mL phytohemagglutinin (PHA) for 72 hours, from which point they were maintained in R10 containing IL-2 (30 U/mL) without PHA for 24 hours. Cells were then infected as described below.

Macrophages derived from primary human monocytes

Human monocytes were purified from PBMCs by negative selection by University of Pennsylvania Human Immunology Core. Monocytes were cultured at $2x10^6$ cells/mL for 6 days in R10 media containing 100 ng/uL macrophage colony stimulating factor (M-CSF), were washed twice with PBS, then were lifted from the plate with cold unsupplemented RPMI. Cells were re-plated at 10^5 cells/well in D10 in a 96-well plate, allowed to adhere overnight, then were infected as described below.

Macrophages derived from primary human and rhesus macaque PBMCs

PBMCs were purified by Ficoll gradient and allowed to rest overnight in R10 without stimulation. Cells were then brought to $5x10^6$ cells/mL in R10 containing M-CSF and were plated onto 12-well plates, 1 mL/well. Following 6 days of incubation, wells were washed 5 times with PBS and media was replaced with D10. After incubating overnight again, MDMs in the 96-well plated were infected as described below.

Infection of target cells for luciferase readout

MDMs were infected with Env-pseudotyped NL4.luc (*env*$^-$, *vpr*$^+$; 20 ng p24 antigen) by spinoculation at 1200xg for 2 hrs. Cells were then incubated for 72 hrs at 37°C and infection was quantified by measuring luciferase content in cell lysates as previously described (14).

Infection of target cells for BlaM readout

500 uL media was removed from the 1 mL in the 12-well plates containing ~$2x10^6$ MDMs or PBMCs and cells were infected with Env-pseudotyped NL4.luc containing BlaM-vpr (100 ng p24 antigen) by spinoculation at 1200xg for 2 hrs as previously described (3). Cells were then washed twice with CO_2-independent media, 100 uL CCF2 loading solution was added to each well (2 uL CCF2-AM dye, 8 uL of solution B (Invitrogen), and 990 uL CO_2-independent media), and plates were incubated at 25° C for 1 hr. Cells were washed 2 more times with CO_2-independent media, and then blocking solution was added (5 uL 0.5M probenecid, 100 uL FBS, 900 uL CO_2-independent media). Plates were incubated overnight, and cells were harvested either by pipeting and centrifugation (for suspension cells) or by trypsonization (for adherent cells). Control cells that had been mock-infected were loaded with CCF2-AM dye. Flow cytometry analysis was used to detect infection as conversion from uncleaved to cleaved CCF2-AM with compensation between these colors determined using beads stained with IgG conjugated with AM-Cyan or Pacific Blue.

Infection using replication-competent virus

Plasma-derived SIVmac251 *env* genes were cloned into p239SpE3' provided by the NIH AIDS repository through J. Hoxie, J. Romano, and A. Jordan as described (7, 13). This particular clone of p239SpE3' had been modified by JH, JR, and AJ to remove a HindIII site in the 5' fragment of the *tat*/*rev* gene by silent mutation, allowing excision of the majority of mac239 *env* gene (a.a. 70 to the c-terminus) using 5' HindIII and 3' SacI sites within *env*. This *env* fragment was replaced with a HindIII/SacI fragment from 2 env clones in our study: RZu4.d42.1.1 and RPe6.d42.7.2. pVP2 clones were then cut at the SphI restriction site (in the *vpr* gene), and ligated to the large SphI fragment of p239SpSp5' to form full-length replication-competent chimeric SIVmac239 containing plasma-derived *env* genes. 2 ug of each plasmid was tranfected into 10^6 293T cells, and virus was harvested and quantified by p27. 100 ng p27 was used to infect $2x10^6$ target cells (cell lines or stimulated human CD4+ T cells) in R10 media. Cell lines were generated by George Leslie and James Hoxie and included supT1-rmCCR5 (expresses human CD4 and RM CCR5), BC7-rmCCR5 (expresses RM CCR5 but not CD4), and BC7 (control cells expressing neither CD4 nor CCR5). Media was collected at various intervals and p27 was detected by ELISA.

Results

Env from d42 CD4-depleted animals mediates increased entry into human MDMs than does Env from control animals

In order to test whether plasma-derived CD4-independent Env variants mediate entry into macrophages, we used luciferase reporter pseudotype virus containing d42 Env from control or CD4-depleted animals to infect human monocyte-derived macrophages (MDMs) from 3 independent donors. As shown in **Figure 5.1**, luciferase levels were significantly higher in MDMs infected with viruses carrying Env from CD4-depleted animals than from control animals for 2 out of 3 donors tested. We fit the data from the 3 donors to a linear model that tested whether Env from CD4-depleted animals mediated entry more efficiently than control Env in MDMs across all donors and found this relationship to be statistically significant. Thus, plasma-derived CD4-independent SIV Envs mediate slightly enhanced entry into human MDMs compared to control Envs.

A virus-cell fusion assay does not reveal infection of MDMs or PBMCs by virus carrying CD4-independent Env

Since the above luciferase-based assay requires viral entry, reverse transcription and integration in order to detect infection, we thought that post-entry restriction might be influencing the readout. We also knew from our work in **Chapter IV** that these CD4-independent Env variants use human CCR5 inefficiently. To circumvent both of these challenges, we generated rhesus macaque MDMs and employed an assay using viral particles pseudotyped with Env and loaded with HIV Vpr fused to β-lactamase (BlaM-vpr; (3)), and measured conversion of a fluorescent dye in infected cells rapidly following infection. The assay effectively detected infection of human MDMs by viruses containing either HIV.BaL Env and VSVg, which are known to infect macrophages highly-efficiently (**Figure 5.2**). However, we were unable to defect robust infection of either human or RM MDMs by a limited panel of our control and CD4-independent Envs using this assay (**Figures 5.2, 5.3**). We also tested whether the E84K and D470N mutations, which modulate the CD4-independent phenotype in these Env variants, could alter macrophage infection, but found no effect (**Figures 5.2, 5.3**). One noteable exception

was Env RUt5.4.3, for which the parental Env mediated entry into human MDMs at a rate of 2%, but whose N470D.K84E mutant mediated entry at 20%, perhaps suggesting that enhanced coreceptor use plasticity allowed increased infection in the mutant Env. Since extensive macrophage infection was observed *in vivo* in CD4-depleted animals, our results suggest that rhesus macaque MDMs from the blood may not accurately reflect infection of mucosal and CNS resident macrophages.

To ensure that the BlaM-vpr assay could indeed detect SIV Env-mediated entry, we also tested the ability of our CD4-independent and control plasma-derived Env variants to mediate fusion to primary RM PBMCs. Fusion mediated by control CD4-dependent SIV Env was detected as cleavage of CCF2-AM dye in PBMCs, which represented *bona fide* Env-mediated entry because it could be blocked by #19 antibody (**Figure 5.4**). However, only one out of 2 CD4-independent parental Envs tested (RPe6.d42.7.2) mediated detectable fusion to PBMCs. A second CD4-independent parental Env (RUt5.d42.4.3) did not mediate detectable fusion in PBMCs, but the CD4-dependent K84E N470D mutant form did. Furthermore, as was the case with MDM infection, D470N E84K mutants of parental control Env did not mediate fusion with target PBMCs. These observations demonstrate that in this particular system, CD4-independent Envs are mostly unable to mediate fusion to target cells, whereas CD4-dependent control Envs can mediate entry into primary PBMCs.

Full-length SIV containing CD4-independent Env replicates in CD4-negative cells but not primary human PBMCs

Pseudotype virus carrying Env may not be fully predictive of a replication-competent virus, so we generated chimeric SIVmac239 containing either a representative control Env (RZu4.d42.1.1) or CD4-independent Env (RPe6.42.7.2) from our studies. These viruses could both replicate in SupT1 cells expressing human CD4 and rmCCR5, although RPe6.42.7.1 was attenuated (**Figure 5.5**). Consistent with its ability to mediate CD4-independent entry in the single-round pseudotype assay, RPe6.42.7.2 could also replicate in BC7-rmCCR5 cells, which lack CD4 (although not to the same levels as in

CD4+ cells), whereas RZu4.d42.1.1 could not. This confirmed that RPe6 is CD4-independent in the context of a replication-competent virus, although replication was attenuated overall, at least in the context of a Mac239 chimera. However, in primary human cells, RPe6.42.7.2 failed to replicate at detectable levels whereas RZu4.d42.1.1 exhibited normal growth (**Figure 5.6**). This data is consistent with our findings in **Chapter IV** that suggest more limited entry pathways and attenuation among CD4-independent Env variants compared to controls.

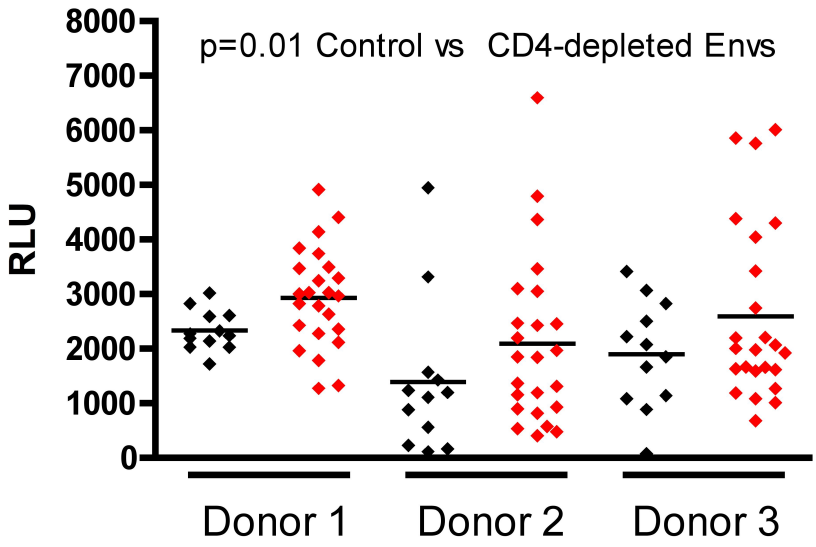

Figure 5.1. Pseudotype virus infection of human monocyte-derived macrophages. Pseudotype viruses carrying either control (**black**) or CD4-depleted (**red**) Env were used to infect monocyte-derived macrophages from 3 healthy human donors, and infection was read by measuring relative light units (RLU). Each diamond represents a single Env clone. Data was fit to a linear model comparing control versus CD4-depleted Env infection across donors to determine statistical significance.

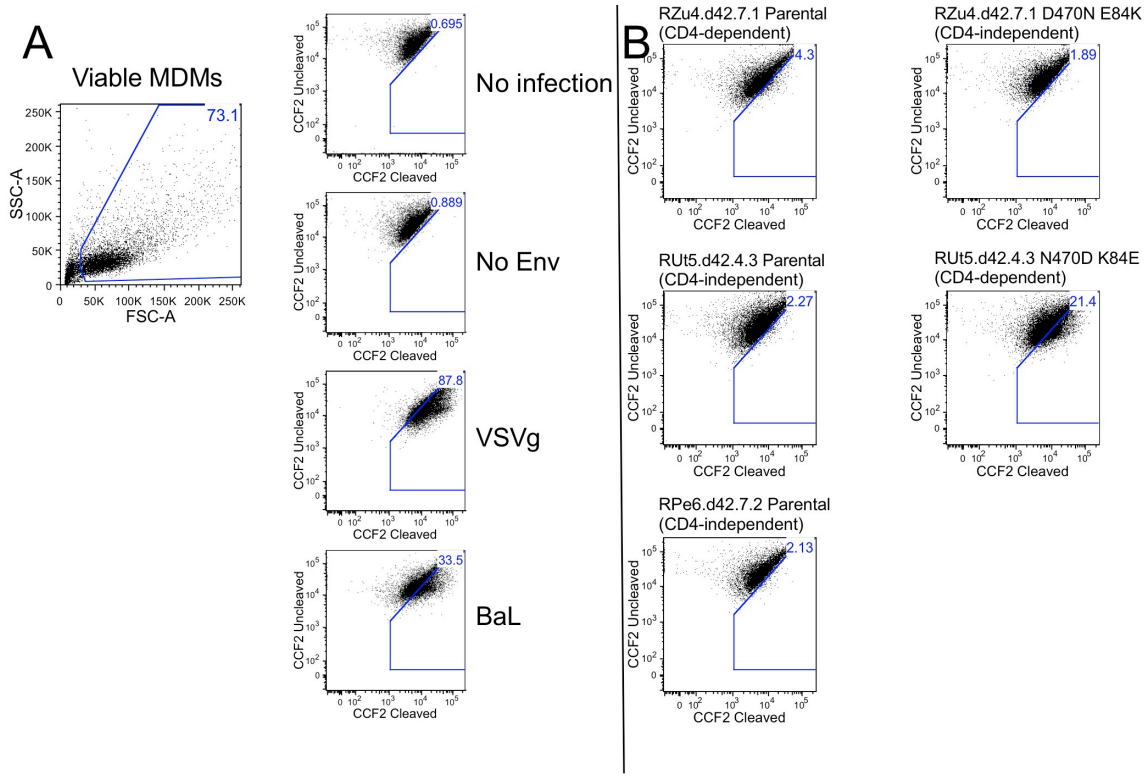

Figure 5.2. BlaM assay can detect virus fusion to human MDMs. Env pseudotype viruses loaded with BlaM-vpr were used to infect human MDMs. **A**) Gating of viable MDMs (**right**) and detection of infection by gating against and un-infected control. Percent infected cells is indicated by the number inside of the gate, with VSVg and BaL used as positive controls. **B**) A select panel of Env pseudotype viruses and their infection of human MDMs in the BlaM assay is shown.

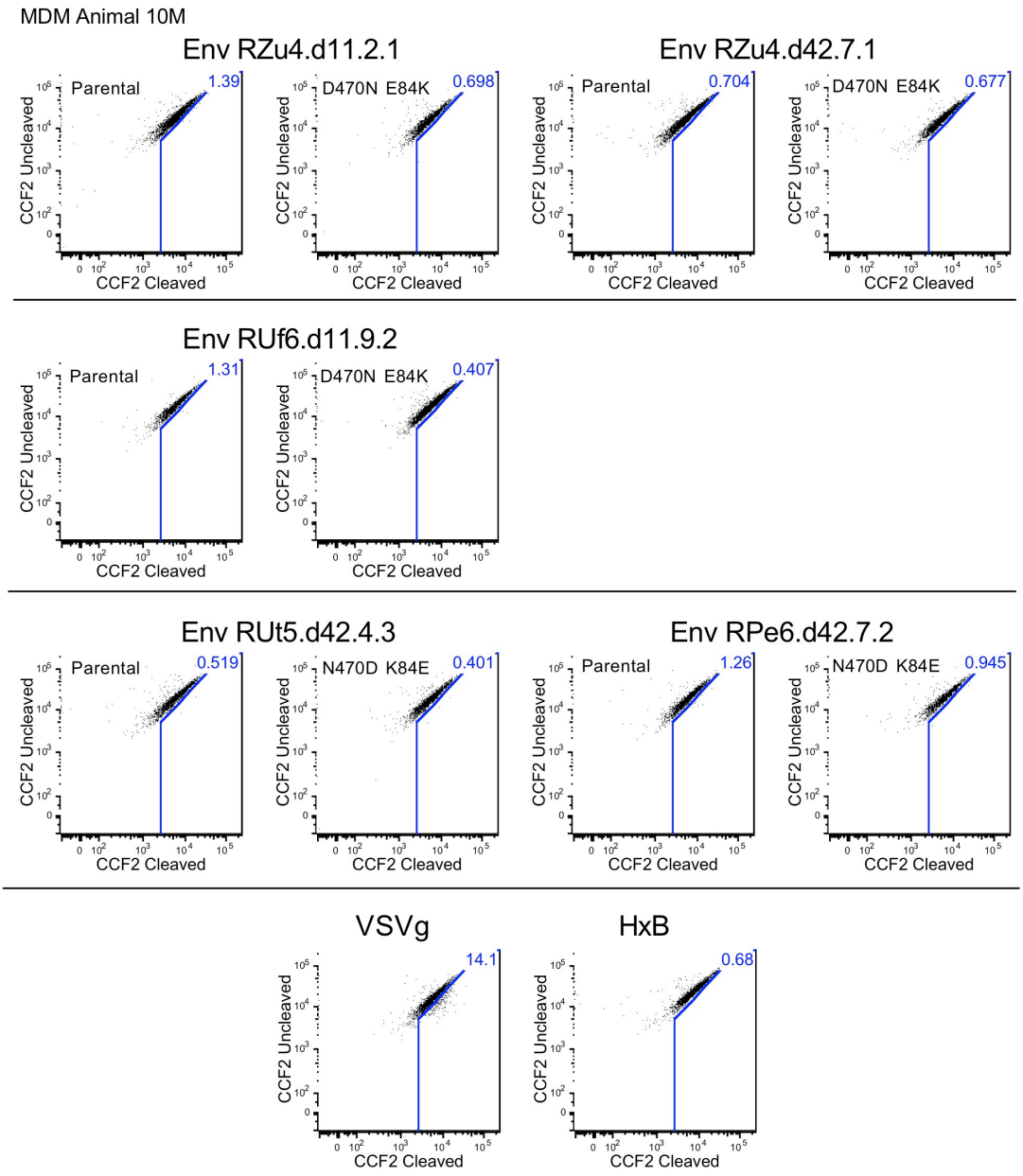

Figure 5.3. BlaM assay detection of infection of RM MDMs. Env pseudotype viruses loaded with BlaM-vpr were used to infect RM MDMs from animal 10M, and percent infected cells for each panel is indicated within the gate. A panel of CD4-dependent Envs and their respective CD4-independent mutant D470N E84K forms (**top 2 rows**) were tested along with 2 CD4-independent d42 CD4-depleted Envs and their respective CD4-dependent N470D K84E mutants (**3rd row**). VSVg was used as a positive control and HxB was used as a negative (**bottom row**).

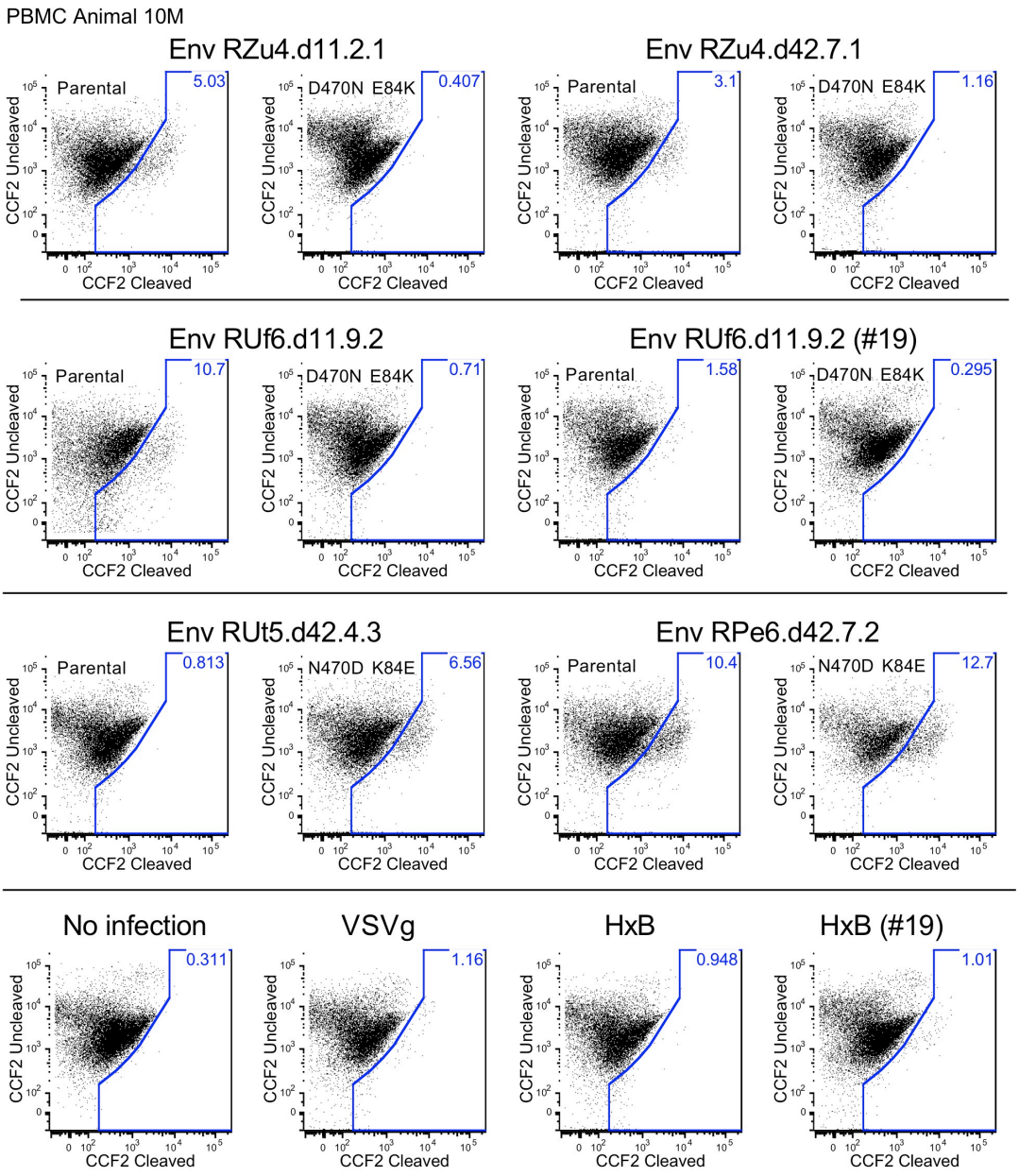

Figure 5.4. BlaM assay can detect virus fusion to RM PBMCs. Env pseudotype viruses loaded with BlaM-vpr were used to infect RM PBMCs from animal 10M, and percent infected cells for each panel is indicated within the gate. A panel of CD4-dependent Envs and their respective CD4-independent mutant D470N E84K forms (**top 2 rows**) were tested along with 2 CD4-independent d42 CD4-depleted Envs and their respective CD4-dependent N470D K84E mutants (**3rd row**). For one CD4-dependent Env, #19 antibody was included as a control to block infection. VSVg was used as a positive control and mock-infected cells as a negative (**bottom row**). Surprisingly, VSVg and HxB were not able to mediate infection into RM PBMCs.

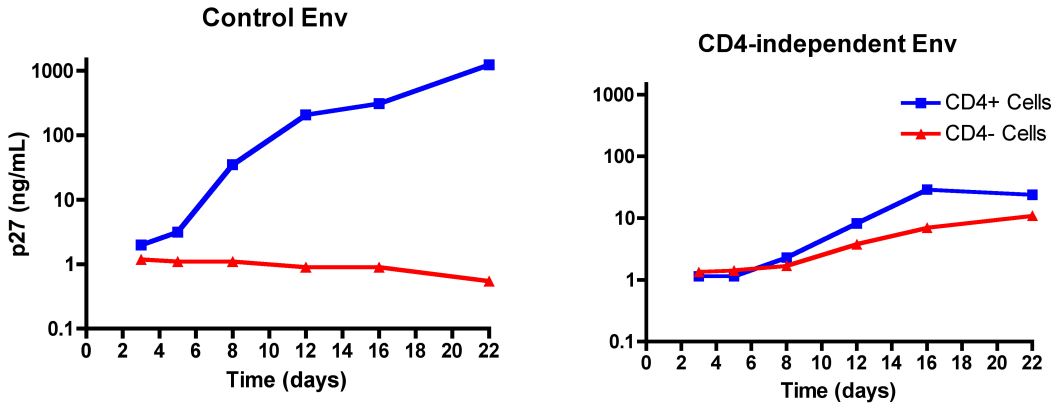

Figure 5.5. Growth of chimeric SIVmac239 in cell lines. SIVmac239 carring either Env RZu4.d42.1.1 (**left**) or RPe6.d42.7.2 (**right**) was used to infect an rmCCR5+ cell line that expresses human CD4 (SupT1-rmCCR5; **blue**) or has had the CD4 gene removed by a zinc finger nuclease (BC7-rmCCR5; **red**).

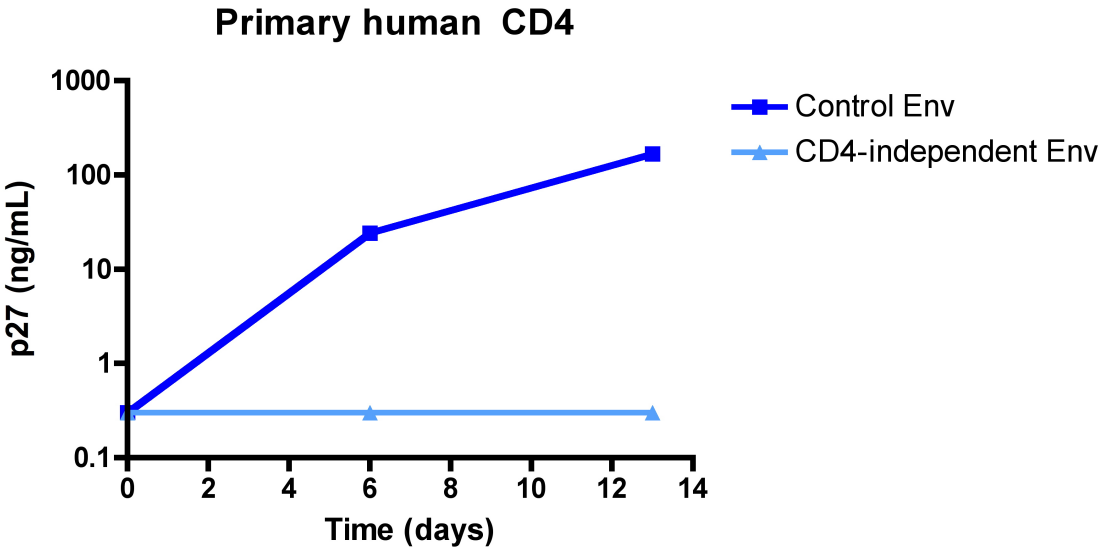

Figure 5.6. Growth of chimeric SIVmac239 in primary human CD4+ T cells. SIVmac239 carring either Env RZu4.d42.1.1 (**dark blue**) or RPe6.d42.7.2 (**light blue**) was used to infect primary human CD4+ T cells and p27 ELISA was performed to assess viral growth.

Conclusion

Here we sought to further characterize the *in vitro* phenotype of plasma-derived Env variants from CD4-depleted or control animals. As expected, CD4-independent Envs from CD4-depleted animals were better able to infect macrophages than were Envs from control animals (**Figure 5.1**). Puzzlingly however, these Env variants did not fuse to rhesus MDMs despite the functionality of the assay (**Figures 5.2, 5.3, 5.4**), suggesting that MDMs may not be the best model for accurately predicting macrophage tropism *in vivo*. This concept has been suggested before (2, 10), and may be related to one or more of several factions: 1) the lack of co-receptor plasticity in these Env variants, which we described in **Chapter IV**; 2) differences in cellular response to stimulants between rhesus and human monocytes; or 3) our cellular isolation and purification processes, which may not completely reflect *in vivo* cells.

We also compared growth in T cell lines and human CD4+ T cells of SIVmac239 carrying either a CD4-independent or control *env* gene (**Figures 5.5, 5.6**). We were surprised at three of our observations: 1) Growth of the CD4-independent virus was attenuated compared to the control virus in cells expressing CD4 and CCR5; 2) While the CD4-independent virus grew in CD4-negative cells, p27 levels did not reach the levels that were observed in CD4-positive cells; and 3) The CD4-independent virus did not grow in human CD4+ T cells. Again, these observations could be related to our studies in **Chapter IV**, where CD4-independent Envs required very high levels of CCR5 in order to mediate robust entry. This could also be related to the binding avidity of the virus for the cells – perhaps bypassing CD4 engagement prevents the virus from engaging CCR5 when cells have not been spinoculated in a single-round infection. It may be necessary in future studies to use tissue-derived macrophages as targets for infections (as opposed to the monocyte-derived macrophages used here). Alternatively, in order to conclude more definitively that D470N and E84K mediate macrophage tropism *in vivo*, infected macrophages and other cell types from infected animals could be isolated, and virus from these cell types could be tested for the presence of D470N and E84K.

Despite the surprising general lack of macrophage and PBMC entry mediated by our CD4-independent Env variants, we are still confident that when these variants are indeed mediating robust infection of macrophages *in vivo*, as CD4-depleted animals experienced extremely high viral load and widespread infection of macrophages. Also, our newly-derived replication-competent SIVmac239 chimera will be useful tools for future studies related to this work. It would be interesting to test whether our full-length CD4-independent virus could mount a productive infection of either control or experimentally CD4 depleted rhesus macaques.

Acknowledgement

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CHAPTER VI

DISCUSSION AND IMPLICATIONS

This thesis has focused on the role of cellular tropism in HIV/SIV infection and how antibodies and coreceptor use plasticity act to enforce CD4-dependent infection *in vivo*. This final chapter places these novel findings into the context of other current research and presents a series of models based on our findings. It will conclude with a proposal for future lines of experiments that will test the accuracy of these models and may provide further insight into the current challenges to HIV-1 eradication and the development of vaccines.

Why do primate lentiviruses use CD4 as a receptor?

This thesis has presented an analysis of an *in vivo* model system in which CD4, often considered the "primary" receptor for HIV and SIV, is remove as a requirement for infection. Results from this work, along with those from previous studies of other CD4-independent variants, suggest that it is the 7TM receptor, not CD4, that is the actual receptor that carries out indispensible functions for HIV and SIV entry (23, 24, 26, 45). This theory is also supported by evidence from non-primate lentiviruses, all of which require 7TM receptors, but not CD4, for entry (12, 13, 51). Most lentiviruses do bind an initial receptor prior to 7TM receptor engagement, which means that the 2-step entry process is evolutionarily conserved; CD4 use is conserved among primate lentiviruses in particular (13, 51). We have examined at least two mechanisms that may underlie this conservation of CD4 use:

1. Abundant antibodies produced during normal infection potently neutralize virus whose Env protein exists constitutively in a CD4-bound conformation, preventing the emergence of CD4-independent viruses (**Chapter III**).
2. Viruses that do not require CD4 for entry also lack plasticity in use of 7TM receptors, potentially limiting the range of potential target cells (**Chapter IV**).

In addition to the mechanisms that we have suggested here, there are multiple other hypotheses to explain primate lentivirus use of CD4 as a receptor, which are neither mutually exclusive of each other nor of ours listed above:

3. Binding to CD4 improves the avidity of the virus-cell interaction, enhancing entry (52).
4. Destruction of CD4+ T cells through direct infection dampens the host adaptive immune response to HIV/SIV infection (15, 39).
5. Limiting infection to CD4+ cells helps the virus avoid innate and adaptive immune responses that are specifically triggered in other cells types upon viral infection (29, 34).

The common theme among each of these theories is that although the requirement for two receptors for entry may superficially appear to be an evolutionary disadvantage to the virus in that it limits potential targets to a relatively narrow cell type (CD4+ T cells), there are in fact more nuanced evolutionary forces at play which keep cellular tropism in check.

How does our novel finding in point [1] above relate more broadly to the concept of host control of CD4 tropism? Immune regulation as a mechanism regulating viral tropism has been previously described and published before our work presented in Chapter III. The relationship between macrophage tropism and CD4-independent entry is well-established, as is the concept of neutralization-sensitivity of CD4-independent variants. Our work was the first to demonstrate this mechanism *in vivo*, and suggested that elimination of the CD4-inducible neutralizing activity normally present in the systemic compartment allows relaxation of CD4-dependent entry and adaptation to infect macrophages. This extensive infection of macrophages in animals carrying high levels of CD4-independent virus adds at least two further layers of complexity to immune regulation of CD4 use. First, direct cytolytic activity specifically targeting SIV-infected macrophages has been previously ascribed to CD4+ T cells (39, 46), a phenomenon which may be related to CNS disease observed in macaques depleted either of naïve or total CD4+ T cells prior to SIV infection and therefore unable to mount a CD4+ T cell-dependent adaptive response (38). Second, macrophages and other myeloid cell types are known to specifically mediate innate immune recognition and antiviral effector functions

in the context of HIV and SIV infection (described in **Chapter II**). It is therefore clear that multiple components of the host immune response to HIV and SIV prevent the widespread emergence of CD4-independent entry: antibodies target the viral particles, myeloid-specific innate factors target the virus in infected cells, and cytolytic CD4+ T cells destroy infected cells.

The second possible mechanism we presented as potentially contributing to the evolutionary conservation of CD4 use in primate lentiviruses, a lack of co-receptor plasticity in CD4-independent SIV variants (**Chapter IV**), is perhaps more surprising. **Figure 6.1** illustrates a model that summarizes our findings and their potential implications. We initially expected that Env from CD4-depleted animals would have an expanded range of co-receptor use, similar to what is seen in SIV-infected sooty mangabeys infected with an SIVsmm strain that induces CD4+ T cell depletion but does not result in immunodeficiency (16, 21, 35). Instead we found that at least in the context of experimentally CD4-depleted macaques, the CD4-independent variants that dominate in plasma lack the ability of to use GPR15, human CCR5, and low levels of macaque CCR5. When we tested the ability of our CD4-independent Env variants to mediate entry into CD8 T-cells (human derived) or PBMCs (human or RM derived), we again observed only modest entry (**Figures 4.1, 5.3** and data not shown), and no drop in CD8+ T cell counts was observed in CD4-depleted animals infected with SIV (39). We can speculate that this finding is at least partially related to our observation in **Chapter IV** that CD4-independent Envs are impaired in use of human CCR5 and more generally lack the coreceptor use plasticity necessary for engagement of CCR5 in various primary cell types.

Using our assays, it is difficult to know whether these primary cells reflect the cell types infected *in vivo* (**Chapter V**), but it would appear that CD4+ T cells and macrophages are the principal targets for infection by CD4-independent SIV *in vivo*, and infection is further limited to cells expressing extremely high levels of CCR5. This is in contrast to virus that requires CD4 for entry, which may not infect macrophages or other CD4-low

cells, but could infect CD4+ T cells expressing GPR15 or low levels of CCR5. Previous studies suggest that even in rhesus macaques, generally considered to have higher lymphocyte expression of CCR5 than other primate species, detectable expression is still limited to fewer than half of circulating T cells. Furthermore, CCR5-positive cells are characterized not by a discretely high-level expression population, but rather by a range of expression levels ranging from extremely low to moderately high (36, 40). So the ability to scavenge relatively low or undetectable levels of CCR5 on the cell surface is presumably an important characteristic of SIV Env. Efficient use of CCR5 may also reflect the ability of Env to engage CCR5 expressed in ligand-bound, drug-bound, or alternate conformations on the cell surface, features which would allow broad cellular targeting (2, 9, 30). Furthermore, the capacity to use alternative (non-CCR5) co-receptors, such as GPR15 or others may additionally increase the potential range of target cells for the virus. Finally, the fact that CD4-dependent SIV Envs were able to use human CCR5 more efficiently to enter cells than CD4-independent Envs, suggests that cross-species transmission may be strictly involve CD4-dependent variants, an idea that is in line with the general consensus that transmitted viral variants are non-macrophage-tropic but may be characterized by efficient CCR5 engagement (41, 42, 55).

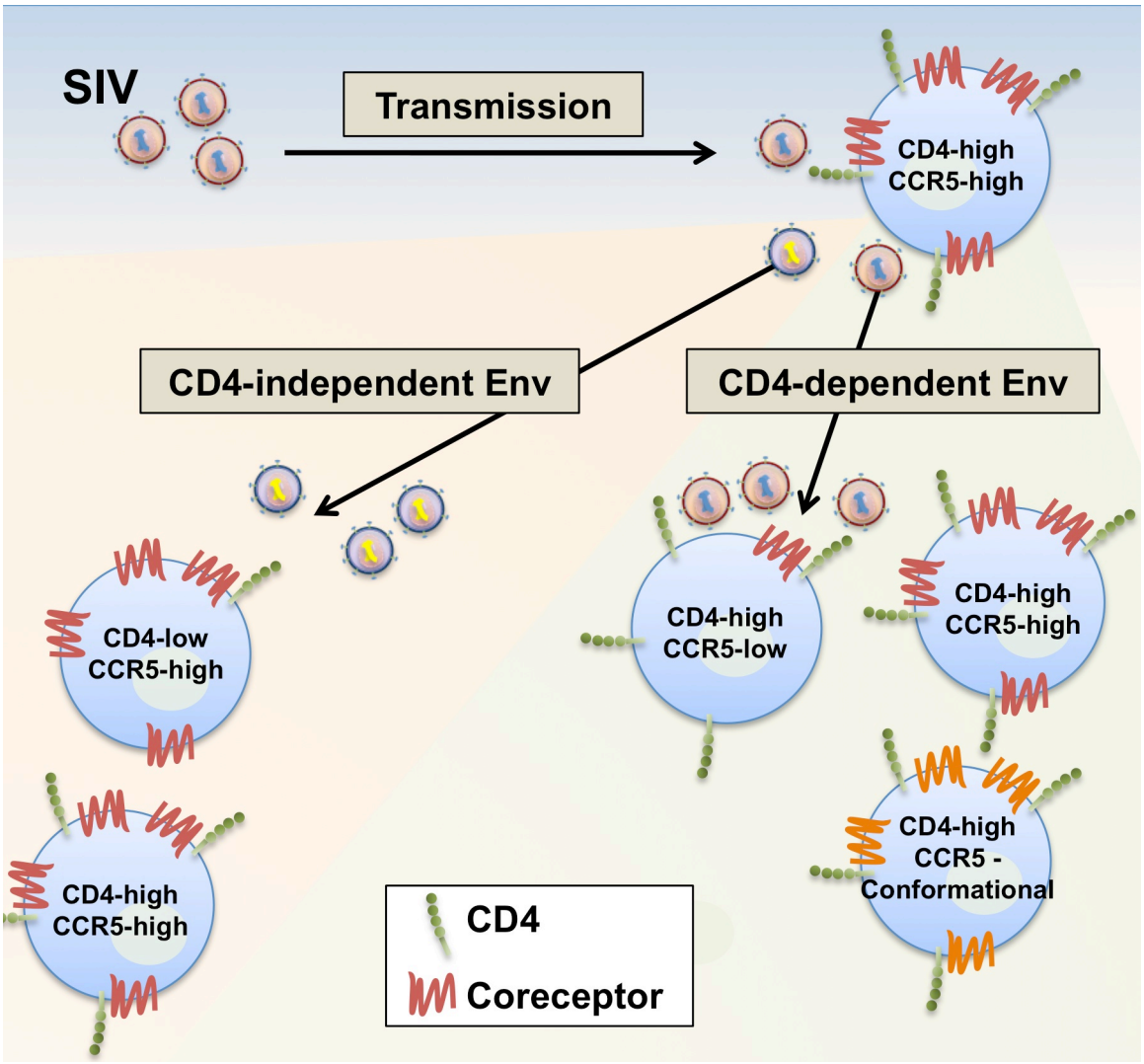


Figure 6.1. Model of CD4-independent entry and plasticity of co-receptor use. This model begins with SIV transmission at the mucosa to the initial target cell thought to be a CD4+ T cell expressing CCR5. As the viral swarm replicates and diversifies, the target cell availability, host response, and compartmentalization may direct individual lineages towards either a CD4-dependent (right) or CD4-independent (left) pathway. Since the CD4-dependent virus is capable of using a diverse range of co-receptors in conjunction with CD4, the number of potential cell targets is increased, and there is even a potential for cross-species transmission.

Macrophages as a latent CNS reservoir and target for eradication

Despite effective highly active antiretroviral therapy (HAART) for the treatment of HIV infection and ever-expanding access to such treatment, an estimated 30 million people worldwide are currently living with HIV (1). HAART, while generally effective in reducing viral load and restoring CD4 T cell counts and immune function, is not curative and is often associated with long-term complications and sub-optimal levels of patient adherence (37). Cessation of HAART or poor patient adherence invariably results in a rebound of viral load and a drop in CD4 T cell levels (6, 18, 57). A significant barrier to a complete and sterilizing cure for HIV is the long-term survival of latently-infected cells which harbor the reservoir of integrated viral DNA and contribute to the rebound in viral load upon cessation of HAART (3, 17, 18). The identification and targeted eradication of this viral reservoir is therefore an important and ongoing topic of research.

Multiple models, each supported by a wealth of evidence beyond the scope of this dissertation, implicate unique mechanisms for the establishment and long-term survival of a latent HIV reservoir (3, 5):

1) Partially- or fully-activated CD4+ T cells are infected during acute infection, but unlike most infected cells, do not undergo apoptosis, and instead return to a resting state, where they survive and persist without producing viral proteins and thus remain undetected by CD8-mediated immunity. Reactivation of the cells in response to pathogens or other stimuli results in the production of viral particles and subsequent spreading infection. This model is attractive because it involves the virus infecting its canonical target, an activated CD4+ T cell. Its shortfall is that it doesn't explain how an infected cell at a mucosal site of inflammation deactivates and returns to an anatomical compartment where it can survive long-term.
2) Resting CD4+ T cells are directly infected, and similarly to above do not attract a cellular immune response. Again, in response to antigen, these cells become activated and produce infectious virus. The strength of this model is that that it doesn't involve deactivation and long-term survival of effector cells, which are generally thought to be terminally differentiated and subject to rapid apoptosis. The

limitation of this model is the general lack of cellular co-factors for viral infectivity in resting cells, which don't express high levels of CCR5 or post-entry factors involved in integration and reverse transcription.

3) Macrophages, either in the CNS, lymph nodes, mucosal compartments, or as circulating monocytes, become infected, and due to their relative senescence remain undetected by the immune system while harboring integrated viral DNA. Since macrophages are terminally differentiated and are known to survive long-term in vivo, no dramatic phenotypic changes are required to explain cell survival or virus reactivation (7).

Current dogma favors CD4+ T cells as the principal HIV latent reservoir, and that at least one of numbers "1" and "2" accounts for the establishment of such a reservoir (3, 5). However, it is also clear that in many HAART-suppressed HIV-1 patients, CNS macrophages are a significant source of persistent, if low-level, viral replication (43, 49, 50). Furthermore, it is also believed that the virus establishes this CNS infection relatively early during the course of infection via infection of circulating monocytes that then cross the blood-brain barrier and differentiate into macrophages (47, 48, 56). Our data presented in **Chapter III** on the timing of emergence of CD4-independent SIV variants in CD4-depleted macaques as well as of CD4-inducible neutralizing activity in control SIV-infected animals, suggests that the acute phase of HIV-1 infection should provide a permissive environment for peripheral monocyte/macrophage infection, possibly at sub-detectable levels. Thus it is likely that macrophage infection, likely in the CNS but possibly in other tissues, is established early during the disease course by virus originating from the periphery (**Figures 6.2, 6.3**). A limited line of evidence additionally suggests that this macrophage-tropic virus may reemerge in the periphery during the late stages of untreated disease, when most CD4+ T cells have been depleted and the antibody response to the virus has waned (31). It is also possible that establishment of the CNS reservoir and the accompanying long-term complications can be prevented in new infections, a concept which is explored below.

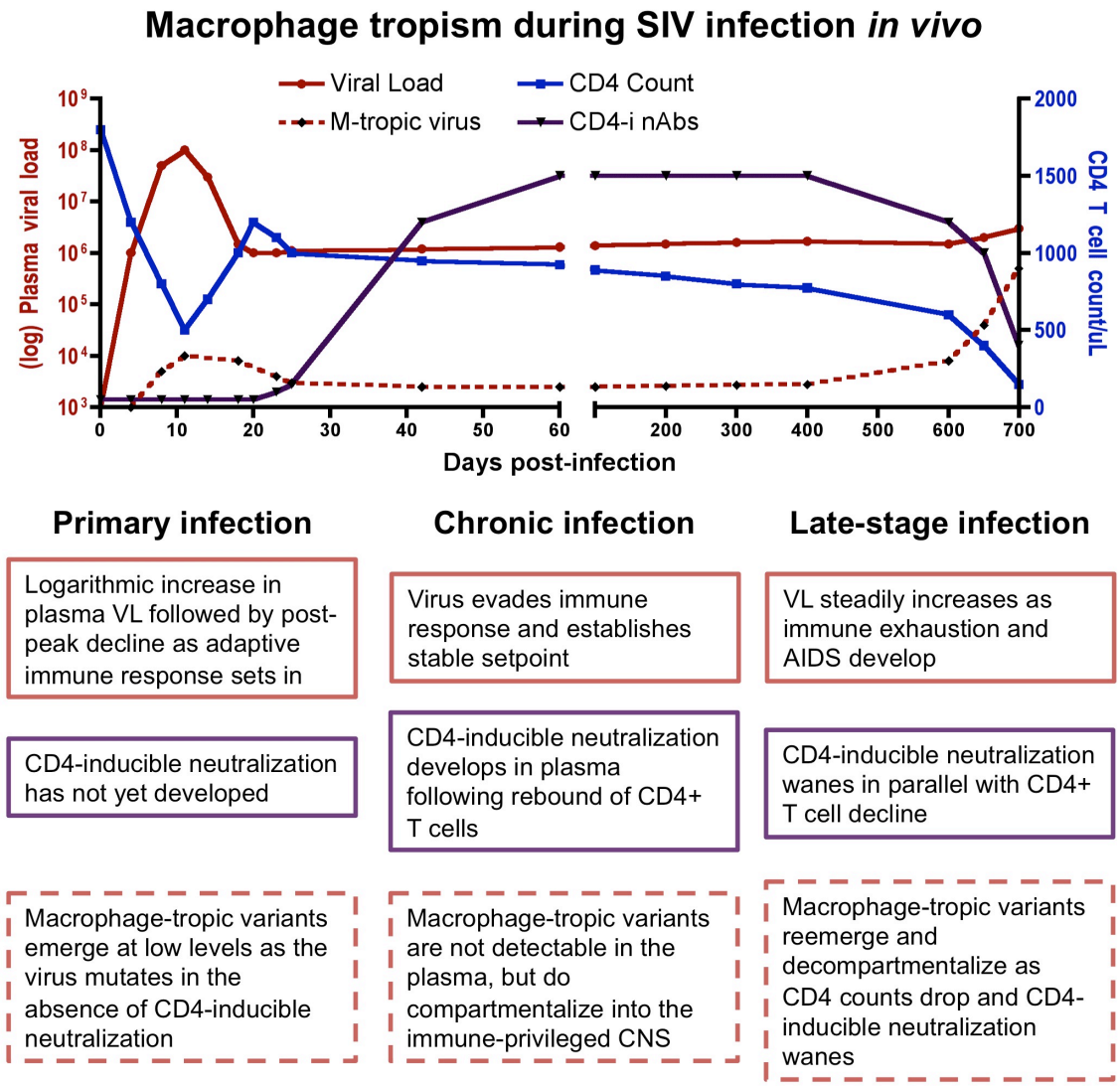
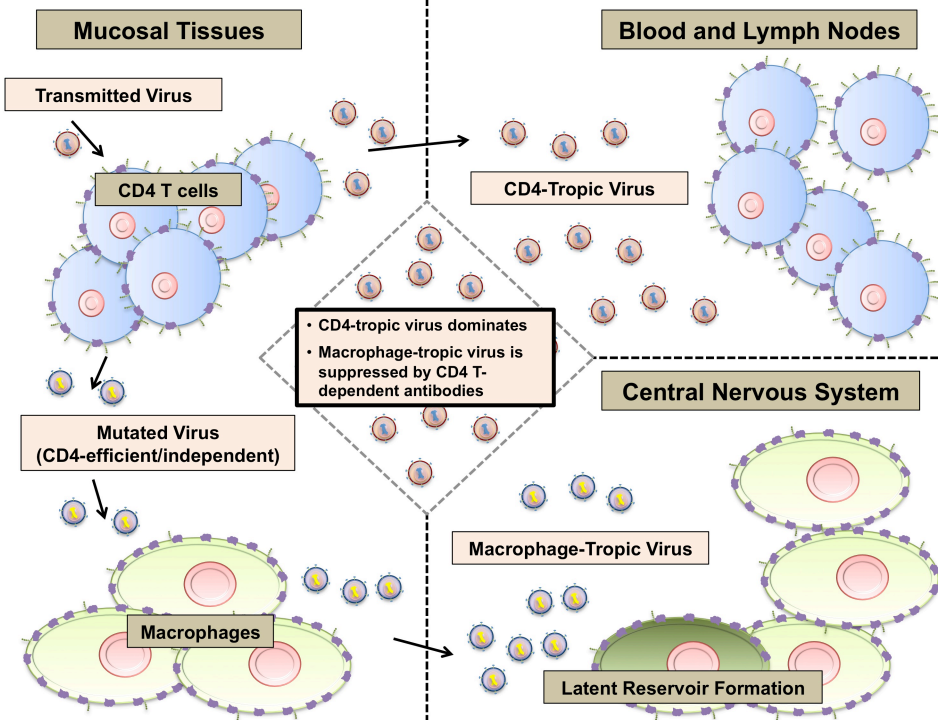
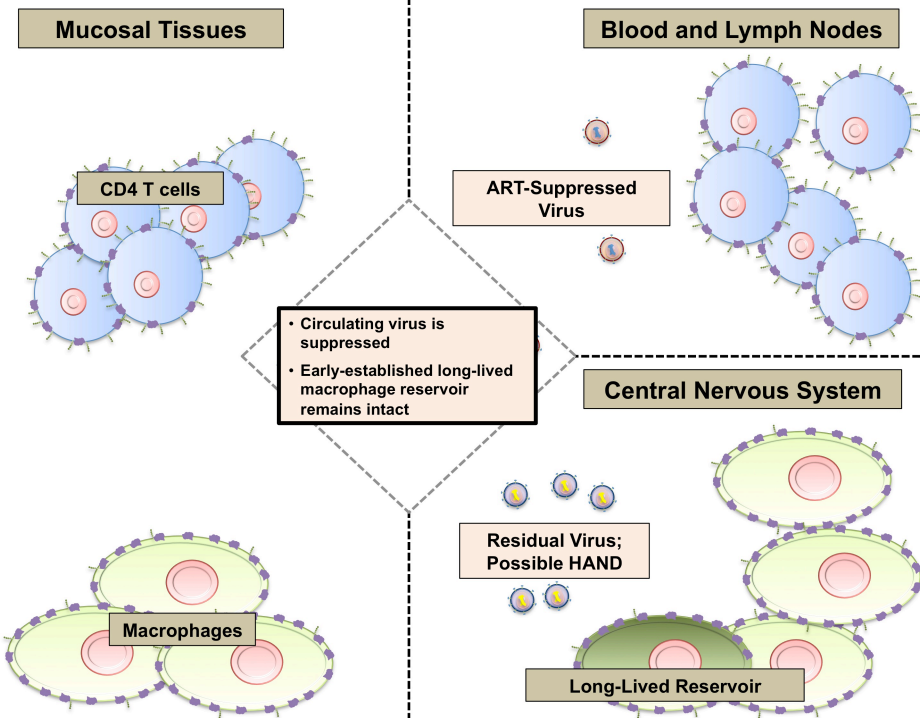


Figure 6.2. **Natural history of untreated SIV infection of rhesus macaques**, with viral load and CD4+ T cell counts from previous studies (see **Chapter I**) indicated on the left and right axes, respectively. Also added is an estimate of the production of plasma antibodies that neutralize SIV in a CD4-inducible manner (CD4-i nAbs), based on our data in **Chapter III** as well as estimated relative levels of macrophage-tropic virus based on ours and previous studies of HIV and SIV. The curves representing CD4-i nAbs and M-tropic virus are not intended to indicated precise quantities, but rather relative levels compared to baseline. This particular model represents SIV infection but could also be applicable to HIV-1 infection of humans.

A. Early untreated infection



B. ART suppression after early untreated infection



C. Possible prevention of CNS reservoir formation

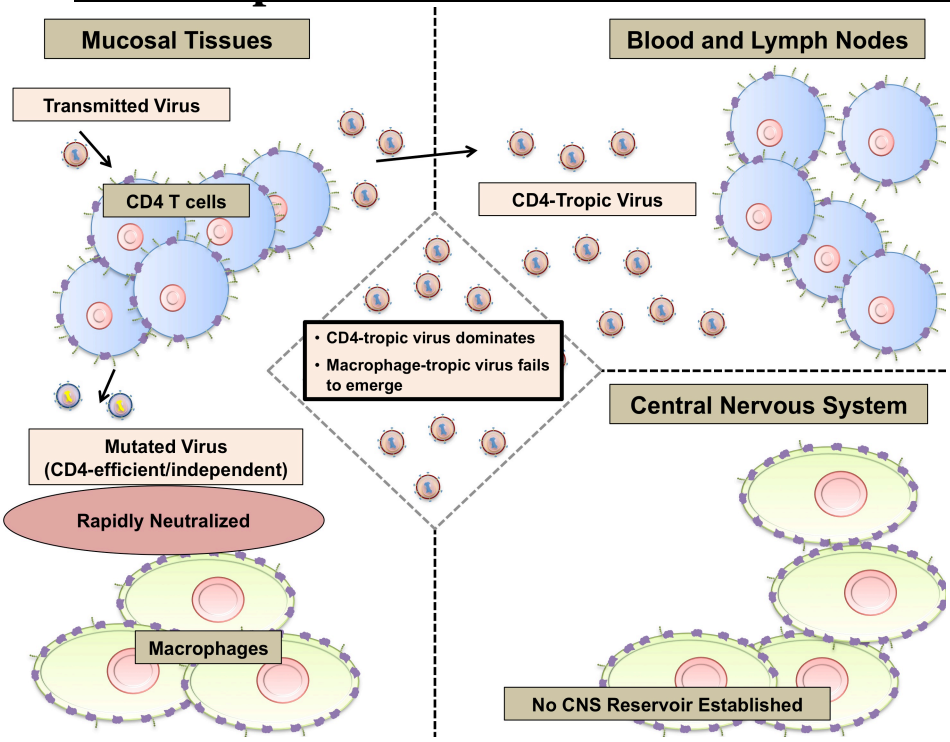


Figure 6.3. (beginning on previous page) **Model of macrophage reservoir formation and persistence during ART therapy**. Low levels of macrophage-tropic virus may be present early during primary infection, prior to the host production of antibodies with CD4-inducible neutralizing activity. These variants may be largely inconsequential during peak infection when they are suppressed by the antibody response (**A**), but they persist during anti-retroviral therapy (ART) and continue to replicate in the CNS, in conjunction with possible HIV-associated neurologic disorders (HAND). Macrophage-tropic variants can later emerge if ART is discontinued (**B**). However, intervention early in infection – i.e. vaccination to induce Env-binding antibodies - could neutralize the early macrophage-tropic variants and prevent reservoir formation (**C**).

Can antibodies that neutralize in a CD4-inducible manner prevent the formation of an HIV-1 macrophage reservoir?

Our model, in which neutralization-sensitive SIV variants dominate and replicate at extremely high levels in animals that have been experimentally depleted of CD4+ T cells, reveals a function for antibodies that are non-neutralizing except against virus that has been pre-triggered with sCD4. Based on our ELISA data (**Figure 3.9**) and similar studies of plasma from HIV-1-infected individuals, it appears that at least from a standpoint of neutralization potential, antibodies that neutralize in a CD4-inducible manner are the major component of the antibody repertoire generated in response to HIV/SIV infection (11, 14, 19). While anti-HIV effector functions have been ascribed to non-neutralizing anti-Env antibodies (20, 25, 33), the novel function we describe (enforcement of CD4 tropism) is intriguing in that it appears to be so universal and readily induced (4). This is in sharp contrast to broadly neutralizing antibodies, which make up an extremely small fraction of the Env antibody repertoire (4, 28). The relative abundance of CD4-inducible neutralizing activity during chronic infection (but possibly not late stage infection) explains the relative lack of macrophage infection observed during this time (see model **Figure 6.2**). Furthermore, data from longitudinally monitored HIV-1 infected individuals has suggested that a long-lived macrophage viral reservoir is established relatively early in infection, presumably prior to the emergence of CD4-inducible or other neutralizing activity (47, 48). A line of research based on clinical data could answer whether CD4-inducible neutralizing antibodies are able prevent formation of this reservoir, and a proposal for future studies is described below.

Clinical trials testing the vaccine efficacy of recombinant and vector-expressed HIV-1 Env have demonstrated either a weak protective effect or no effect against acquisition, and no protective effect in reducing viral load or slowing disease progression (44). In these trials, subjects at a high risk for HIV acquisition were vaccinated with either a pox vector encoding HIV-1 gp160 or recombinant HIV-1 gp140 protein, or both. Most vaccinated individuals developed only a weakly-neutralizing or non-neutralizing anti-Env response, but did develop relatively high binding titers of IgG targeting gp120 V3 and

other epitopes (8, 58), specificities which we have demonstrated neutralize in a CD4-inducible manner (**Figure 3.5**). Detailed structural studies of intact Env spikes on virions have in fact suggested that the soluble recombinant forms of Env used for vaccine trials do not expose the same epitopes as native Env, but instead exist constitutively in a state that resembles the CD4-bound conformation (27, 32, 53, 54), likely making CD4-inducible epitopes constitutively available for induction of antibodies specific to these regions. We therefore would hypothesize that although the plasma of vaccinated subjects lacks broad and potent neutralizing activity, it does contain CD4-inducible neutralizing activity and should therefore prevent or delay macrophage infection and accompanying reservoir formation and HIV-associated neurologic disorders.

To test this hypothesis, we can pursue several lines of investigation using banked samples from gp160/gp140 vaccine trials:

1) Does plasma from vaccinated individuals neutralize HIV-1 that has been pre-triggered with soluble CD4? Does the neutralizing activity in this plasma target macrophage-tropic and/or CNS-derived variants, and does it specifically prevent macrophage infection in the context of a heterogeneous swarm containing macrophage-tropic variants? These questions can be answered by simply obtaining banked plasma samples from phase I clinical trials and testing its activity against a panel of HIV-1 Env variants. As controls, unvaccinated, chronically (but not late-stage) infected individuals would be expected to have high levels of CD4-inducible neutralizing activity in their plasma.
2) In vaccinated individuals who later became infected and were treated with HAART, do the viral load second-order decay rates upon treatment suggest lower rates of viral infection in a long-lived cells compared to controls? Again, this information could be found in the medical records of patients who were closely monitored, without the need for any wet lab work.
3) In vaccinated individuals who later became infected, can macrophage and monocyte infection be detected in tissues and in the CNS? What is the incidence of HIV-associated neurologic disorders (HAND) in these individuals? Moderate levels of

tissue and CNS macrophage infection and high rates of HAND are expected in unvaccinated, untreated individuals, but macrophage infection and CNS disease may be more rare in vaccinated individuals due to the prevention of early establishment of a CNS reservoir.

If the above lines of experimentation revealed either potent plasma CD4-inducible neutralizing activity in vaccinated, uninfected subjects and/or decreased rates of macrophage infection among vaccinated, infected subjects, then it could be concluded that the non-neutralizing antibodies induced by the vaccine shape viral tropism early in infection, preventing macrophage infection and potentially altering the disease course. The shortcoming of this line of investigation is that studies that have investigated the genotype and phenotype of the dominant circulating viral variants in vaccinated individuals who later became infected have found no difference from the virus in unvaccinated infected individuals (10, 22). However, macrophage-tropic viruses are relatively minor variants during normal HIV-1 infection and nevertheless establish a CNS reservoir detectable late in infection, so absent deep-sequencing data from early in infection, the presence or absence of these variants cannot be concluded. Even with deep-sequencing analysis, macrophage tropism can be conferred by a wide array of genetic changes and might not be predictable based on sequence. Another problem we would encounter is that even if vaccination appears to alter viral tropism, all other data suggests an equally severe overall disease course with or without vaccination, which simultaneously reinforce the conclusions that 1) current vaccine strategies lack a protective effect; and 2) macrophage infection does not affect HIV-1 pathogenesis. However, if vaccination is found to prevent HIV-associated neurologic disorders, then we will have available a novel mechanism through which prophylaxis can reduce HIV-1 morbidity – antibody-mediated prevention of CNS reservoir formation.

In summary, we have demonstrated that the CD4 use and tropism for CD4+ T cells that is generally the rule for primate lentiviruses *in vivo* is maintained by the target cell availability and CD4-inducible neutralization present during normal infection. Whereas

CD4-independent entry and macrophage tropism are viable adaptive pathways for the virus, the forces maintaining CD4 tropism generally prevent these pathways from dominating. Using this knowledge as a foundation, we have proposed a line of investigation that may help in efforts to eradicate the macrophage HIV reservoir.

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