DISTINCT PATTERNS OF CCR5 VERSUS ALTERNATIVE CORECEPTOR DEPENDENCE IN NON-NATURAL HOST VERSUS NATURAL HOST SIMMIAN IMMUNODEFICIENCY VIRUS INFECTION

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Dedication

To my parents, Asa C. Elliott and Valerie J. Elliott, who always encouraged me to learn. And to my grandmother, Ruth L. Hoffman-Marshall, who taught me to be inquisitive.

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ABSTRACT

DISTINCT PATTERNS OF CCR5 VERSUS ALTERNATIVE CORECEPTOR DEPENDENCE IN NON-NATURAL HOST VERSUS NATURAL HOST SIMMIAN IMMUNODEFICIENCY VIRUS INFECTION

Sarah T.C. Elliott

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Natural host sooty mangabeys infected with simian immunodeficiency viruses (SIV) exhibit high plasma viral loads without widespread $CD4^+$ T cell loss. By contrast, non-natural host rhesus macaques experimentally infected with related SIV exhibit high viral loads but display subsequent $CD4^+$ T cell loss and progression to AIDS, analogous to the effects of HIV-1 infection in humans. Several mechanisms have been proposed to explain these discrepant outcomes, including infection of distinct target cells *in vivo*. Cell targeting is substantially determined at the level of viral entry. Prior work demonstrated that sooty mangabey infection occurs in the absence of functional coreceptor CCR5, implicating alternative SIV entry pathways in this natural host. In this thesis, I identified host-dependent patterns of SIV coreceptor use that distinguish viral entry in sooty mangabeys from viral entry in rhesus macaques. I cloned and characterized a panel of putative sooty mangabey coreceptors. Transfected target cells expressing species-specific CD4 and coreceptors identified sooty mangabey CCR5, CXCR6, and GPR15 as functional coreceptors of SIV *in vitro*. While rhesus macaque CCR5 and GPR15 also supported robust SIV infection *in vitro*, rhesus macaque CXCR6 was a remarkably poor coreceptor of SIV. Using CCR5 antagonist Maraviroc to block CCR5-mediated infection in primary peripheral blood mononuclear cells (PBMCs), I determined that SIV were highly CCR5-dependent in rhesus macaque PBMCs, whereas SIV were partially CCR5-independent in sooty mangabey PBMCs, implicating alternative coreceptors in infection of the natural host primary cells. A chemokine ligand of CXCR6, CXCL16, partially attenuated SIV infection of sooty mangabey PBMCs, indicating that sooty mangabey CXCR6 was indeed an alternative coreceptor of some SIV in primary sooty mangabey

cells. Collectively, these data define coreceptor use as a host determinant distinguishing sooty mangabey SIV infection from rhesus macaque SIV infection in primary cells. Because $CD4^+$ T cells from sooty mangabeys express exceedingly low levels of CCR5 relative to $CD4^+$ T cells from rhesus macaques, the expression patterns of CXCR6 and other alterative coreceptor(s) in sooty mangabey $CD4^+$ cell subsets may classify host target cells that maintain high levels of SIV replication without leading to loss of $CD4^+$ T cell homeostasis.

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CHAPTER 1

GENERAL INTRODUCTION

Despite major advances in therapy, the disease burden of human immunodeficiency type 1 (HIV-1) remains high. In 2013, 2.1 million people became newly infected with HIV-1 and there were approximately 35 million people living with HIV-1. The impacts of HIV-1 are especially critical in developing nations where up to 70% of new infections occur. In 2013, 11.7 million patients were receiving antiretroviral therapy in low- and middle-income countries, representing only 36% of people living with HIV in these regions (1). There remains a strong need for inexpensive and accessible therapies, new ways to modulate pathogenesis, a preventative HIV-1 vaccine, and/or a cure for HIV-1. Despite the ability of modern combination antiretroviral therapy (ART) to control HIV-1 viral loads and substantially lower the rate of progression to AIDS (acquired immunodeficiency syndrome), viral rebound inevitably occurs in patients on long-term ART with undetectable peripheral plasma viral loads when therapy is discontinued. Identifying and targeting persistent reservoirs of HIV-1 remains a major hurdle to treating and curing HIV-1 infection.

Efforts to further understand HIV-1 pathogenesis are also needed. Importantly, not all $CD4^+$ T cell loss following HIV-1 infection is a result of direct viral infection and killing (4, 33, 44, 45, 108). Chronic immune activation caused by HIV-1 infection is one of the major drivers of $CD4^+$ T cell loss and progression to AIDS. Chronic immune activation is a broad term encompassing many observed consequences in HIV-1 infection arising from the host pro-inflammatory immune response to the virus, the immune-modulatory functions of viral proteins, the immune response to opportunistic pathogens, the loss of gastrointestinal mucosal integrity and subsequent microbial translocation, the altered balance of critical $CD4^+$ T cell subsets, and the increased homeostatic proliferation of $CD4^+$ T cells (83). Some measures of immune activation correlate better with progression to AIDS than HIV-1 plasma viral loads (108). Furthermore, even patients with controlled viremia on successful ART experience chronic immune activation and consequent increased morbidity and mortality (93), highlighting the need for a better understanding of mechanisms leading to this activation. Given the multifactorial nature of chronic immune

activation, the relative contributions of each mechanism of chronic immune aviation to $CD4^+$ T cell loss and progression to AIDS remain largely undefined.

Strikingly, natural SIV (simian immunodeficiency virus) infection in nonhuman primate sooty mangabeys (*Cercocebus atys)* leads to ongoing viral replication without chronic immune activation or disease progression to AIDS. It is critical to understand how SIV infection and replication can occur in these primates without resulting in chronic immune activation. My goal for this thesis was to define SIV viral entry coreceptors in sooty mangabeys and to compare these to SIV viral entry coreceptors in rhesus macaques, primate hosts that progress to AIDS following infection with SIV. The identification of sooty mangabey target cells that express functional coreceptors of SIV may inform cellular reservoirs of viral infection in sooty mangabeys that maintain SIV replication without leading to chronic immune activation, loss of immune homeostasis, and disease progression in these natural hosts.

Origins of HIV and SIV

HIV-1 is responsible for the current worldwide AIDS epidemic. Multiple cross-species transmissions of SIV from nonhuman primates gave rise to HIV-1 (34, 103). Specifically, a cross-species transmission of SIV from chimpanzees (*Pan troglodytes troglodytes*, SIVcpz) resulted in HIV-1 Group M (**Figure 1.1**), by far the most prevalent HIV-1 globally. Recent studies demonstrate that SIVcpz is pathogenic in chimpanzee hosts (55). SIVcpz originated from cross-species transmission and recombination of viruses from red-capped mangabeys (*Cercocebus torquatus*, SIVrcm) and greater spot-nosed monkeys (*Cercopithecus nictitans,* SIVgsn) (5, 24, 36). In contrast to HIV-1 and SIVcpz, SIVrcm and SIVgsn infections are presumed to be non-pathogenic in their cognate hosts.

My research determined the viral entry characteristics of SIVsmm, a virus found in nonhuman primate sooty mangabeys. SIVsmm infection does not lead to AIDS or death over the typical

lifetime of its sooty mangabey host (94) (**Figure 1.2**). Sooty mangabey SIVsmm is of special interest because direct cross-species transmissions of SIVsmm to humans gave rise to HIV-2, another pathogenic HIV in humans (21, 51, 65) (**Figure 1.1**). Crucially, cross-species transmission of SIVsmm into Asian nonhuman primate rhesus macaques (*Macaca mulatta*) leads to simian AIDS (72). Multiple passages of SIVsmm in rhesus macaques gave rise to SIVmac, a group of viruses used in rhesus macaque challenge studies as models for vaccine development, new treatments, and AIDS pathogenesis (50). It is critical to define how SIVsmm can replicate in sooty mangabeys without leading to AIDS, whereas the same virus or very closely related viruses cause AIDS in rhesus macaques (107).

Natural and Non-Natural Hosts of SIV

Sooty mangabeys are referred to as natural hosts of SIV. SIV is endemic in these natural hosts. SIV-infected natural hosts typically exhibit high viral loads without a consequent loss of $CD4^+$ T cells or progression to AIDS (94, 104). (**Table 1.1**). Rhesus macaques are referred to as *non*-natural hosts of SIV. SIV infections of non-natural host macaques are experimental and generally lead to high viral loads, widespread $CD4^+$ T cell loss, and rapid progression to AIDS (104). We hypothesize that discrepancies in viral entry and consequent cell subset tropism are mechanisms whereby SIV infection of non-natural host rhesus macaques leads to widespread $CD4^+$ T cell loss while SIV infection of natural host sooty mangabeys does not.

Non-natural host infections, most commonly SIVmac 239 and SIVmac 251 infection of macaques, are used to model HIV-1 infection and pathogenesis in humans. The outcomes in HIV-1-infected humans are much like those in SIVmac-infected rhesus macaques. Without ART intervention, HIV-1 infection typically leads to high viral loads, loss of peripheral $CD4^+$ T cells, and progression to AIDS. It should be noted that disease progression in HIV-1-infected humans is much slower (8-10 years from infection to AIDS) than disease progression in SIVmac 239- or SIVmac 251-infected rhesus macaques (1-2 years from infection to AIDS).

Peripheral HIV and SIV plasma viral loads typically spike in the first weeks of the acute phase of infection and then settle to a set point viral load during the chronic phase of infection (**Figure 1.3**). Viral loads reach a high set point in SIV-infected natural host sooty mangabeys as well as non-natural host rhesus macaques. Immune control of SIVsmm viral replication is not responsible for the lack of pathogenesis in sooty mangabeys; ongoing SIVsmm infection and replication clearly occur in these animals. This distinguishes natural host sooty mangabeys from HIV-1 elite controllers, infected patients that do not progress to AIDS even in the absence of therapy, who display frequently undetectable set point plasma viral loads (105).

Peripheral $CD4^+$ T cell counts are a measure of disease progression, and AIDS can be clinically defined by a drop in $CD4^+$ T cells below a threshold at which patients become increasingly susceptible to opportunistic infections. In natural host SIV infections, $CD4^+$ T cells generally decline transiently during the acute phase of infection and reach a relatively stable set point $CD4^+$ T cell count during the chronic phase of infection (**Figure 1.3B**). By contrast, HIV-1 infection in humans and SIV infection in non-natural hosts results in an acute drop in $CD4^+$ T cell counts, transient recovery to an unstable set point, and a steady decline over time with progression to AIDS (**Figure 1.3A**). In this study, I defined SIV entry in natural host sooty mangabeys and non-natural host rhesus macaques. An understanding of viral entry and tropism may determine how SIVsmm infection and replication in natural host sooty mangabeys fail to induce widespread CD4+ T cell loss, while closely-related SIVmac infection and replication in rhesus macaques lead to rapid peripheral $CD4^+$ T cell loss.

Chronic Immune Activation

Chronic immune activation drives $CD4^+$ T cell loss and progression to AIDS following human HIV-1 infection and non-natural host SIV infection. Viremia alone is clearly not sufficient to induce AIDS as demonstrated by the lack of disease progression in SIV-infected natural hosts,

as well as the lack of disease progression in rare HIV-1 long-term non-progressors with high viral loads. Many mechanisms leading to chronic immune activation have been proposed, but the casual relationship between infection and immune activation remains largely undefined. The importance of studying these mechanisms is highlighted by the observation that successfully ART-treated patients with undetectable HIV-1 viremia exhibit higher levels of T cell activation than uninfected people, and that this residual immune activation is associated with increased morbidity and mortality even in patients receiving effective ART (99). Therapies targeting the inflammatory aspects of HIV-1 infection may limit progression to AIDS and HIV-1-associated morbidity in patients on ART.

In HIV-1 infection, chronic immune activation, measured by HLA-DR, CD38, CD69, and other molecules on $CD4^+$ and $CD8^+$ T cells, is a correlate of pathogenesis and mortality; the level of T cell activation is often more predictive of disease progression than viral load or $CD4^+$ T cell count (26, 37, 38, 108, 112). Pro-inflammatory cytokines (including IFNγ, IL-2, and TNF) are found at high levels in plasma during the chronic stage of pathogenic HIV-1 infection. Microbial translocation across the gastrointestinal barrier, evidenced in part by increased plasma levels of bacterial and fungal products such as peptidoglycan, lipopolysaccharide (LPS), and flagellin, leads to innate immune activation and further release of pro-inflammatory cytokines. There is also marked lymph node hyperplasia in chronically HIV-1 infected patients, including infiltration of $CD8^+$ T cells, macrophages, and other immune cells (92). Like HIV-1-infected humans, SIV-infected non-natural hosts also display signs of chronic immune activation. SIVmac-infected rhesus macaques exhibit high levels of activation markers on T cells, cell turnover, microbial translocation, chronic innate immune responses, and lymph node pathology (12, 13, 16, 28, 53, 74, 106).

In contrast to human HIV-1 and non-natural host SIV infection, chronic immune activation is absent in SIV infection of sooty mangabeys and other natural hosts. While the immune response

is high in the acute phase of sooty mangabey SIVsmm infection, it resolves to baseline levels during the chronic phase of infection (**Figure 1.3B**). A number of factors underlie discrepancies between SIVsmm natural host sooty mangabey infection and SIVmac non-natural host rhesus macaque infection (summarized in **Table 1.1**). Sooty mangabeys show no increase in the percentage of $CD4^+$ T cells expressing activation markers CD25 or HLA-DR as well as no significant increase in bystander cell turnover or apoptosis post-infection (74, 106). The innate immune response during the chronic phase of SIV infection in natural hosts is tempered relative to the response in non-natural hosts; for example, chronic type I interferon-stimulated gene expression is lower in SIVsmm-infected sooty mangabeys than in SIVmac-infected non-natural host rhesus macaques (12, 53). The magnitude and breadth of adaptive SIV-specific T cell responses (including production of inflammatory cytokines IFNγ, IL-2, and TNF) are lower in chronically SIVsmm-infected sooty mangabeys than in chronically HIV-1-infected humans (28). Studying differences between SIV infection in pathogenic and non-pathogenic hosts can lead to further identification of the mechanisms underlying chronic immune activation.

Critical Cell Subsets

The loss of critical $CD4^+$ T cell subsets likely contributes to chronic immune activation, $CD4^+$ T cell loss, and progression to AIDS in pathogenic hosts whereas protection of critical $CD4^+$ T cell subsets may protect natural hosts from disease progression. Short-lived, activated $CD4^+$ T cells support the majority of viral replication in both natural and non-natural hosts (42, 58). These cells are primarily terminally differentiated effector cells from the gastrointestinal mucosa-associated lymphoid tissue (GALT), the predominant tissue site of viral replication in both natural and non-natural hosts (48, 87). HIV and SIV infection also occur in additional $CD4^+$ T cell subsets and tissue sites. Natural host sooty mangabeys exhibit protection of specific and likely critical $CD4^+$ T cell subsets relative to non-natural hosts and humans. These protected cell subsets include sooty mangabey $CD4^+$ T-helper 17 (Th17) cells, T central memory cells (T_{cm}), and T follicular helper (Tfh) cells.

Some studies maintain that chronic immune activation in infected humans and non-natural hosts is largely supported by microbial translocation from the gastrointestinal tract (15, 31). Maintenance of the sooty mangabey gastrointestinal immune milieu likely provides a barrier to microbial translocation and chronic immune activation in these natural hosts. GALT $CD4^+$ T cells are rapidly depleted upon acute HIV-1 infection of humans, SIVmac infection of rhesus macaques, and SIVsmm infection of sooty mangabeys (16, 71). However, sooty mangabeys maintain GALT $CD4^+$ T cells during the chronic phase of infection whereas human and rhesus macaque GALT $CD4^+$ T cells are further depleted (**Figure 1.3**) (87). As a subset of GALT cells, Th17 cells support the physical and immunological barrier to microbial translocation by maintaining antibacterial defenses and intestinal epithelial architecture. Crucially, humans and rhesus macaques exhibit a loss of critical gastrointestinal Th17 cells post-infection whereas sooty mangabeys do not (14, 19).

Chronic immune activation is also marked by lymph node pathology in humans and non-natural hosts. By contrast, SIV infection of natural hosts does not lead to lymph node pathology or immune cell infiltration (27, 106). Viral replication is low in lymph nodes of natural hosts relative to lymph nodes of non-natural hosts and humans (7, 18, 25, 40, 75). Critical $CD4^+$ Tcm cells reside in the lymph node and maintain T cell homeostasis by replenishing short-lived $CD4^+$ effector T cells lost to infection (17). $CD4^+$ Tcm cells are a large reservoir of HIV-1 in humans and are progressively depleted in SIV-infected rhesus macaques (13, 23, 63, 80, 88). Infection and depletion of Tcm leads to homeostatic proliferation and the location within the lymph node, a compartment where many immune responses are initiated, may contribute to widespread immune activation. Importantly, $CD4^+$ Tcm from sooty mangabeys are protected from infection, high rates of turnover, and depletion relative to $CD4^+$ Tcm from humans and rhesus macaques (18, 73, 82). Another subset of lymphoid T cells, $CD4^+$ Tfh cells, reside in the lymph node germinal center and support survival and activation of B cells. CD4+ Tfh cells are infected at high frequencies in

chronic human HIV-1 and non-natural host SIV infection relative to natural host SIV infection (18, 70, 120). The relative protection of Tfh cells may also protect sooty mangabeys from chronic immune activation.

For this study, I defined host-dependent viral entry pathways in sooty mangabeys and rhesus macaques that likely underlie the differential infection and depletion of critical cell subsets such as $CD4^+$ Th17, Tcm and Tfh. Identification of these entry pathways may define additional critical cell subsets and mechanisms leading to chronic immune activation and disease progression.

HIV and SIV Virology

HIV and SIV are lentiviruses from the family *Retroviridae*. Each virion is enveloped by a lipid membrane and associated matrix, inside of which is a capsid containing two copies of single-stranded RNA genome. The surface of the virion lipid membrane contains trimers of Envelope (Env) protein, each monomer of which is composed of gp120 and gp41 subunits. HIV and SIV Env trimers initiate viral entry by binding to receptor CD4 on the surface of T cells, macrophages, monocytes, dendritic cells, and other target cells (**Figure 1.4**). This induces a conformational change in gp120 that allows binding to the coreceptor, a cell-surface 7-transmembrane chemokine receptor. Binding to this chemokine receptor induces further conformational changes exposing the gp41 fusion subunit of Env, initiating fusion and entry into the target cell.
The expression of CD4 and functional coreceptor initially define HIV and SIV cellular tropism. The vast majority of HIV-1 transmitter/founder Env and early primary isolates are CCR5-tropic (32, 122). In up to half of untreated HIV-1 infections (dependent on the subtype), the virus evolves the ability to infect cells via CXCR4 leading to the emergence of dual CCR5/CXCR4-tropic and singly CXCR4-tropic primary isolates (113). Importantly, most SIV do not use CXCR4 (29, 77, 101). Previous dogma held that, like HIV-1, most SIVsmm and SIVmac viruses utilize coreceptor CCR5. However, in this study, I identified non-CCR5, non-CXR4, *alternative* coreceptors of SIV that support SIV infection *in vitro* and *ex vivo*. These alternative coreceptors are particularly important in natural host sooty mangabey SIVsmm infection.

Post-fusion and entry, the viral capsid is shed and the viral reverse transcriptase converts viral RNA into DNA. HIV and SIV reverse transcriptase enzymes make frequent nucleotide mis-incorporations and can facilitate recombination of the two RNA genomes in each capsid. These frequent errors allow HIV and SIV to quickly evolve resistance to antiretroviral drugs, restriction factors, and the host immune response. Following reverse transcription, viral DNA integrates into the host cell genome and early viral gene transcription is driven off viral long terminal repeats (LTR) by host cell proteins.

The viral genomes of HIV-1 and SIVsmm/SIVmac are very similar (**Figure 1.5**). Both carry critical *gag*, *pol*, and *env* genes that encode the matrix and capsid, the reverse transcriptase and integrase, and the surface envelope glycoproteins, respectively. Early viral products Tat and Rev facilitate transcription and nuclear export of viral mRNA into the cytoplasm, respectively. Viral product Nef supports cell activation and immune evasion. HIV-1 Vpr mediates nuclear import of the HIV-1 pre-integration complex and facilitates infection of non-dividing cells.

Following viral fusion and entry initially dictated by the expression of CD4 and coreceptor, multiple host cell mechanisms can further restrict which cells are ultimately infected with and produce HIV or SIV. Cytoplasmic TRIM5 (tripartite-motif-containing protein 5) proteins mediate early post-entry restriction by interacting with viral capsid and blocking un-coating (102, 110). Cytoplasmic host restriction factor SAMHD1 (SAM domain-and HD-domain-containing protein 1) hydrolyzes dNTPs and thereby decreases the rate of reverse transcription in cells with naturally low levels of dNTPs, such as myeloid cells and dendritic cells (6, 41, 91). The SIV protein Vpx overcomes this restriction by targeting SAMHD1 for proteasomal degradation (52, 62). Notably, there is no Vpx protein in HIV-1 to counteract SAMHD1. Host cell membrane protein tetherin (also known as BST2) acts late in infection to anchor mature virions to the cell surface and prevent viral release (79). Most SIV counteract nonhuman primate tetherin with viral Nef protein,

whereas HIV-1 and HIV-2 overcome human tetherin restriction with Vpu and Env, respectively (54, 100). The restriction factor APOBEC3 (apolipoprotein B-editing catalytic subunit-like 3) is incorporated into nascent virions and induces guanine to adenine hyper-mutations in the proviral DNA genome during reverse transcription (49, 68). HIV and SIV Vif proteins counteract APOBEC3 restriction (22).

Notably, these host restriction factors can inhibit cross-species transmission of HIV and SIV. For example, rhesus macaque Trim5 restricts HIV-1, thus HIV-1 does not infect rhesus macaques (109). The HIV-1 Vif protein easily counteracts human APOBEC3 but not other primate APOBEC3 (69, 117), also presenting a barrier to HIV-1 infection of nonhuman primates. SIV Vif proteins counteract APOBEC3 from multiple species, including other nonhuman primates and humans.

Coreceptors and Cellular Tropism

HIV and SIV cellular tropism is initially dictated by the expression of CD4 and functional coreceptors on the surface of host cells. Coreceptor expression can clearly delineate subsets of human $CD4^+$ cells that are targeted by HIV-1. CCR5 is expressed at high levels on human peripheral $CD4^+$ memory T cells and expression levels generally increase in response to immune activation (81, 97, 119). CCR5 is also found at high levels on human $CD4^+$ T cells in the gastrointestinal mucosa, which likely explains the rapid depletion of gastrointestinal $CD4^+$ T cells in acute infection (46, 115). Human peripheral naïve $CD4^+$ T cells do not express CCR5 and are therefore not directly targeted in early HIV-1 infection. CXCR4, the coreceptor of some late-stage HIV-1, is expressed on these $CD4^+$ naïve T cells as well as memory T cells. The acquisition of CXCR4 use by HIV-1 is associated with accelerated peripheral $CD4^+$ T cell loss and progression to AIDS as HIV-1 acquires the ability to infect $CD4^+$ T naïve cells (10, 11). It is not clear what portion of this accelerated $CD4^+$ T cell loss is mediated by direct infection of $CD4^+$ T naïve cells

versus indirect consequences on immune activation. Nevertheless, this is a clear example of how viral coreceptor use impacts the outcome of infection.

Like HIV-1, most SIV utilize coreceptor CCR5 *in vitro*. However, SIV do not typically evolve the ability to use CXCR4 (29, 77, 101). SIV-uninfected natural hosts, such as sooty mangabeys, display exceedingly low levels of CCR5 expression on peripheral, lymph node, and mucosal $CD4^+$ T cells relative to uninfected non-natural hosts and humans (86). The broadly low expression of CCR5 in sooty mangabey $CD4^+$ T cells first led us to ask if coreceptors aside from CCR5 support robust SIVsmm replication made evident by high plasma viral loads in sooty mangabeys. Such alternative coreceptors may define distinct SIV tropism in sooty mangabeys. Limited CCR5 and alternative coreceptor expression on critical $CD4^+$ cell subsets in the natural host may protect sooty mangabeys from $CD4^+$ T cell loss, chronic immune activation, and disease progression. For example, exceedingly low CCR5 expression on critical $CD4^+$ T_{cm} cells in sooty mangabeys relative to $CD4^+$ T_{cm} in rhesus macaques was associated with 10-fold lower levels of viral DNA in sooty mangabey $CD4^+$ T_{cm} relative to rhesus macaque $CD4^+$ T_{cm} (73, 80, 82). These $CD4^+$ T_{cm} cells are critical to maintaining $CD4^+$ T cell homeostasis and the relative lack of SIVsmm infection of this subset has been hypothesized to protect sooty mangabeys from widespread $CD4^+$ T cell loss. Ultimately, both the $CD4^+$ cell subsets that are targeted and those that are preserved likely play an important role in immune homeostasis versus widespread $CD4^+$ T cell loss and disease progression (13, 46, 47, 80, 105).

Alternative Coreceptors of SIV

Interestingly, multiple primate host species carry truncated dysfunctional alleles of CCR5 ($CCR5^{\Delta}$) that are not expressed at the cell surface and/or do not support HIV or SIV infection. A 32 base pair deletion in human CCR5 abrogates cell surface expression and coreceptor function. Approximately 21% of Caucasian North Americans carry a $CCR5^{\Delta 32/\Delta 32}$ genotype, though the prevalence of this genotype is much less common in African-Americans, Asian-Americans,

Hispanic North Americans, and Native North Americans (123). Humans with this $CCR5^{\Delta32/\Delta32}$ genotype are highly resistant to infection and those with a heterozygous $CCR5^{wt/\Delta32}$ genotype display slower progression to AIDS (9, 67, 98). African primate red-capped mangabeys have an exceptionally high allelic frequency of $CCR5^{\Delta24}$ in the wild ($CCR5^{\Delta24}$ allelic frequency 76-86.6%, $CCR5^{\Delta24/\Delta24}$ genotype 62-73% of surveyed animals from Nigeria and Gabon) (8, 20). Our lab previously identified a $CCR5^{\Delta2}$ allele in natural host sooty mangabeys ($CCR5^{\Delta2}$ allelic frequency 26% in captive sooty mangabeys at the Yerkes National Primate Research Center YNPRC) (96). Both this allele and a previously described sooty mangabey $CCR5^{\Delta24}$ allele (85) ($CCR5^{\Delta24}$ allelic frequency 3% in captive sooty mangabeys at the YNPRC) are not expressed at the cell surface and do not support SIVsmm infection. The discovery of sooty mangabey $CCR5^{\Delta2}$ most importantly led to the discovery of captive CCR5-null sooty mangabeys with $CCR5^{\Delta2/\Delta24}$ and $CCR5^{\Delta2/\Delta2}$ genotypes ($CCR5^{\Delta/\Delta}$ genotype 8% of captive sooty mangabeys). Notably, no $CCR5^{\Delta}$ allele has been observed in non-natural host rhesus macaques (118). The co-evolution and prevalence of $CCR5^{\Delta}$ alleles in African nonhuman primate species lend support to the hypothesis that endemic SIV infections in natural hosts are partially to entirely CCR5-independent.

Crucially, our lab previously discovered that robust SIVsmm infection and replication occur in sooty mangabeys with a $CCR5^{\Delta/\Delta}$ genotype (96) (**Figure 1.6**). There is no significant difference in the prevalence of SIVsmm infection between sooty mangabey CCR5 genotypes (96). Robust SIVsmm plasma viral loads greater than 10^4 RNA copies per mL are observed in sooty mangabeys lacking functional CCR5. Although these levels are slightly lower (by 0.5 $\log_{10}$ RNA copies per mL) in animals with a $CCR5^{\Delta/\Delta}$ genotype than in animals with a $CCR5^{wt/wt}$ genotype (96), this robust infection of $CCR5^{\Delta/\Delta}$ sooty mangabeys demonstrates that SIVsmm can use alternative, non-CCR5 coreceptors for initial infection of and replication within natural host sooty mangabeys. We previously observed that transfected cells expressing human CD4 with human CCR5, CXCR6, GPR1, and GPR15 support SIVsmm Env-mediated infection (96). However, species-specific amino acid differences between primate coreceptor molecules may affect

coreceptor function (89). Thus, in this thesis, I extended these findings to a larger set of SIVsmm Env to determine which coreceptors of sooty mangabey origin support diverse SIVsmm Env-mediated infection. I also defined the species-specific alternative coreceptor use of SIVsmm Env from a very intriguing set of SIVsmm viruses that induce widespread $CD4^+$ T cell loss in sooty mangabeys (76, 77).

Other natural hosts are infected with SIV that have unique receptor and alternative coreceptor use patterns. Natural host red-capped mangabey SIVrcm primarily uses alternative coreceptor human CCR2 but not CCR5. This SIVrcm CCR5-independence is consistent with the high allelic frequency of $CCR5^{\Delta}$ alleles and the high prevalence of $CCR5^{\Delta/\Delta}$ genotypes amongst red-capped mangabeys (20). When SIVrcm is passaged in macaque cells, SIVrcm gains the ability to use CCR4, not CCR5 (35). SIVrcm also enters cells expressing human CXCR6 *in vitro* (8, 20). SIVagm from natural host African green monkeys (*Chlorocebus*) utilize human alternative coreceptors CXCR6 and GPR15, in addition to human CCR5, to infect cell lines *in vitro* (39, 59, 60).

While SIV from non-natural host rhesus macaques were found to use alternative receptors *in vitro*, the role of these coreceptors in infection of rhesus macaques *in vivo* is not entirely clear. SIVmac 239 uses rhesus macaque CCR5 and GPR15 *in vitro*, but GPR15 appears to play a negligible role *in vivo* (56, 90, 121). A mutant SIVmac 239 Env that lacks the ability to use GPR15 (but still uses CCR5 *in vitro*) replicated in rhesus macaques and induced AIDS *in vivo* comparable to parental SIVmac 239 (90), revealing that GPR15 may not contribute to substantial infection or disease progression in rhesus macaques. Plasma viral loads decreased when rhesus macaques chronically infected with SIVmac 251 were treated with CCR5 antagonist CMPD167 *in vivo* (114), indicating that CCR5 supports some viral replication in rhesus macaques. A limited number of experiments also demonstrate the importance of CCR5 in rhesus macaque peripheral blood mononuclear cell (PBMC) infections; CCR5 antagonist Maraviroc blocked the majority of

SIVmac 239 infection and replication in rhesus macaque PBMCs (78) and high concentrations of CCR5 antagonist TAK-779 blocked all SIVmac 239 and SIVmac 251 infection and replication in rhesus macaque PBMCs (121). These findings indicate that CCR5 is likely the primary coreceptor of SIVmac in rhesus macaque PBMCs.

No one has reported a comprehensive analysis of SIVsmm/SIVmac coreceptors from both sooty mangabeys and rhesus macaques or a systematic study of SIVsmm/SIVmac CCR5 and alternative coreceptor use in primary cells *ex vivo*.

Simian Immunodeficiency Viruses in This Study

To date, serological evidence of SIV infection in the wild has been observed in over 40 nonhuman primate species (57, 103). All naturally occurring SIV discovered to date were found in African primate species, though fully exhaustive surveys of Asian and American primates have not been performed (103). The estimated prevalence of naturally occurring SIV in African primate hosts varies widely by species, ranging from 1% to over 50% (2). Some natural host SIV infections are well studied in captivity and are known not to induce AIDS, including SIVsmm infection of sooty mangabeys and SIVagm infection of African green monkeys (specifically the subspecies *Chlorocebus sabeaus* and *Chlorocebus pygerythrus*). Other SIV infections in African primates, for example SIVgsn in greater spot-nosed monkeys and SIVrcm in red-capped mangabeys, are not often studied in captivity and are only presumed to be widely non-pathogenic in their cognate hosts.

The origin and naming conventions of SIV used in this study can be confounding. In this thesis, I examined nonpathogenic SIVsmm from natural host sooty mangabeys that were naturally infected. I also examined pathogenic SIV that are commonly used to experimentally infect non-natural host rhesus macaques and drive progression to AIDS.

There are at least nine clades of SIVsmm circulating in captive sooty mangabeys in the United States. These clades represent a magnitude of diversity close to that of the HIV-1 Group M subtypes (3, 65) (**Figure 1.7**). Each of these clades likely arose from independent introductions of infected animals from Sierra Leone and Côte d'Ivoire. I investigated SIVsmm Env from sooty mangabeys infected with SIVsmm Clade 1, the most prevalent SIVsmm at the Yerkes National Primate Research Center. I found that SIVsmm Env from Clade 1, 2, 3, and 5 exhibited the same pattern of sooty mangabey CCR5 and alternative coreceptor use *in vitro* (Chapter 2). Therefore, subsequent *in vitro* studies were performed using Clade 1 Env alone. I also used a primary isolates from Clade 6 (SIVsmm D215) and a primary isolate from an unknown clade (SIVsmm M935) to infect primary sooty mangabey PBMCs (Chapter 3).

Rhesus macaque SIVmac viruses were generated via cross-species transmissions and multiple passages of SIVsmm in rhesus macaques (50) (**Figure 1.8**). In this study, I examined SIVmac 239 and SIVmac 251, a related infectious provirus and viral swarm frequently used to experimentally challenge rhesus macaques. SIVmac 239 and SIVmac 251 are most closely related to Clade 8 SIVsmm (3) (**Figure 1.7**). I also examined the rhesus macaque virus SIVsmm E660. Importantly, SIVsmm E660 was derived separately from the two SIVmac mentioned above and is more closely related to SIVsmm Clade 1 (3). SIVsmm E660 was derived via multiple *in vivo* passages and the virus induces pathogenesis in non-natural host rhesus macaques. The nomenclature is misleading; this SIVsmm E660 virus in rhesus macaques is not to be confused with non-pathogenic primary SIVsmm viruses in sooty mangabeys.

Goals of This Thesis

I tested the hypothesis that natural host SIVsmm/sooty mangabey infection and non-natural host SIVmac/rhesus macaque infection are sustained by different coreceptor-mediated entry pathways. Specifically, I proposed that SIVsmm infection and replication in sooty mangabey cells is largely mediated by alternative (non-CCR5) coreceptors, making SIVsmm

partially CCR5-independent. I also proposed that SIVmac infection and replication in rhesus macaque cells is mediated primarily by CCR5, making SIVmac largely CCR5-dependent.

To address these hypotheses, I first examined a panel of putative alternative coreceptors cloned from sooty mangabeys and defined sooty mangabey CXCR6 as a robust alternative coreceptor of a diverse array of SIVsmm Env *in vitro* (Chapter 2). Sooty mangabey GPR15 and GPR1 also supported moderate to low levels of SIVsmm infection *in vitro*. Next, I analyzed approaches to block viral entry and demonstrated that small-molecule CCR5 antagonist Maraviroc and recombinant human chemokine CXCL16 are specific inhibitors of CCR5-mediated and CXCR6-mediated SIV infection, respectively (Chapter 3). Ultimately, I demonstrated that even through SIVmac uses alternative coreceptors *in vitro*, SIVmac infection and replication in primary rhesus macaque PBMCs *ex vivo* is largely CCR5-dependent (Chapter 4). By contrast, I found that SIVsmm infection and replication in sooty mangabey PBMCs *ex vivo* is partially CCR5-independent. I also provided the first direct evidence that alternative coreceptor CXCR6 supports some SIVsmm replication in primary sooty mangabey PBMCs.

These discoveries lend support to SIVmac infection of rhesus macaques as a model of CCR5-tropic HIV-1 infection. Crucially, given the ubiquitously low expression of CCR5 on sooty mangabey $CD4^+$ T cells, the expression of functional alternative coreceptors of SIV in these hosts may delineate target cell subsets that support natural host SIV infections without leading to an overall loss of $CD4^+$ T cell homeostasis.

FIGURES

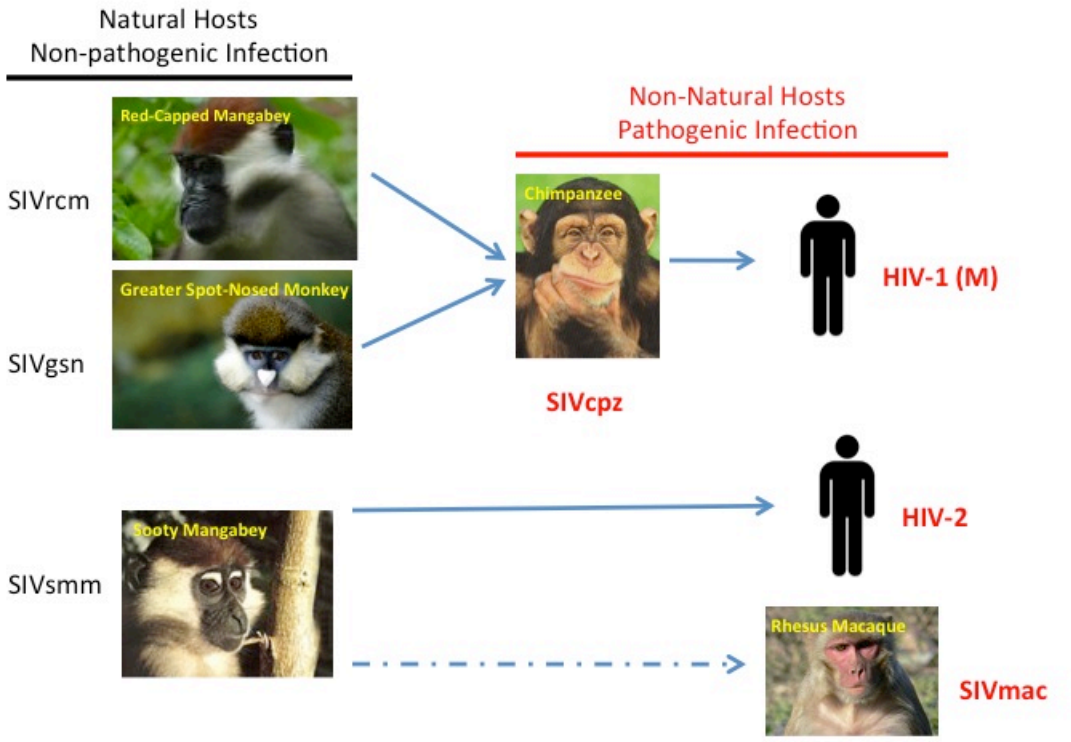


Figure 1.1. The natural host origins of HIV-1 Group M and SIVmac. Solid arrows represent cross-species transmissions that occurred between nonhuman primates in the wild, or incidentally between humans and nonhuman primates. Broken arrow represents SIVsmm cross-species transmission to rhesus macaques in captivity and the experimental derivations of SIVmac in rhesus macaques. Non-pathogenic natural host sooty mangabeys and other presumed natural hosts of SIV are on the left (black); pathogenic non-natural hosts of SIV and human hosts of HIV are on the right (red).

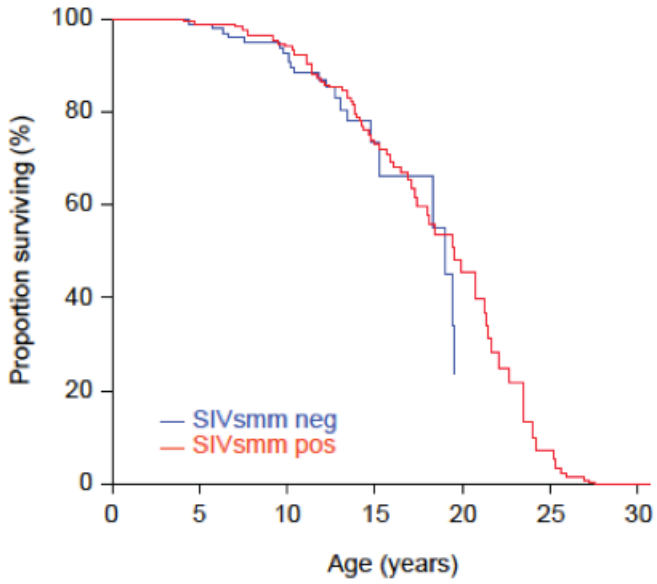


Figure 1.2. Lack of SIVsmm-associated mortality in infected natural host sooty mangabeys. Kaplan-Meier survival curves of SIVsmm-infected (N=167, red) and uninfected (N=62, blue) sooty mangabeys from the Yerkes National Primate Research Center. The proportion of surviving animals is shown in relation to the animals' age in years. Mortality rates do not differ significantly between groups (p=0.55). *Figure from Keele, B., et al. Nature, 2009*. (55). Reprinted by permission from Nature Publishing Group.

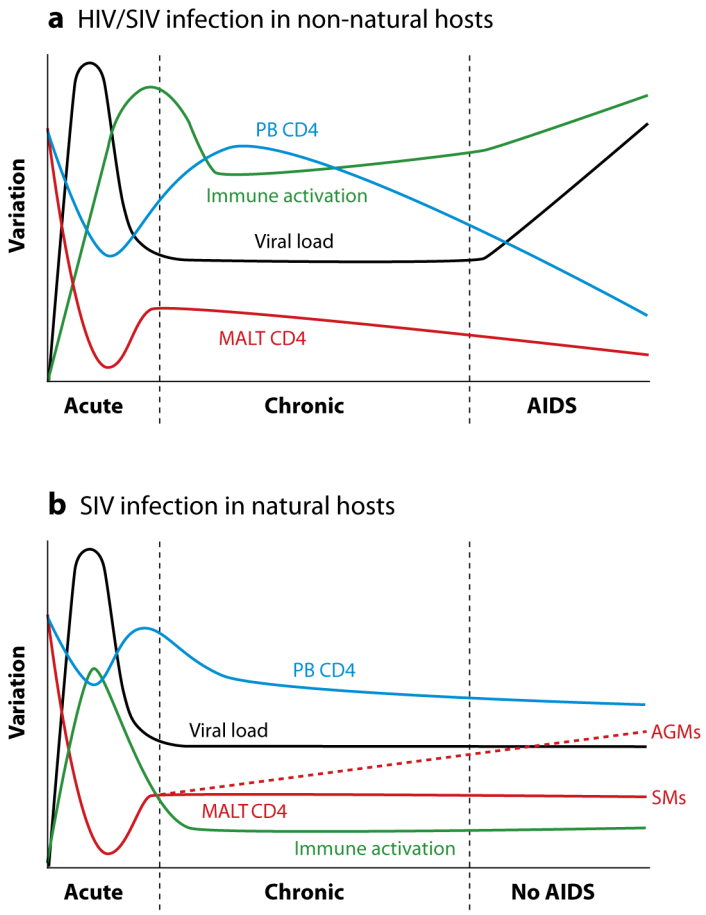


Figure 1.3. Characteristics of HIV and SIV infection in natural and non-natural hosts. Variation in plasma viral loads (black), peripheral CD4+ T cell counts (blue), immune activation (green), and gastrointestinal mucosa-associated lymphoid tissue (MALT) CD4+ T cells (red) during the course of **A)** human and non-natural host infections and **B)** natural host infections. The relative changes in each parameter are represented for the acute, chronic, and (in A) AIDS stages of infection. SMs, sooty mangabeys; AGMs, African green monkeys. *Figure from Paiardini, M., et al. Annual Review of Medicine, 2009.* (84). Reprinted by permission from Annual Reviews.

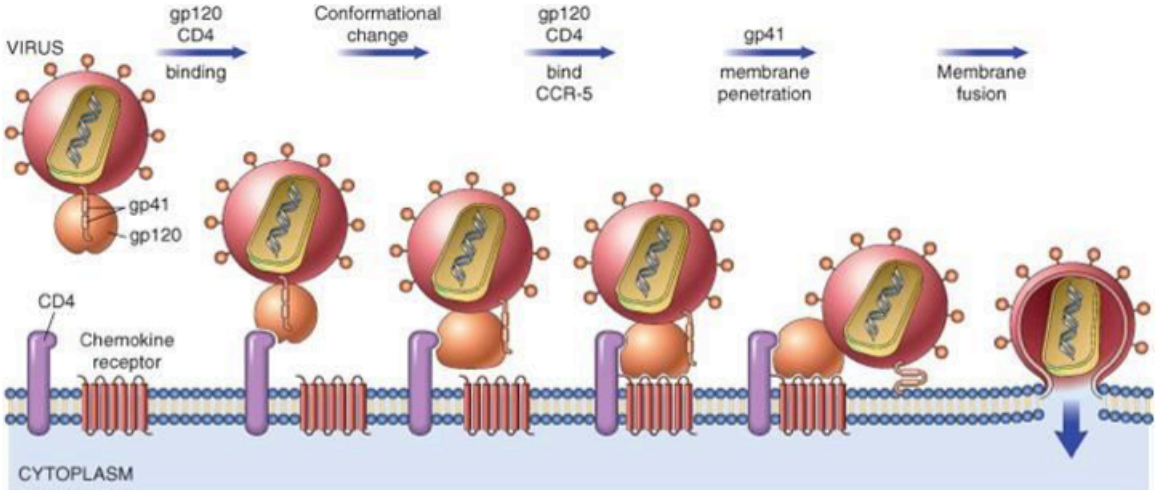


Figure 1.4. HIV and SIV entry. HIV Env trimer subunit gp120 (or SIV Env trimer subunit gp140) binds to receptor CD4 on the surface of target cells, initiating a conformational change and subsequent binding to a 7-transmembrane coreceptor (represented here as CCR5). Coreceptor binding induces further conformational changes, exposing the gp41 subunit and initiating 6-helix bundle formation, fusion, and ultimately viral entry. *Adapted from Kumar, V., et al. Robbins Basic Pathology, 8th Edition, 2007.* (61). Reprinted by permission from Elsevier.

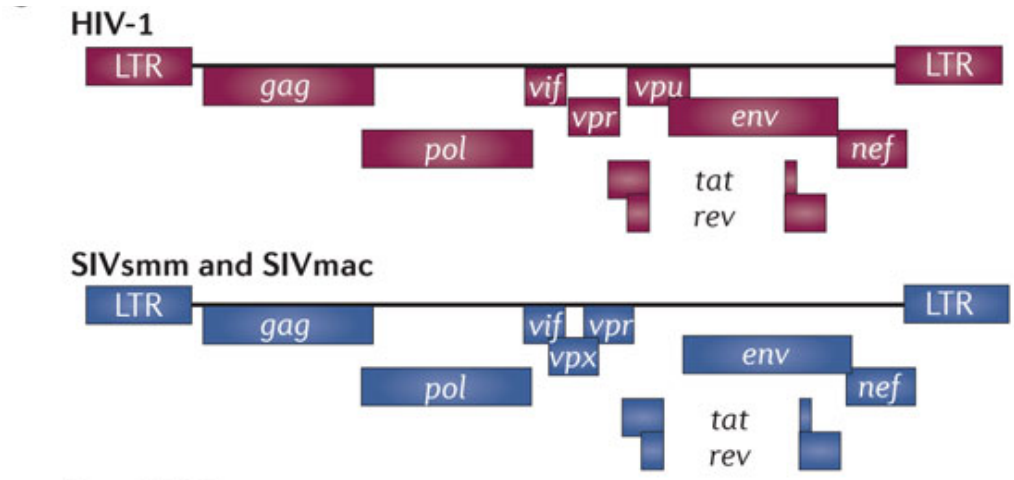


Figure 1.5. DNA structure of HIV-1 and SIVsmm/SIVmac. A schematic representation of HIV-1 and SIVsmm/SIVmac viral DNA genomes. LTR, long terminal repeats. *Adapted from Hatziioannou, T., and Evans, D. Nature Reviews Microbiology, 2012.* (50). Reprinted by permission from Nature Publishing Group.

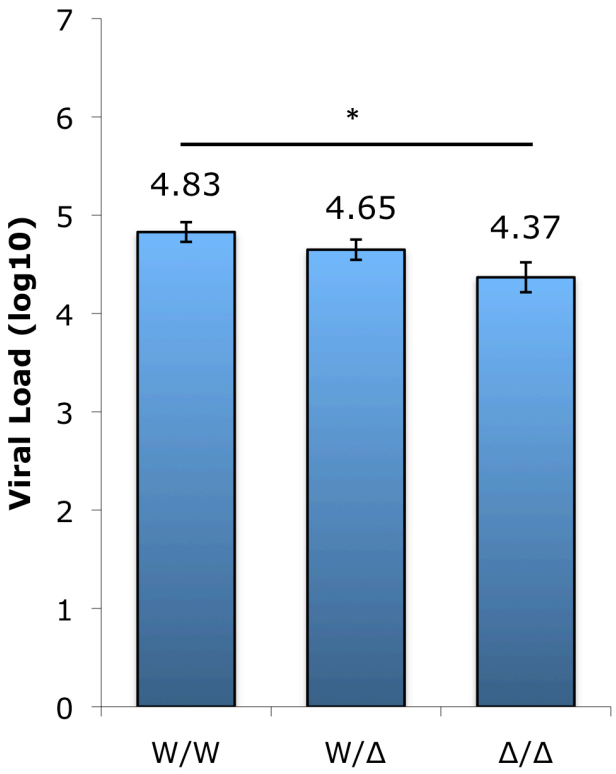


Figure 1.6. SIVsmm infection and replication in the absence of functional CCR5 *in vivo*. Plasma viral loads (log_{10} RNA copies per mL; mean ± SEM) were collected in three surveys of infected sooty mangabeys between 2004 and 2009. Viral loads are plotted by CCR5 genotype: $CCR5^{wt/wt}$ (n = 60), $CCR5^{wt/\Delta}$ (n = 49), and $CCR5^{\Delta/\Delta}$ (n = 7). The difference in viral loads between $CCR5^{wt/wt}$ and $CCR5^{\Delta/\Delta}$ sooty mangabeys is statistically significant ($p<0.05$; Dunn's multiple comparison test) whereas other comparisons do not reach statistical significance. *Adapted from Riddick, N., et. al. PLoS Pathogens, 2010* (96).

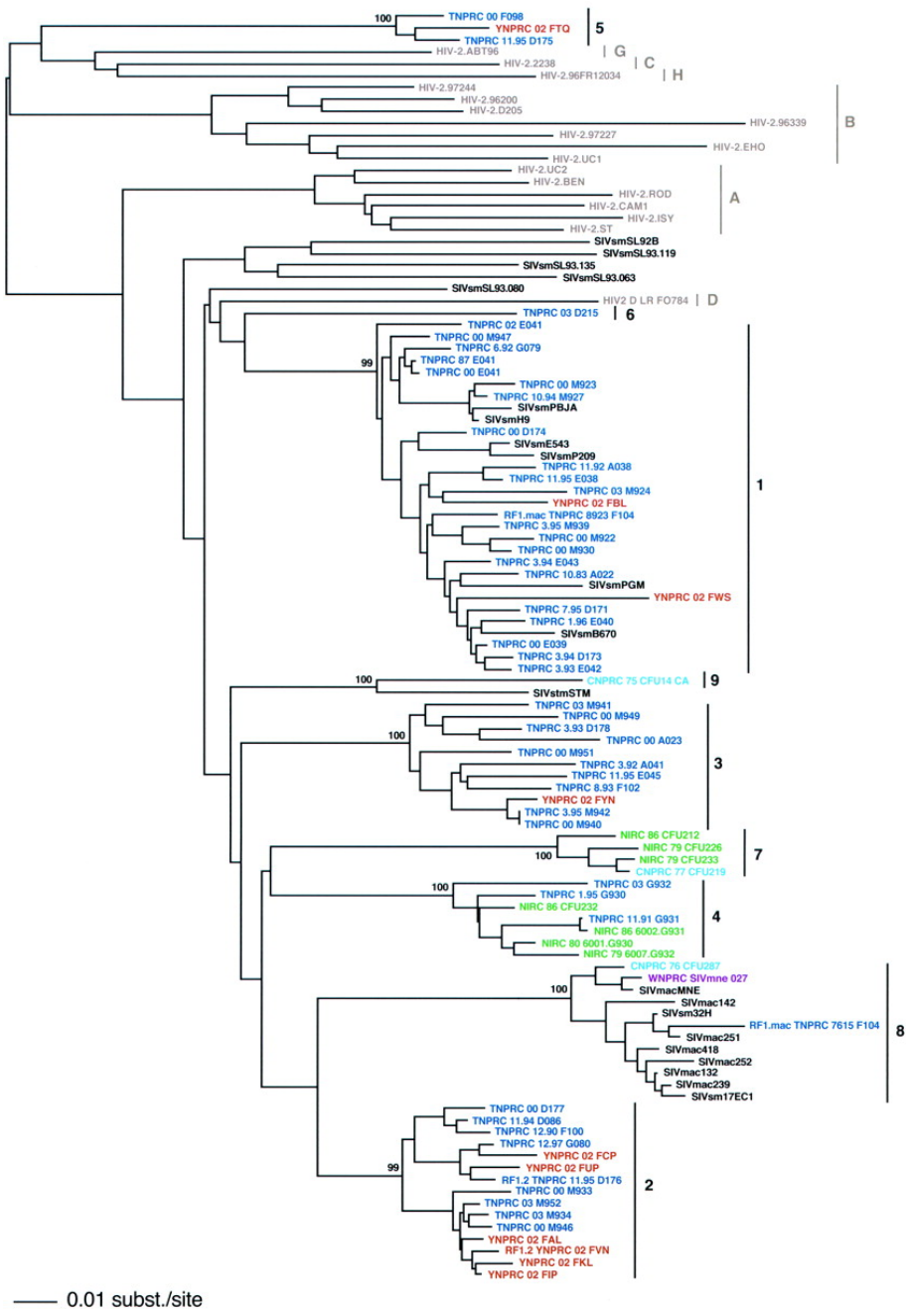


Figure 1.7. SIVsmm and SIVmac Env phylogeny. Neighbor-joining phylogeny of 405 base pair *env* predicted amino acid sequences. The scale represents 0.01 amino acid substitutions per site. Strain nomenclature includes the primate center of origin (YNPRC, TNPRC, CNPRC, NIRC), the collection year, and the animal identification number. Reference laboratory SIVsmm strains, laboratory SIVmac strains, and strains from sooty mangabeys living in the wild are shown in black. HIV-2 strains (Subtypes A, B, C, D, H, D) are shown in gray. SIVsmm strains (Clades 1-9) identified from captive sooty mangabeys are in color. *Adapted from Apetrei, C., et al. Journal of Virology, 2005.* (3)

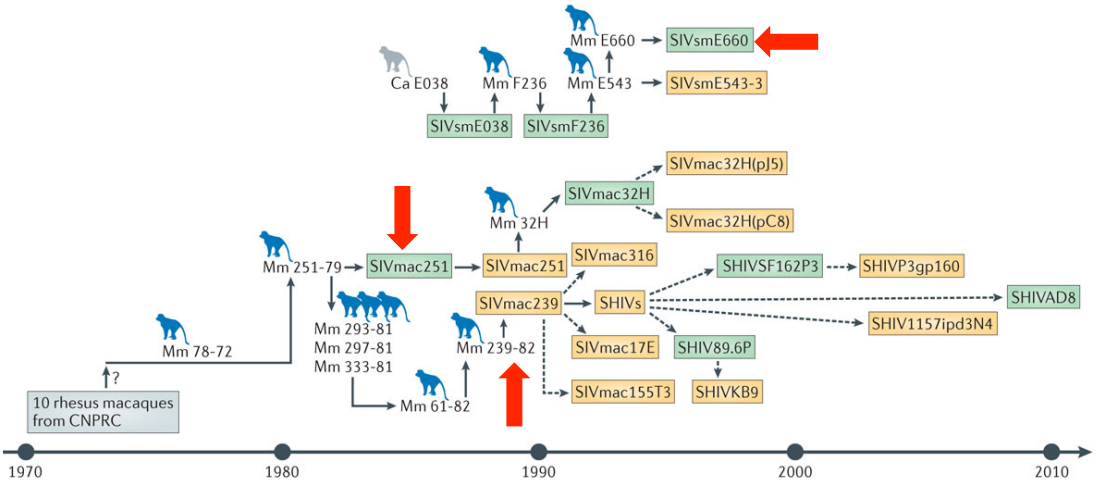
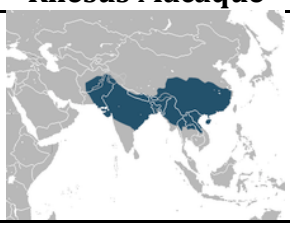


Figure 1.8. The separate origins of SIVsmm E660 versus SIVmac 251 and SIVmac 239. Rhesus macaque challenge viruses SIVmac 251, SIVmac 239, and SIVsmm E660 were derived via multiple passages of SIVsmm in rhesus macaques. Large red arrows identify SIVsmm E660, SIVmac 251, and SIVmac 239. Ca, *Cercocebus atys* sooty mangabey (gray). Mm, *Macaca mulatta* rhesus macaque (blue). Green boxes represent viral isolates and yellow boxes represent infectious molecular clones. Solid arrows represent direct transmission events and broken arrows represent multiple cloning steps. CNPRC, California National Primate Research Center. *Adapted from Hatziioannou, T., and Evans, D. Nature Reviews Microbiology, 2012.* (50). Reprinted by permission from Nature Publishing Group.

Table 1.1. Characteristics of rhesus macaque and sooty mangabey SIV infections.

	Non-Natural Host Rhesus Macaque	Natural Host Sooty Mangabey
Natural Habitat		
Virus	SIVmac	SIVsmm
Endemic SIV	No	Yes
Set point viral load (log_{10})	6.66 ± 1.51[a]	4.83 ± 0.10[b]
Peripheral $CD4^+$ T cell loss	Yes	Very Rare[c]
Progression to AIDS	Yes	No
Time to AIDS[d]	1–2 years	N/A
CCR5 on $CD4^+$ T cells[e]	High	Low
CCR5 on $CD4^+$ T_{cm} cells[f]	High	Low
$CD4^+$ T_{cm} viral DNA[f]	High	Low
Gut Th17 Depletion[g]	Yes	No
Acute GALT $CD4^+$ T cell loss[h]	Yes	Yes
Chronic GALT $CD4^+$ T cell loss[i]	Yes	No
Microbial translocation	Yes	No
Chronic immune activation[j]	Yes	No
Lymph node pathology[k]	Yes	No
Chronic CTL response[l]	High	Low
Chronic Type I IFN response[m]	High	Low
Frequency of DN T cells[n]	Low	High
Viral replication[o]	Short-lived $CD4^+$ T cells	Short-lived $CD4^+$ T cells

For comprehensive reviews pertaining to this figure, see (105, 107).

[a] RNA copies per mL, Mean ± SEM plasma viral loads: days 35-77 of SIVmac 239 infection in Indian Rhesus Macaques (50, 66). [b] RNA copies per mL, Mean ± SEM plasma viral loads: three surveys of chronically SIVsmm-infected $CCR5^{wt/wt}$ sooty mangabeys (95). [c] In rare instances, SIVsmm infection is associated with peripheral $CD4^+$ T cell loss. These include animals infected by an atypical SIVsmm with CXCR4 tropism (30, 76, 77), and aging SIVsmm-infected sooty mangabeys with declining $CD4^+$ T cell counts (64, 111). [d] Time between SIV infection and AIDS. Note the typical lifespan of a captive sooty mangabey is 14-18 years (64). [e] (74, 106). [f] CCR5 expression is low on $CD4^+$ T cells from the periphery, lymph node, and GALT of sooty mangabeys (86). [g] Central memory T cells (T_{cm}) (82). [h] $CD4^+$ T-helper 17 cell (Th17) (14, 19). [i] Gut mucosal associated lymphoid tissue (GALT) (43, 71). [j] (87). [k] Lymph node pathology includes collagen deposition and/or an increased number of effector-type T cells (16, 106). [l] (28). [m] An acute increase in Type I IFN genes in natural and non-natural hosts returns to baseline in natural hosts but remains high in non-natural hosts (12, 53). [n] The frequency of double-negative (DN) $CD4^-CD8^-$ T cells is higher in uninfected sooty mangabeys (and other natural hosts) than in uninfected rhesus macaques (116). [o] Primary source of viral replication *in vivo* (42).

[ϕ] Pathogenic rhesus macaque viruses used in this study included SIVmac 239, SIVmac 251, and SIVsmm E660 (not to be confused with natural non-pathogenic SIVsmm in sooty mangabeys, see Figure 1.8).

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CHAPTER 2

CLONING AND ANALYSIS OF SOOTY MANGABEY ALTERNATIVE CORECEPTORS THAT SUPPORT SIMIAN IMMUNODEFICIENCY VIRUS SIVsmm ENTRY INDEPENDENTLY OF CCR5

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ABSTRACT

Natural host sooty mangabeys infected with SIVsmm do not develop AIDS despite high viremia. Sooty mangabeys and other natural hosts express very low levels of CCR5 on $CD4^+$ T cells and SIVsmm infection and robust replication occur *in vivo* in sooty mangabeys genetically lacking functional CCR5, indicating use of additional entry pathways. SIVsmm use several alternative coreceptors of human origin *in vitro*, but it is not known which molecules of sooty mangabey origin support infection. We cloned a panel of putative coreceptors from sooty mangabeys and tested their ability to mediate infection, in conjunction with smCD4, by pseudotypes carrying Envs from multiple SIVsmm subtypes. Sooty mangabey smCXCR6 supported efficient infection by all SIVsmm pseudotypes with infection levels comparable to smCCR5; smGPR15 enabled infection by all pseudotypes at modest levels. smGPR1 and smAPJ supported low and variable infection whereas smCCR2b, smCCR3, smCCR4, smCCR8, and smCXCR4 were not used by most pseudotypes. Infection was similar with both known alleles of smCD4. In contrast to typical SIVsmm, SIVsmm from rare infected sooty mangabeys with profound $CD4^+$ T cell loss used smCXCR4, smCXCR6 and smCCR5 efficiently and also exhibited robust infection through smCCR3, smCCR8, smGPR1, smGPR15, and smAPJ. Alternative coreceptors of typical SIVsmm, particularly smCXCR6 and smGPR15, may support virus replication in sooty mangabeys that display restricted CCR5 expression as well as sooty mangabeys genetically lacking CCR5. Defining expression of these molecules on sooty mangabey $CD4^+$ subsets may delineate distinct natural host target cell populations capable of supporting SIVsmm replication without consequent $CD4^+$ T cell loss.

INTRODUCTION

HIV-1 infection of humans and SIVmac infection of non-natural host rhesus macaques result in high viral loads, peripheral $CD4^+$ T cell loss and progression to AIDS. By contrast, SIVsmm infection of natural host sooty mangabeys rarely leads to peripheral $CD4^+$ T cell loss or disease despite viral loads comparable to those measured in non-natural hosts. Identifying mechanisms responsible for the different outcomes of infection between natural hosts and non-natural simian or human hosts has become a central focus of efforts to understand AIDS pathogenesis. Although many models of natural host infection exist, SIVsmm is unique as direct cross-species transmission of SIVsmm from sooty mangabeys to humans and rhesus macaques gave rise to pathogenic HIV-2 and SIVmac (2, 31). Similarly, experimental transmission of SIVsmm from infected sooty mangabey hosts results in AIDS in non-natural host rhesus macaques (43). While the factors regulating pathogenic versus nonpathogenic outcomes of infection are complex and not fully understood (9), comparison of infected sooty mangabeys with rhesus macaques recently revealed differential targeting of $CD4^+$ T cell subsets (51). Thus, an understanding of SIVsmm cellular tropism may identify cells in natural host sooty mangabeys that maintain viral replication without leading to $CD4^+$ T cell depletion or AIDS.

Classically, SIVsmm was thought to exclusively use the entry coreceptor CCR5 *in vivo*. Highly restricted expression of CCR5 on $CD4^+$ T cells is characteristic of SIV natural hosts, including sooty mangabeys, compared with non-natural host species (52, 53) and may contribute to distinct cellular targeting *in vivo* (51). However, SIVsmm use of non-CCR5 coreceptors *in vivo* was revealed recently by the identification of common CCR5 deletion alleles among sooty mangabeys ($CCR5^{\Delta 2}$ and $CCR5^{\Delta 24}$) that abrogate CCR5 cell surface expression and entry coreceptor function (58). A substantial minority of captive sooty mangabeys in US primate centers possess homozygous null ($CCR5^{\Delta/\Delta}$) genotypes, but the absence of functional CCR5 expression does not restrict SIVsmm replication *in vivo* as $CCR5^{\Delta/\Delta}$ animals are susceptible to natural as well as experimental infection and exhibit high viral loads (58). These findings indicate that SIVsmm

infection, replication and cell targeting occur *in vivo* through alternative pathways in addition to CCR5.

A number of 7-transmbrane receptors (7TMR) of human origin were shown to function as alternative coreceptors of HIV and SIV *in vitro*. SIVsmm can enter target cells through human CXCR6, GPR1 and GPR15 in addition to CCR5 (11, 50, 58). Natural host red-capped mangabeys, the majority of which carry a $CCR5^{\Delta/\Delta}$ genotype, are naturally infected with SIVrcm that enter cells through human CCR2b but not CCR5 (4, 12, 74). Experimental transmission of SIVrcm to pigtailed macaques resulted in expanded SIVrcm *in vitro* tropism for human CCR4 (27). Additional human 7TMR molecules that support entry or infection by subsets of HIV-1 or SIV include APJ, CCR3 and CCR8 (13, 14, 19, 50, 60, 64). Although CXCR4 is a common coreceptor in late stages of HIV-1 infection, SIVsmm, like other SIV strains, does not use CXCR4 except in rare cases (16, 46, 50, 62).

Notably, the majority of alternative coreceptor studies to date employed 7TMR molecules of human origin and rarely utilized homologous species-derived molecules. Species-specific amino acid differences in 7TMRs may markedly affect coreceptor function (55). Therefore, in order to define the sooty mangabey molecules that mediate SIVsmm infection and that might be involved in CCR5-independent cell targeting and replication *in vivo*, we cloned sooty mangabey smAPJ, smCCR2b, smCCR3, smCCR4, smCCR8, smCXCR4, smCXCR6, smGPR1 and smGPR15. Because sooty mangabeys have two distinct alleles of CD4 that differ in their putative gp120 binding regions (10, 24, 25), we utilized both smCD4 alleles in conjunction with sooty mangabey 7TMRs for functional coreceptor analysis. Finally, we compared coreceptor use by a variety of SIVsmm pseudotypes. While infected sooty mangabeys do not progress to AIDS, SIVsmm exists in at least nine viral genetic lineages (2) and sooty mangabeys at the Yerkes National Primate Research Center (YNPRC) infected with subtype 5 SIVsmm generally display lower peripheral $CD4^+$ T cell counts than uninfected sooty mangabeys or those infected with subtype 1, 2 or 3

SIVsmm (1). In addition, a small number of infected sooty mangabeys developed rapid profound $CD4^+$ T cell loss and were found to harbor SIVsmm variants with expanded use of human coreceptors in fusion assays, including CXCR4 (45, 46). SIVsmm use of sooty mangabey coreceptors has not been described in these animals. Thus, we assessed sooty mangabey-derived coreceptor use by a variety of SIVsmm strains in pseudotype infection.

METHODS

Cloning sooty mangabey receptor molecules

Full-length 7TMRs were cloned from sooty mangabey genomic DNA (APJ, CCR3, CCR8, CXCR6, GPR1, GPR15) or sooty mangabey cDNA (CCR2b, CCR4, CXCR4) from animals housed at the Yerkes National Primate Research Center (YNPRC). Primers were designed to anneal outside the 7TMR open reading frame 5' start codons and 3' stop codons based on primers previously used to clone receptors from rhesus macaques (22, 42) or sequences matched to human and rhesus macaque mRNA published in GenBank. Genomic DNA was extracted from unfractionated sooty mangabey PBMC. cDNA was obtained from sooty mangabey $CD4^+$ T cells stimulated for 120 hours with Concanavalin-A/IL-2 and subjected to mRNA purification (RNEasy Mini Kit; Qiagen, Valencia, CA) and reverse transcription (SuperScript First-Strand Synthesis System for RT-PCR; Invitrogen, Carlsbad, CA, USA). The 50 uL PCR reactions contained 200 ng genomic DNA or 0.1 uL cDNA, 1 unit high-fidelity Phusion polymerase in HF buffer (Finnzyme, Thermo Scientific, Waltham, MA), primers (0.5 mM), and dNTPs (0.2 mM). Thermocycling conditions were: initial denaturation 98 °C for 45 seconds, followed by 30 cycles of 98 °C for 10 seconds, annealing for 30 seconds, 72 °C for 1.5 minutes, and a final extension at 72 °C for 10 minutes. Annealing temperatures were 53 °C (CCR2b), 60 °C (GPR1), 62 °C (CCR4, CXCR4), 64 °C (APJ, CCR3) and 72 °C (CCR8, CXCR6, GPR15). PCR products were column purified (QIAquick PCR Purification; Qiagen). CCR8, CXCR6, GPR15 were then cloned into the expression plasmid pcDNA3.1+ (Invitrogen) using dual restriction digests with *HindIII*, *BamHI* and *XhoI* (22). APJ, CCR2b, CCR4, CXCR4 and GPR1 were TA-cloned using a pcDNA3.1 Directional TOPO Expression Kit (Invitrogen). Clones were screened by restriction analysis and nucleotide sequencing. Sequences of the sooty mangabey CCR2b, CCR3, CCR4, CCR8, CXCR4, GPR1, GPR15 and APJ clones were submitted to GenBank (Accession numbers: CCR2b, JN701018; CCR3, JN701013; CCR4, JN701019; CCR8, JN701014; CXCR4, JN701020; GPR1, JN701016; GPR15, JN701017; APJ, JN701012). Cloning of wild-type smCCR5 from SM genomic DNA has been previously described (58). SM CD4

alleles (smCD4 2.1 and smCD4 4.3) were PCR amplified from cDNA by T. Vanderford and TOPO-cloned into pcDNA3.1 (Invitrogen). Individual clones were screened and verified by sequence analysis. Because our smCXCR4 clone differed from a previously published smCXCR4 sequence (11) at two predicted amino acids (residues 32 and 171), the relevant region was PCR-amplified and sequenced from genomic DNA of ten additional sooty mangabeys using primers within the CXCR4 gene (forward 5'-AGT GAA TGT CCA TTC CTT TG-3'; reverse 5'-GAC AAT ACC AGG CAG GAT AA-3'). Nucleotide and amino acid alignments were performed using the ClustalW algorithm in MacVector 12.0 (Cary, NC, USA). 7TMR membrane topology predictions were made using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html).

SIVsmm envelope clones and pseudotyped viruses

SIVsmm *env* genes were cloned by C. Derdeyn and B. Li (Emory University) from virions isolated from plasma of infected sooty mangabeys employing single genome amplification (SGA) methods previously described (38, 39, 61). Briefly, viral RNA was purified from plasma using the QIAamp Viral RNA purification kit (Qiagen) and cDNA was prepared using Superscript III (Invitrogen) and the primer SM-ER1 (5'- CTA TCA CTG TAA TAA ATC CCT TCC AGT CCC-3'). First round PCR amplification was performed on endpoint diluted cDNA using primers H2SM-EF1 (5'- CCC TTG AAG GMG CMR GAG AGC TCA TTA-3') and SM-ER1 (5'-ATA AAA TGA GAC ATG TCT ATT GCC AAT TTG-3'), followed by a second round of amplification using primers H2SM-EF2 (5'– CAC CTA AAA ART GYT GCT AYC ATT GCC AG-3') and SM-ER2 (5' -ATA AAA TGA GAC ATG TCT ATT GCC AAT TTG-3'). PCR amplicons were cloned into the pcDNA3.1V5HisTOPO-TA expression vector (Invitrogen), and transformed colonies that contained an insert were identified by PCR screening. SIVsmm Env-mediated infection was assessed using luciferase-expressing viral pseudotypes as previously described (58). Pseudotyped virions were generated in 293T cells by co-transfecting pNL-Luc- E^-R^+ plasmid encoding an NL4-3-based *env*-deleted luciferase-expressing HIV-1 viral backbone (15) along with plasmids carrying SIVsmm *env* genes. Pseudotypes lacking Env (pNL-Luc- E^-R^+ co-transfected with empty pcDNA3.1+) served as a

negative control and virions carrying VSV-G served as a positive control. Cells were transfected overnight using Fugene (Promega; Madison, WI, USA) and washed the following day to remove DNA and transfection reagent. Supernatants were collected 3 days post-transfection and stored at -80C in 5% sucrose to enhance stability. Prior to use in infection assays, pseudotype stocks were treated with DNase (50 units/mL x 15 min). Pseudotype virion stocks were quantified by HIV-1 Gag p24 antigen content as measured by ELISA (PerkinElmer, Waltham, MA, USA) and by infectivity based on luciferase output (RLU) measured in U87 cells stably expressing human CD4 and CCR5 (5).

Infection and coreceptor function analyses

Coreceptor function was evaluated by infection of 293T cells transfected with CD4 and 7TMR molecules of interest as described (58). Target cells were co-transfected with plasmids encoding CD4 and 7TMR plasmids using Fugene, washed the next day to remove transfection reagent and re-plated at 2×10^4 cells/well in 96 well plates. CD4 and 7TMR expression was confirmed by flow cytometry (CCR2b, CCR4) and/or viral infection with promiscuous CD4-low SIVsmm. The next day, cells were infected with pseudotype virus using equal amounts of each virus based on luciferase activity (RLU) measured in U87/CD4/CCR5 cells or based on p24 Gag antigen content. Infections were carried out by spin inoculation (1200×g for 2 hours at 25C). Three days later, cells were lysed (0.5% Triton-X 100 in PBS) and luciferase was quantified by adding an equal volume of luciferase substrate (Luciferase Assay System; Promega) and measuring activity on either a MLX Microplate Luminometer (Dynex Technologies, Chantilly, VA) or a Luminoskan Ascent Microplate Luminometer (Thermo Scientific). For infections in which blocking of endogenous human CXCR4 expressed by 293T cells was necessary, cells were treated for 1 hour pre-infection with 20μM AMD3100 (Sigma-Aldrich, St. Louis, MO, USA), which was also maintained over the course of infection.

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) and the Washington National Primate Research Center (WaNPRC). Studies were also reviewed and approved by the University of Pennsylvania IACUC.

RESULTS

Cloning and sequence analysis of sooty mangabey 7-transmembrane receptors

We amplified the full open reading frames of smAPJ, smCCR2b, smCCR3, smCCR4, smCCR8, smCXCR4, smCXCR6, smGPR1, and smGPR15 using primers upstream and downstream of the coding regions. Primers we designed to match both human and rhesus macaque genomic and mRNA sequences in order to maximize likely homology to sooty mangabey sequences (22). Sooty mangabey genomic DNA was used for cloning 7TMRs that lack introns in the open reading frame: smAPJ, smCCR3, smCCR8, smCXCR6, smGPR1, and smGPR15. Complimentary cDNA from sooty mangabey $CD4^+$ PBMCs was used for cloning of smCCR2b, smCCR4 and smCXCR4, molecules which contain introns within the open reading frames. Amplified receptors were cloned into mammalian expression plasmids for sequencing and functional studies.

We aligned the predicted amino acid sequences of these sooty mangabey 7TMR molecules to sequences of human and rhesus macaque molecules. Sooty mangabey molecules showed greater than 91% identity to human and greater than 98% identity to rhesus macaque sequences (**Table 2.1**). While the domains of these 7TMR molecules that might interact with Env to facilitate entry have not been mapped, studies with human CCR5 and CXCR4 show that all extracellular regions may contribute to entry with the N-terminal domain (NTD) and extracellular loop 2 (ECL2) as the most critical (20). Thus, we aligned the predicted extracellular regions between human and sooty mangabey 7TMR molecules (**Figure 2.1**). 7TMR molecules CCR4 and GPR1 were highly conserved between sooty mangabeys, rhesus macaques, and human within extracellular domains and differ by only two extracellular residues between sooty mangabey and human. CXCR6, CCR3, and CCR8 were particularly divergent between the species in extracellular domains (**Table 2.1** and **Figure 2.1**).

Sequences were not described previously for smAPJ, smCCR2b, smCCR3, smCCR4, smGPR1 and smGPR15, but smCXCR6 and smCXCR4 have been reported (11, 55). Our clones matched the published smCXCR6 sequence, but differed at two predicted amino acids from the previously published smCXCR4 sequence (E32 instead of K in the NTD, and D171 instead of E in transmembrane domain 4). These alleles differed in a region critical to CXCR4 antagonist AMD3100 binding (59). To confirm that our smCXCR4 clone was not an artifact of PCR amplification or a unique sequence limited to one animal, we sequenced CXCR4 genes from ten additional sooty mangabeys. Genomic sequencing revealed E32/D171 in all ten sooty mangabeys, indicating that the allele of smCXCR4 we cloned was neither an artifact nor rare among sooty mangabeys in the YNPRC colony.

Both sooty mangabey CD4 alleles mediate SIVsmm pseudotype infection through smCCR5 but not smCXCR4

Studies of SIV Env-mediated infection *in vitro* were traditionally performed using human CD4 and coreceptors. Sooty mangabeys are known to carry two distinct alleles of CD4, smCD4 2.1 and smCD4 4.3, whose putative gp120 interaction domains differ from both human huCD4 and from each other (24, 36). Thus, we determined whether both alleles of smCD4 support SIVsmm infection through smCCR5, and whether they differ from huCD4 in this respect. The two smCD4 alleles were independently cloned from cDNA and found to match the sequences previously reported (24).

We tested function of the two smCD4 alleles in conjunction with smCCR5. Virions were generated using a pNL-Luc- $E^{-}R^{+}$ HIV-1 viral backbone lacking Env and carrying luciferase in place of Nef. This backbone was pseudotyped with subtype 1 SIVsmm Envs cloned by single genome amplification (SGA) from infected SM with CCR5-wild-type ($CCR5^{wt/wt}$) or CCR5-deficient ($CCR5^{\Delta/\Delta}$) genotypes (FFv and FNp, respectively) as previously described (58). Target 293T cells were transfected with huCD4, smCD4 2.1, and smCD4 4.3, with or without smCCR5,

then infected with pseudotype virions. Luciferase output was measured as an indication of entry and infection. Flow cytometry confirmed that huCD4, smCD4 2.1, and smCD4 4.3 were expressed at similar levels on the cell surface (data not shown). Virions lacking Env served as a negative control and VSV-G pseudotypes served as a positive control (**Figure 2.2** and data not shown).

Both smCD4 2.1 and smCD4 4.3 enabled SIVsmm infection when transfected with smCCR5 (**Figure 2.2**). While modest quantitative differences were seen within individual experiments, there was no consistent difference in efficiency between smCD4 2.1, smCD4 4.3, and huCD4 over repeated experiments. CD4-independent SIV Env were described previously (26, 56, 73). In our experiments, target cells expressing smCCR5 in the absence of CD4 did not permit SIVsmm pseudotype infection, confirming that SIVsmm Env-mediated infection does not occur through smCCR5 independent of CD4. Thus, despite sequence differences, both smCD4 molecules support efficient SIVsmm pseudotype infection in conjunction with smCCR5. These results also showed that 293T cells expressing CD4 alone were unable to support infection by these pseudotypes. Because 293T cells express endogenous human CXCR4 (17), the lack of infection following transfection of huCD4, smCD4 2.1 or smCD4 4.3 alone demonstrates that human CXCR4 is not a coreceptor for these SIVsmm. Furthermore, given the breadth of human alternative coreceptors used by many SIV strains, lack of infection in CD4-transfected cells establishes 293T as a suitable cell line for our further coreceptor assays of SIVsmm.

Multiple studies show that most naturally-occurring SIV, including SIVsmm, do not use human CXCR4. One study reported that SIVsmm did not enter cells through a previously described but distinct allele of smCXCR4 (11). Therefore, we asked if SIVsmm could utilize the smCXCR4 we cloned in conjunction with species-matched CD4 (**Figure 2.3**). Neither the SIVsmm Envs derived from infected $CCR5^{wt/wt}$ nor those from $CCR5^{\Delta/\Delta}$ SM were capable of infecting cells through smCXCR4, regardless of which smCD4 allele was used. A similar lack of infection through

smCXCR4 was also seen using an extended panel of additional SIVsmm pseudotypes (discussed further below, see Figure 2.5). Efficient infection through smCXCR4 was seen with virions carrying the dual CCR5- and CXCR4-tropic HIV-1 89.6 Env (data not shown), confirming smCXCR4 expression and functionality. These results confirm SIVsmm does not typically use smCXCR4, in contrast to HIV-1 infection where acquisition of CXCR4-tropism is relatively common.

Alternative coreceptors from sooty mangabey support subtype 1 SIVsmm pseudotype infection

Small differences in alternative coreceptor sequences between species can markedly alter coreceptor function (55), suggesting that prior studies using human 7TMR molecules may not necessarily reflect SIVsmm coreceptor use in natural host sooty mangabey infection. Therefore, we determined the capacity of eight additional 7TMR molecules cloned from sooty mangabey to mediate SIVsmm infection in conjunction with both smCD4 alleles. These coreceptors included smCXCR6, smGPR15, and smGPR1, since we previously showed that SIVsmm frequently use human CXCR6, GPR15 and GPR1 (58); smCCR2b and smCCR4, since the human homologs of these receptors support infection by SIVrcm derived from natural host red-capped mangabey or following passage into pigtail macaques (4, 12, 27, 74); and smAPJ, smCCR3, and smCCR8, since the human molecules are used by limited subsets of HIV or SIV (13, 14, 19, 50, 60, 64).

Alternative coreceptor smCXCR6 acted as a robust entry pathway for SIVsmm in conjunction with either smCD4 4.3 or smCD4 2.1, yielding infection levels generally comparable to smCCR5 (**Figure 2.4**). smGPR15 also supported infection, although to a lesser extent than smCXCR6 or smCCR5. These results are similar to those seen previously using transfected human CXCR6 and GPR15 (58). smGPR1 and smAPJ supported low levels of infection as luciferase output is above the background in mock transfected cells for most Envs but well below output levels in

cells expressing smCCR5, smCXCR6, or smGPR15. Surprisingly, neither smCCR2b nor smCCR4 supported infection by SIVsmm Env pseudotypes, even though these 7TMR support SIVrcm infection. smCCR3 and smCCR8 also failed to mediate infection by these SIVsmm. smCD4 2.1 and smCD4 4.3 supported similar levels of infection (**Figure 2.4A** and **Figure 2.4B** respectively), whereas none of the alternative coreceptors enabled infection in the absence of CD4 (data not shown). Similar coreceptor-mediated entry patterns were seen between Envs from CCR5-deficient and CCR5-wild-type sooty mangabeys, consistent with the notion that SIVsmm alternative coreceptor use is not a feature exclusive to virus from CCR5$^{\Delta/\Delta}$ sooty mangabeys.

Multiple subtypes of SIVsmm show similar alternative coreceptor use in vitro

SIVsmm circulating in U.S. primate centers cluster in several genetic subtypes (2). Infected sooty mangabeys generally do not display CD4$^+$ T cell loss, but, collectively, animals at YNPRC infected with subtype 5 SIVsmm display lower peripheral CD4$^+$ T cell counts relative to sooty mangabeys infected with other subtypes (1). Therefore, we asked if coreceptor use patterns differ among SIVsmm subtypes and, in particular, whether subtype 5 SIVsmm exhibit a distinct pattern that might lead to expanded tropism and consequent CD4$^+$ T cell loss. Pseudotyped virions were generated with SGA-derived Envs from sooty mangabeys infected with SIVsmm subtype 5, as well as subtypes 2 and 3, and tested for infection of 293T cells transfected with sooty mangabey-derived coreceptors and smCD4. Since we previously observed no difference in infection between the two smCD4 alleles, we utilized smCD4 4.3 to test this extended panel of SIVsmm Envs.

SIVsmm subtypes 2, 3, and 5 exhibited alternative coreceptor use patterns comparable to those observed for subtype 1 SIVsmm (**Figure 2.5**), with robust infection through smCXCR6 and smCCR5. These Envs displayed modest use of smGPR15 and levels of infection through smGPR1 and smAPJ that were variable and generally marginal. Subtype 2, 3, and 5 Envs did not enter cells expressing smCXCR4 in conjunction with smCD4. Notably, the subtype 5

SIVsmm did not display expanded alternative coreceptor use relative to other subtypes of SIVsmm examined, suggesting that factors other than distinct coreceptor use patterns underlie the relatively lower $CD4^+$ T cell counts with this SIVsmm lineage.

Rare SIVsmm that induce rapid $CD4^+$ T cell loss in sooty mangabeys exhibit expanded sooty mangabey coreceptor use

In contrast to the majority of infected animals, rare SIVsmm were described that give rise to profound rapid $CD4^+$ T cell loss in infected sooty mangabey hosts (46). Previous studies employing cell-cell fusion assays and coreceptors of human origin showed these SIVsmm isolates display expanded tropism for several coreceptors, including CXCR4 (45, 46). Therefore, we determined if the expanded use of human coreceptors previously described also reflects expanded use of sooty mangabey-derived 7TMR molecules, and whether these coreceptors can support infection as well as cell-cell fusion. We tested infection by pseudotyped virions carrying SGA-derived Env isolated from three of these very low-CD4 sooty mangabeys (FJv, FPy and FTv) and compared to infection with Env from one animal with Clade 1 typical non-progressive infection (FFv).

Because 293T cells express low levels of endogenous human huCXCR4 (17), we first determined if transfection of smCD4 alone enabled infection. Indeed, smCD4 alone enabled infection by pseudotypes carrying Envs from the CD4-low animals FJv, FPy and FTv (**Figure 2.6**). This pattern differs from that seen with typical Clade 1 to Clade 5 SIVsmm (see Figure 2.3, Figure 2.4, Figure 2.5) where there is no infection in 293T cells expressing CD4 alone. Pre-treatment with the CXCR4 antagonist AMD3100 completely abrogated SIVsmm FJv, FPy and FTv infection in 293T cells transfected with smCD4 alone, confirming that infection is mediated by endogenous human CXCR4 on 293T cells. Additionally, because AMD3100 completely blocks background infection by these pseudotypes, 293T cells treated with AMD3100 can be use to examine other coreceptors.

Transfection of 293T cells with smCD4 and smCXCR4 together resulted in markedly increased levels of infection compared to cells transfected with smCD4 alone (**Figure 2.6**). Thus, sooty mangabey-derived smCXCR4 supports robust infection by these low-CD4 SIVsmm pseudotypes. AMD3100 pre-treatment blocked the majority of infection in smCXCR4-transfected cells, suggesting that AMD3100 has substantial activity against smCXCR4 in addition to human CXCR4. Alignment of the human and sooty mangabey CXCR4 sequences showed amino acid residues previously identified as critical to the interaction between AMD3100 and human CXCR4 (D171, D262, E288) are conserved between the two species (22, 30, 59). SIVsmm FJv, FPy, and FTv retained the ability to infect cells via smCCR5. Infection through smCXCR4 appeared to be at least as efficient as infection through smCCR5 *in vitro*.

We then tested the extended panel of sooty mangabey-derived 7TMR molecules with low-CD4-derived SIVsmm Envs. Target cells were transfected with 7TMR molecules and smCD4, pre-treated with AMD3100 to block infection through endogenous huCXCR4 on 293T, and infected with Env pseudotyped viruses. SIVsmm FJv, FPy, and FTv displayed broad and efficient use of multiple alternative coreceptors (**Figure 2.7**). Like all other SIVsmm studied, these Env used smCXCR6 very efficiently and smGPR15 slightly less so. In addition, while none of the typical SIVsmm Env used smCCR3 or smCCR8 (see Figure 2.4, Figure 2.5, and SIVsmm FFv in Figure 2.7), both of these pathways supported infection by SIVsmm FJv, FPy, and FTv. Finally, while most SIVsmm Envs used smAPJ and smGPR1 to a very limited extent, these 7TMRs supported substantially more robust infection by SIVsmm from these CD4-low sooty mangabeys. Notably, neither smCCR2b nor smCCR4 supported infection, nor did infection occur through any coreceptors in the absence of smCD4 (data not shown). Thus, in contrast to typical SIVsmm Envs, the SIVsmm Envs derived from rare CD4-depleted sooty mangabeys exhibit expanded use of SM alternative coreceptors smCXCR4, smCCR3, and smCCR8 as well as efficient infection though smAPJ, smCXCR6, smGPR1, and smGPR15, while retaining the ability to enter cells through smCCR5. This finding is consistent with earlier results using human CXCR4, CCR8 and

CXCR6 in fusion assays and extends the breadth of coreceptor use to include sooty mangabey-derived APJ, CCR3, GPR1 and GPR15.

FIGURES

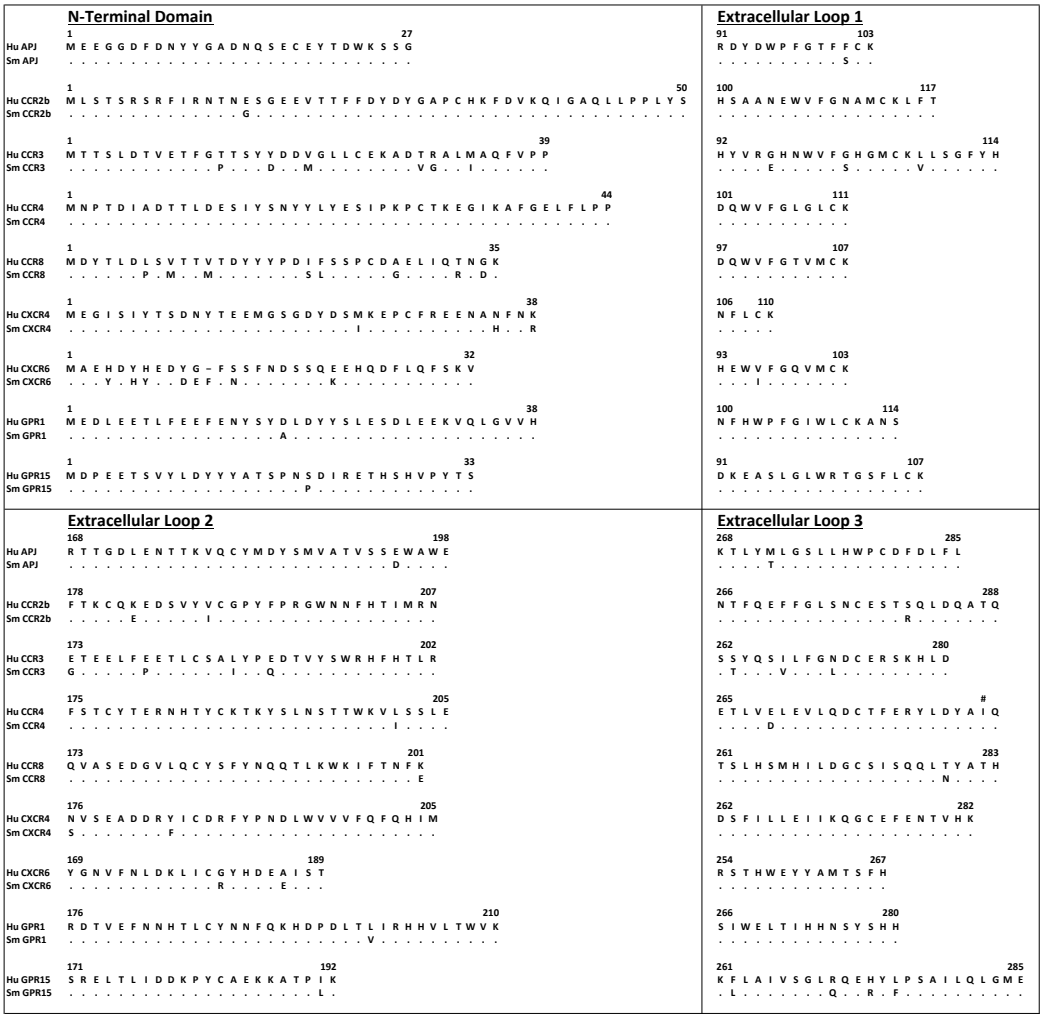

Figure 2.1. Human and sooty mangabey 7-tramsmembrane receptors differ in extracellular domains. Putative alternative coreceptors APJ, CCR2b, CCR3, CCR4, CCR8, CXCR4, CXCR6, GPR1 and GPR15 were cloned and sequenced from sooty mangabey (Sm) and predicted amino acid sequences were aligned to human (Hu) sequences from GenBank. Alignments are shown between all human and sooty mangabey extracellular domains, including the N-terminal domain and extracellular loops.

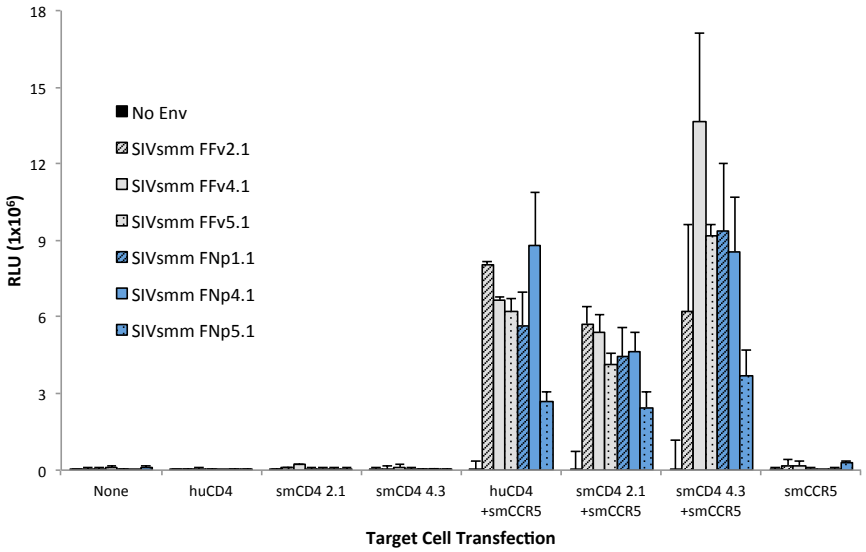

Figure 2.2. Two alleles of sooty mangabey CD4 support SIVsmm Env-mediated infection via CCR5 *in vitro*. Target 293T cells were transfected with human CD4 (huCD4) or each of two sooty mangabey CD4 alleles (smCD4 2.1 and smCD4 4.3), with or without sooty mangabey CCR5 (smCCR5). Cells were infected with luciferase-expressing pseudotype virions carrying Env glycoproteins from two subtype 1 SIVsmm-infected sooty mangabeys using equal amounts of each virus based on infectivity in U87/CD4/CCR5 cells (10^6 RLU of each virus). SIVsmm FFv Env were derived from an infected animal with a $CCR5^{wt/wt}$ genotype (gray) and SIVsmm FNp Env were derived from an infected animal with a $CCR5^{\Delta/\Delta}$ genotype (blue). Virions lacking envelope glycoprotein served as negative controls (No Env, black). Infection was measured by relative light units (RLU) in cell lysates 3 days post-infection (N=3, mean ± SD).

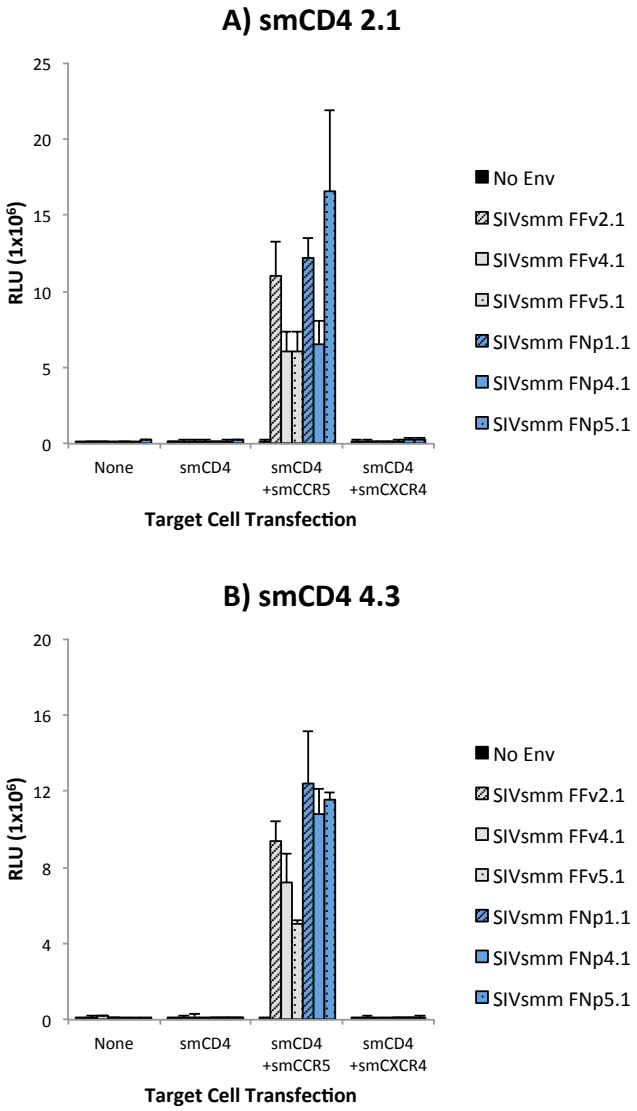

Figure 2.3. Sooty mangabey CXCR4 does not support SIVsmm Env pseudotype infection. Target 293T cells were transfected with empty plasmid (None), smCD4 alone, or smCD4 with smCCR5 or smCXCR4. Both smCD4 alleles, **(A)** smCD4 2.1 and **(B)** smCD4 4.3, were used for transfections. Target cells were infected with luciferase-expressing SIVsmm FFv (gray) and SIVsmm FNp (blue) Env pseudotype virions (5×10^5 RLU of each virus). Infection was measured by relative light units (RLU) in cell lysates 3 days post-infection (N=3, mean ± SD).

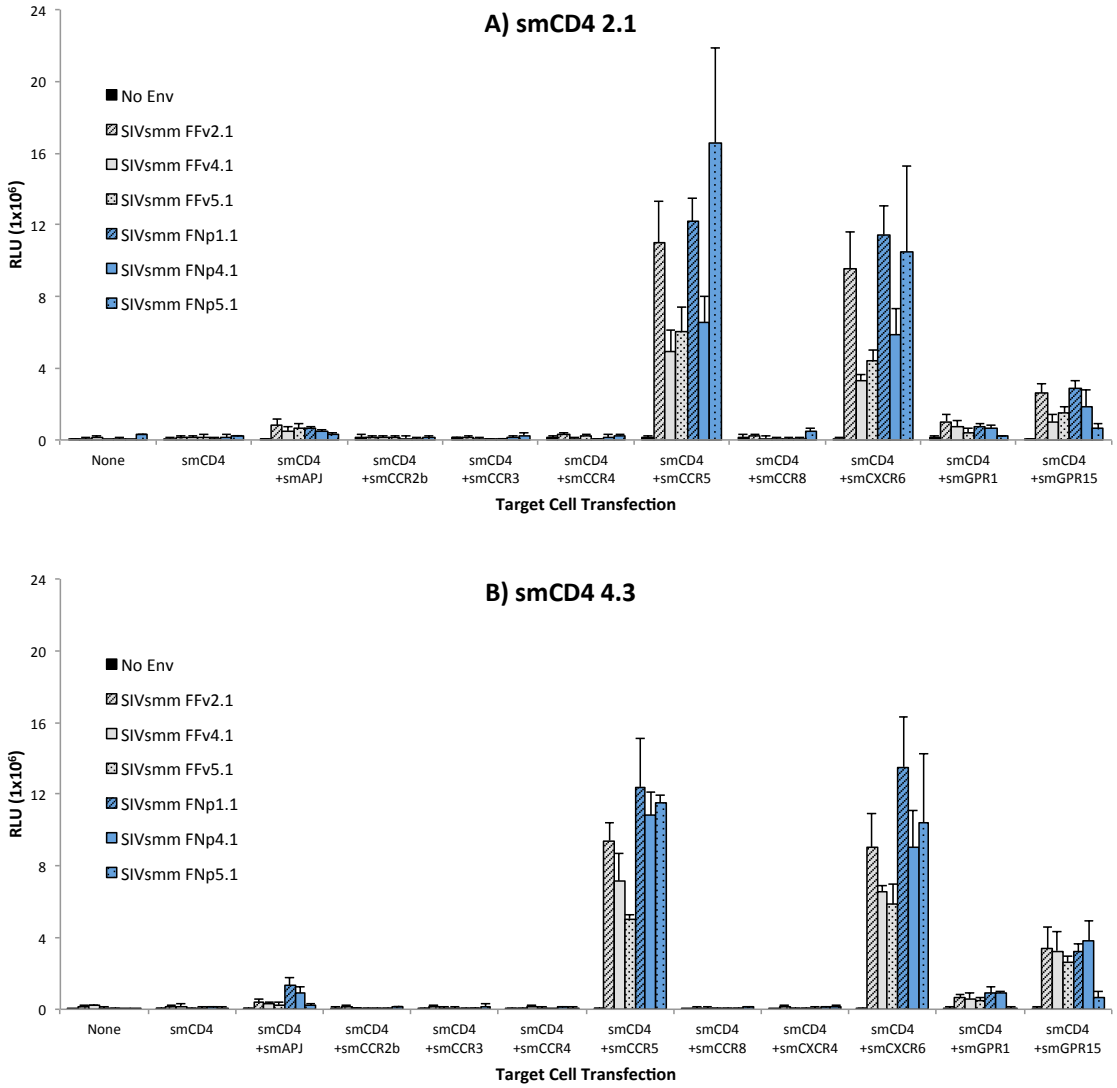

Figure 2.4. Alternative coreceptors of sooty mangabey origin support SIVsmm Env pseudotype infection. Target 293T cells were transfected **(A)** smCD4 2.1 or **(B)** smCD4 4.3. Cells were co-transfected with smCD4 alone (No CoR) or smCD4 with smAPJ, smCCR2b, smCCR3, smCCR4, smCCR5, smCCR8, smCXCR6, smGPR1 and smGPR15. Cells were infected with pseudotypes carrying subtype 1 SIVsmm Env cloned from infected $CCR5^{wt/wt}$ (FFv, gray) and $CCR5^{\Delta/\Delta}$ (FNp, blue) sooty mangabeys (5×10^5 RLU of each virus). Infection was measured by relative light units (RLU) in cell lysates 3 days post-infection (N=3, mean ± SD).

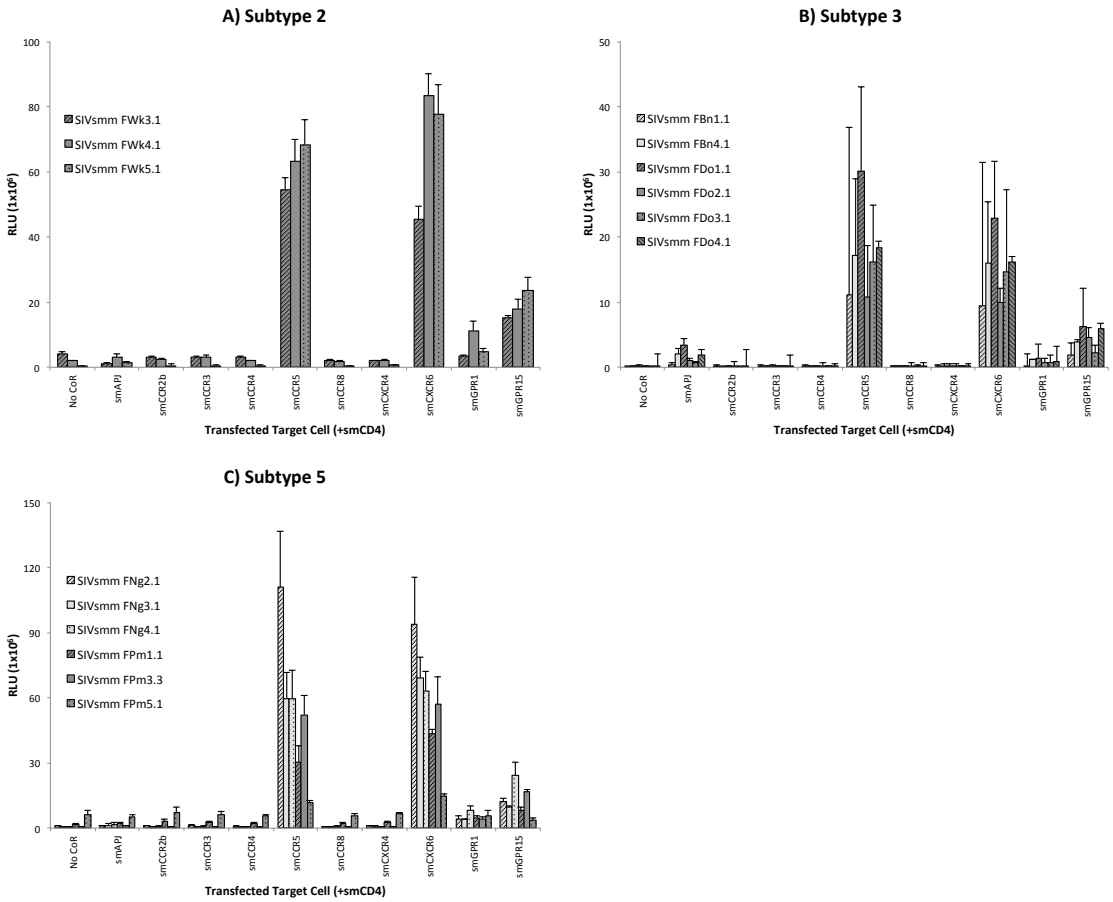

Figure 2.5. Alternative coreceptor use by distinct subtypes of SIVsmm. Target 293T cells were transfected with smCD4 4.3 in conjunction with sooty mangabey-derived 7-transmembrane receptors as described in Figure 2.4. Cells were infected with pseudotyped virions (3.5×10^5 RLU of each virus) carrying Env from SIVsmm **(A)** subtype 2 (FWk), **(B)** subtype 3 (FBn and FDo), or **(C)** subtype 5 (FNg and FPm). Infection was measured by relative light units in cell lysates 3 days post-infection (N=3, mean ± SD).

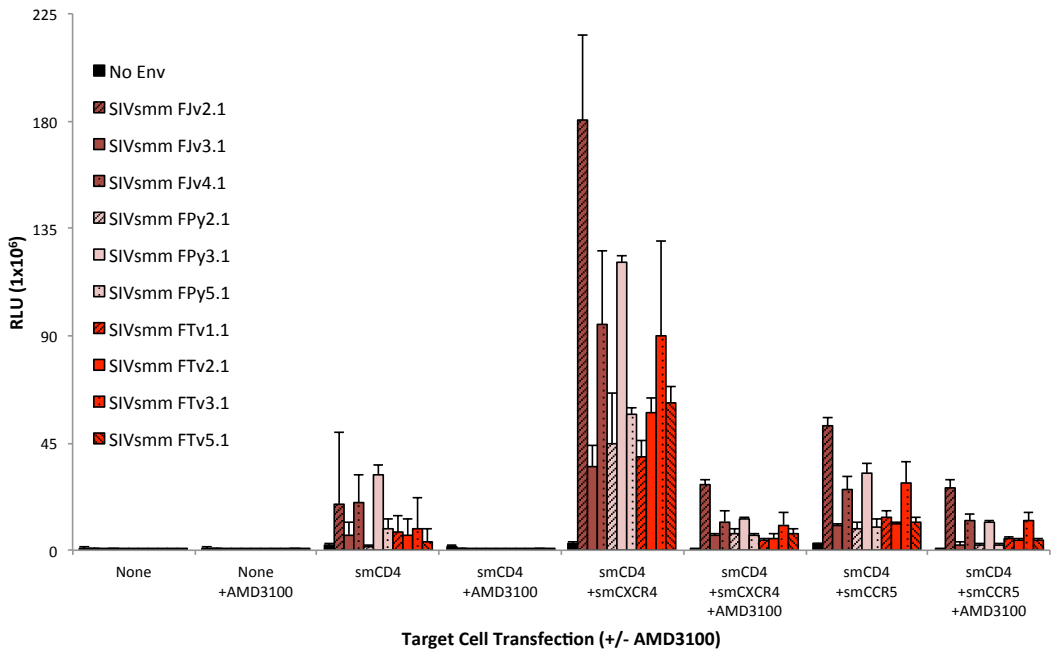

Figure 2.6. SIVsmm Env from CD4-low sooty mangabeys use sooty mangabey CXCR4. Target 293T cells were transfected with or without smCD4 4.3, smCXCR4, and smCCR5 and pre-treated with or without the CXCR4 antagonist AMD3100 (20μM). Cells were infected with pseudotyped virions carrying No Env (black) or SIVsmm Envs from CD4-low sooty mangabeys FJv, FPy, and FTv. Inocula were equilibrated on the basis of P24 Gag antigen content (2ng P24). Infection was measured by relative light units in cell lysates 3 days post-infection (N=3, mean ± SD).

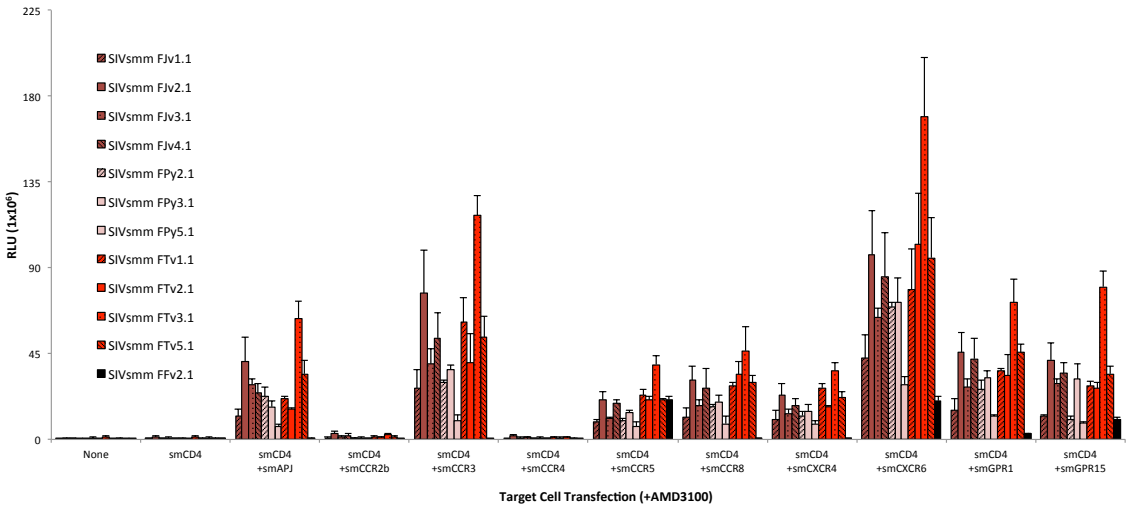

Figure 2.7. SIVsmm Env from CD4-low sooty mangabeys use sooty mangabey alternative coreceptors. Target 293T cells transfected with smCD4 4.3 in conjunction with sooty mangabey 7-transmembrane receptors (smAPJ, smCCR2b, smCCR3, smCCR4, smCCR5, smCCR8, smCXCR4, smCXCR6, smGPR1, smGPR15) were treated with AMD3100 (20μM) to block endogenous CXCR4 and then infected with pseudotyped virions. Pseudotypes carried SIVsmm Envs from CD4-low sooty mangabeys FJv , FPy, and FTv (red), or from one animal with typical clade 1 infection, FFv (black). Inocula were equilibrated on the basis of P24 Gag antigen content (2ng P24). Infection was measured by relative light units in cell lysates 3 days post-infection (N=3, mean ± SD).

Table 2.1. Relatedness of sooty mangabey and human 7TMRs.

7TMR	Percent overall amino acid identity to[a]		Number of residues differing from human receptor[b]	
	Human	*Rhesus Macaque*	*NTD*	*ECL2*
APJ	98.7	99.7	0	1
CCR2b	96.7	99.2	1	2
CCR3	91.3	98.6	6	4
CCR4	98.6	100.0	0	1
CCR8	94.4	99.2	8	1
CXCR4	98.3	99.4	3	2
CXCR6	94.5	98.3	8	2
GPR1	97.7	99.7	1	1
GPR15	97.2	99.4	1	1

[a] Alignment between predicted amino acid sequences of sooty mangabey molecules cloned herein and homologous human and rhesus macaque sequences from GenBank

[b] Number of predicted amino acids differences between sooty mangabey and human molecules in the N-terminal domain (NTD) and extracellular loop 2 (ECL2), two regions most relevant to coreceptor function based on mapping studies of HIV-1 interaction with CCR5 and CXCR4 (20).

DISCUSSION

Natural SIVsmm infection of sooty mangabeys is typically non-pathogenic despite robust virus replication. Key findings associated with the benign nature of this infection include the absence of chronic immune activation and a pattern of infected cells *in vivo* that shows relative sparing of $CD4^+$ central memory T cells (T_{cm}) (51, 66). While the relative sparing of sooty mangabey $CD4^+$ T_{cm} from infection is associated with low CCR5 expression, previous studies from our group showed that a substantial minority of SIVsmm-infected sooty mangabeys possess CCR5-null genotypes (58), indicating that alternative entry coreceptors are used *in vivo* and will need to be defined in order to understand mechanisms underlying viral tropism and targeting *in vivo*. In this study, we cloned multiple potential alternative coreceptors of sooty mangabey origin and found smCXCR6 was used consistently and very efficiently, with infection levels comparable to smCCR5, while smGPR15 use was also broad although somewhat less efficient. Infection through smGPR1 and smAPJ was variable and typically low-level; smCXCR4, smCCR3 and smCCR8 were used only by SIVsmm from rare sooty mangabeys with profound $CD4^+$ T cell loss that previously demonstrated expanded human coreceptor use.

Robust use of the principal sooty mangabey alternative coreceptors CXCR6 and GPR15, along with CCR5, was a feature of all SIVsmm studied including pseudotypes from sooty mangabeys with CCR5 wild-type as well as CCR5-null genotypes. While CCR5-null sooty mangabeys display high viremia indicative of efficient alternative coreceptor function *in vivo* (mean $>10^4$ copies/ml), a modest but significantly higher levels of viremia (~0.5 log difference) was seen in sooty mangabeys with CCR5 homozygous wild-type genotypes, and intermediate viral loads in heterozygous animals (58). Those findings suggest that alternative pathways are exclusively responsible for SIVsmm replication in animals lacking CCR5, while both CCR5 and alternative coreceptors may be used in hosts where both CCR5 and other pathways are available. It was not clear from the amino acid sequences of functional alternative coreceptors what specific components of 7TMR extracellular regions dictate functional coreceptor activity.

The sooty mangabey model of SIV infection is of keen interest given the ability of natural hosts to avoid disease despite robust viral replication. Although multiple mechanisms likely contribute to this resistance, including distinct immune activation patterns and CD4-negative T helper cell function (3, 9, 45, 66, 72), entry coreceptor use by immunodeficiency viruses defines viral target cell tropism and can be an important determinant of $CD4^+$ T cell loss. Acquisition of CXCR4 use by HIV-1 parallels rapid $CD4^+$ T cell decline as HIV-1 gains the ability to infect CXCR4-expressing naïve T cells (6, 7, 35). In SIVmac-infected rhesus macaques, depletion of $CD4^+$ T_{cm} correlates with $CD4^+$ T cell loss and disease progression, suggesting a critical role for T_{cm} in maintaining T cell homeostasis (37, 49, 54). Natural hosts of SIV, including sooty mangabeys, display low CCR5 expression on $CD4^+$ T cells relative to non-natural hosts (52, 53, 65, 70, 71). CCR5 is especially restricted on $CD4^+$ T_{cm} of sooty mangabeys compared with rhesus macaques *ex vivo* and following *in vitro* stimulation (51). T_{cm} of sooty mangabeys are also relatively resistant to SIV infection *in vitro* compared with rhesus macaques, and harbor lower levels of viral DNA *in vivo* (51). Thus, restricted coreceptor expression may protect critical cells from infection and preserve memory $CD4^+$ T cell homeostasis in natural hosts.

Overall levels of viremia in sooty mangabeys are equivalent to or exceed those in rhesus macaques (66), indicating no lack of susceptible target cells in sooty mangabeys despite limited CCR5 expression. Therefore, combined with protection of critical target cells mediated by restricted CCR5 expression, alternative coreceptors may define populations of more dispensable target cells capable of supporting SIV replication in natural hosts without disruption of T cell homeostasis.

In human and mouse cells, CXCR6 is found on subsets of $CD4^+$ TH1, TH17 and TREG memory/effector cells, as well as NKT cells, but is generally not co-expressed with naïve or central memory markers such as CD45RA, CCR7 or L-selectin (28, 34, 41, 63, 69). If patterns

are similar in SIV natural hosts, the exclusion of CXCR6 from less differentiated and self-renewing naïve or T_{cm} cells would be consistent with shifting of viral targets away from subsets critical for T cell homeostasis into potentially more expendable cell targets. While GPR15 is expressed in human blood $CD4^+$ T cells and in lymphoid tissues (18, 21, 23, 32, 40), its distribution on specific subsets has not been defined. CXCR6 and GPR15 are also both expressed in human intestinal tissue (18, 40), which is an important site for viral replication. Antibodies to define surface expression of both sooty mangabey molecules are not presently available, but we found smCXCR6 and smGPR15 RNA transcripts in sooty mangabey $CD4^+$ T cells (unpublished, data not shown) (57). Thus, it is important to define the patterns of expression for these receptors by sooty mangabey $CD4^+$ cell subsets, and their coreceptor function under physiological expression conditions in primary cells responsible for replication *in vivo*.

Coreceptors other than CCR5 and CXCR4 were not thought to be important in human infection with HIV-1. However, a recent report described a transmitter/founder virus from an acutely infected person that was impaired in its ability to use CCR5 and CXCR4 but entered efficiently through GPR15 (32). It remains to be determined if alternative coreceptors including CXCR6 or GPR15 may also play a role in HIV-1 pathogenesis in rare situations, or more broadly in as-yet unrecognized ways.

While the majority of infected sooty mangabeys show neither CD4 loss nor disease, a small number of previously-reported animals had extensive $CD4^+$ T cell depletion, which was associated with expanded SIVsmm use of human coreceptors including huCXCR4, huCXCR6 and huCCR8 (46). We now confirm expanded tropism by Envs from these animals for sooty mangabey-derived coreceptor molecules, and identify smCCR3, smGPR1, smGPR15 and smAPJ utilization as well. Acquisition of CXCR4-mediated tropism for naïve $CD4^+$ T cells is strongly linked to accelerated $CD4^+$ T cell loss in HIV-infected humans, so it is uncertain if the additional coreceptors used by these CXCR4-tropic SIVsmm Envs contribute further to disrupted

$CD4^+$ T cell homeostasis. Unfortunately, we did not have enough Env sequences from either CD4-low- or typically-infected sooty mangabeys to form a strong alignment and identify which region(s) of gp120 might support this expanded tropism. We did not see distinct alternative coreceptor use or smCXCR4-mediated infection by pseudotypes derived from sooty mangabeys naturally infected with subtype 5 SIVsmm, which display more moderate levels of $CD4^+$ T cell loss (1). Thus, $CD4^+$ T cell loss in subtype 5 SIVsmm-infected sooty mangabeys is likely due to factors other than expanded tropism by the virus, at least for the coreceptors tested here.

Notably, SIV-infected sooty mangabeys that display widespread $CD4^+$ T cell loss do not progress to AIDS (45). Additionally, uninfected natural host African green monkey T cells were found to down-regulate CD4 cell surface expression when entering the memory pool such that African green monkeys express a low fraction of $CD4^+$ T cells relative to $CD8^+$ T cells (3, 47, 48). Although we hypothesize that typical SIV alternative coreceptor use may protect natural hosts from $CD4^+$ T cell loss, it is clear that CD4 loss does not necessarily lead to disease progression. Double-negative $CD4^-CD8^-$ T cells may account for the lack of disease progression in natural hosts. A higher proportion of double-negative T cells are found in uninfected natural host sooty mangabeys and African green monkeys relative to uninfected non-natural host rhesus macaques (33, 72). Furthermore, double-negative $CD4^-CD8^-$ T cells in the blood, lymph nodes, and rectal mucosa of sooty mangabeys have effector T cell-like functions (68), and $CD4^-$ T cells from African green monkeys maintain functions normally attributed to $CD4^+$ T cells (3). Natural host double-negative cells may be also be protected from bystander killing post-infection as apoptosis occurs in fewer double-negative sooty mangabey T cells than double-negative rhesus macaque T cells following SIV infection (44). It is critical to define not only mechanisms leading to $CD4^+$ T cell loss in rhesus macaques and humans, but also to define the exact role of $CD4^+$ T cell loss in disease progression.

Multiple complex and non-exclusive mechanisms are likely involved in protecting sooty mangabeys and other natural hosts both from $CD4^+$ T cell loss, as well as from disease in the few animals where $CD4^+$ T cell depletion does occur (3, 8, 9, 45, 51, 66, 67, 72). Thus, both the target cells spared from infection and those supporting replication are critical to understanding the nature of infection and pathogenesis (29). Future studies are needed to define CXCR6 and GPR15 coreceptor expression patterns on sooty mangabey $CD4^+$ cell subsets, the relationship between alternative coreceptor and CCR5 expression, and how alternative coreceptors are regulated. Finally, comparing coreceptor expression patterns between natural and non-natural host species may identify more expendable populations of $CD4^+$ cells in the natural host that are capable of supporting viral replication through use of alternative entry pathways without leading to disease progression.

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CHAPTER 3

MARAVIROC AND CHEMOKINE CXCL16 SPECIFICALLY ATTENUATE CCR5-MEDIATED AND CXCR6-MEDIATED SIV INFECTION *IN VITRO*

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ABSTRACT

Viruses from the SIVsmm/SIVmac lineage generally exhibit robust CD4-dependent infection through coreceptor CCR5 and alternative coreceptor CXCR6 expressed on transfected cells *in vitro*. Other alternative coreceptors also support SIVsmm/SIVmac infection *in vitro*. The role of CCR5 and alternative coreceptors in SIVsmm/SIVmac infection of primary cells is largely undefined. To later identify if CCR5, CXCR6, or other coreceptors support SIV infection and replication in primary sooty mangabey PBMCs and rhesus macaque PBMCs *ex vivo*, we first developed methods to specifically inhibit CCR5-mediated and CXCR6-mediated SIV infection. Small-molecule CCR5 antagonists, including Maraviroc, blocked infection via sooty mangabey CCR5 (smCCR5) and inhibited the majority of, but not all, infection via rhesus macaque CCR5 (rmCCR5) *in vitro*. There are no known small-molecule inhibitors of CXCR6. We discovered that recombinant human CXCL16, a ligand of CXCR6, attenuated sooty smCXCR6-mediated SIV infection at low levels of sooty mangabey CXCR6 expression *in vitro*. Neither Maraviroc nor CXCL16 had appreciable direct off-target effects on infection mediated by non-ligand coreceptors, indicating that Maraviroc and CXCL16 can be used to specifically antagonize CCR5-mediated and CXCR6-mediated SIV infection in primary cells.

INTRODUCTION

Our lab previously demonstrated that natural host sooty mangabey SIVsmm infection and replication occur in the absence of functional CCR5, indicating that alternative coreceptors support SIVsmm infection and replication *in vivo* (28). Furthermore, we defined the genetic and functional nature of sooty mangabey coreceptors of SIVsmm *in vitro* (5) (Chapter 2). SIVsmm Envelopes (Env) cloned from SIVsmm-infected sooty mangabeys display robust use of sooty mangabey CCR5 (smCCR5) and sooty mangabey CXCR6 (smCXCR6), as well as moderate to low use of smGPR15 and smGPR1 *in vitro*. Closely related SIVmac Env derived from pathogenic infections of rhesus macaques display a similar pattern of alternative coreceptor use and have been found to use rhesus macaque CCR5 (rmCCR5) and rhesus macaque GPR15 (rmGPR15), but not rhesus macaque CXCR6 *in vitro* (15, 27, 45) (also see Chapter 4). Here, we define methods to inhibit SIV infection mediated by the robust alternative coreceptor CCR5 cloned from both sooty mangabeys and rhesus macaques, as well as the robust alternative coreceptor CXCR6 cloned from sooty mangabeys.

Small-molecule CCR5 antagonists allosterically block HIV-1 infection by binding within the transmembrane cavity of human CCR5 and modifying the conformation of extracellular loop 2 (ECL2), disrupting interactions between the human CCR5 and HIV-1 Env (9, 18, 35). Each small-molecule CCR5 antagonist displays unique signaling properties and stabilizes unique confirmations of CCR5 (8). Therefore, we examined which CCR5 antagonists specifically block smCCR5- and rmCCR5-mediated SIV infection. We primarily used Maraviroc, a CCR5 antagonist clinically approved for the treatment of HIV-1 (4). We also investigated AD101 (37), Aplaviroc (3, 18), CMPD167 (6), and Vicriviroc (33).

Identifying an inhibitor of smCXCR6-mediated infection was more challenging, as there is no commercially available small-molecule inhibitor of CXCR6. Prior studies found that high concentrations of CCR5 chemokines RANTES, MIP-1α, and MIP-1β block CCR5-mediated HIV

and SIV infection (2). CXCL16 is a natural chemokine ligand of CXCR6 produced in membrane-bound and soluble forms *in vivo* by antigen presenting dendritic cells, monocytes, macrophages, and NKT cells (10, 23). Given the ability of CCR5 ligands to inhibit CCR5-mediated infection, we investigated the ability of recombinant human chemokine CXCL16 to block SIV infection mediated by smCXCR6.

Notably, SIV infection of natural host sooty mangabeys is non-pathogenic, whereas SIV infection of non-natural host rhesus macaques results in rapid progression to AIDS. Ultimately, we are interested in defining the coreceptor use patterns of viruses from both sooty mangabeys and rhesus macaques in primary cells. Therefore, in this this study, we examined the ability of small-molecule CCR5 antagonists and recombinant human CXCL16 to inhibit entry of SIVsmm from naturally infected sooty mangabeys as well as rhesus macaque SIV from the same viral lineage: SIVmac 251, SIVmac 239, and SIVsmm E660. Note that, despite the confounding nomenclature, SIVsmm E660 is a rhesus macaque SIV and should not be confused with sooty mangabey SIVsmm. SIVmac 251, SIVmac 239, and SIVsmm E660 are experimental challenge viruses used to induce pathogenesis in rhesus macaques. SIVmac 239/251 and SIVsmm E660 were derived by separate experimental cross-species transmissions of SIVsmm from sooty mangabeys into rhesus macaques (12) (see Chapter 1, Figure 1.8) and these viruses represent diverse pathogenic strains of SIV in rhesus macaques.

We found that small-molecule CCR5 antagonists completely blocked SIV infection via smCCR5. These compounds also blocked the majority of, but not all, rmCCR5-mediated SIV infection at tolerable concentrations *in vitro*. Maraviroc had no appreciable off-target effects on CXCR6- and GPR15-mediated SIV infection. Recombinant human chemokine CXCL16 was a specific and significant inhibitor of smCXCR6-mediated SIV infection at low levels of smCXCR6 expression *in vitro*; chemokine CXCL16 blocked the majority of, but not all, SIV infection through smCXCR6. Ultimately, these methods to specifically and effectively inhibit CCR5- and CXCR6-mediated SIV

infection can be used to define the relative role of CCR5, CXCR6 and other alternative coreceptors in SIV infection of natural and non-natural host primary cells.

METHODS

CCR5 Antagonists and CXCL16

Maraviroc (Selzentry, Celsentri) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID (Cat #11580) and suspended at 20mg/mL in dimethyl sulfoxide (DMSO). AD101 was a gift from J. Strizki (Merck & Co.) and was suspended in DMSO. AD101 compound was verified by liquid chromatography mass spectrometry (LC-MS/MS) in collaboration with R. Kohli at the University of Pennsylvania Department of Chemistry Mass Spectrometry Facility. Aplaviroc, CMPD167, and Vicriviroc (Merck & Co.) were a gift from Z. Parker in the laboratory of R. Doms (University of Pennsylvania). Lyophilized recombinant human CXCL16 (R&D Systems #976-CX-025) was re-suspended at 25µg/mL in phosphate buffered saline and used within 2 days with ≤1 freeze/thaw cycle.

Viruses and Viral Env Pseudotypes

SIVsmm FFv 5.1 Env was provided by C. Derdeyn (Emory University) and was cloned from a SIVsmm Clade 1infected sooty mangabey with a $CCR5^{wt/wt}$ genotype using single genome amplification (SGA) (5, 17, 28, 30). SIVmac 239 Env was cloned from the full-length provirus SIVmac 239 and was a gift from A. Swanstrom in the laboratory of J. Hoxie (University of Pennsylvania). SIVmac 251 Env RZu4-1.1 Day 11 was cloned by C. Derdeyn by SGA from a control (non-CD4-depleted) rhesus macaque infected with SIVmac 251 as previously described (7). SIVsmm E660 CP3C-P-A8 and SIVsmm E660 CR54-PK-2A5 Env were cloned by SGA from SIVsmm E660 infected rhesus macaques and were a gift from K. Barr, M. Lopker, and G. Shaw (University of Pennsylvania) (13, 29). HIV-1 JR-FL Env was cloned previously from the CCR5-tropic proviral clone. Proviral infectious molecular clone (IMC) SIVsab 92018ivTF was provided by F. Bibollet-Ruche and B. Hahn (University of Pennsylvania) (11). Sabaeus African Green Monkey SIVsab 92018ivTF Env was cloned from this IMC using nested primers designed by alignment of SIVsab 92018ivTF and SIVsab-1 sequences: 3'-half SIVsab-a796r-stce (5'-CTC-CWC-CCT-GGA-AAG-TCC-CKC-T-3'), SIVsab-a787f-stce (5'-TGY-TGG-TGG-GGA-AAR-ATA-

GAG-CAC-WC-3'); Env SIVsabA-Fwd (5'-CAC-CCC-SCT-CCA-GGC-CTG-TRN-CAA-TA-3'), SIVsabB-Rev (5'-CCA-RCC-ATC-SAC-WAT-DCC-CC-3'). The SIVsab 92018ivTF Env PCR product was TA cloned into pcDNA3.1 using a directional TOPO expression kit (Invitrogen). Env pseudotypes were generated by co-transfecting SIV Env plasmids and pNL-Luc-E^-R^+ into human embryonic kidney 293T cells as previously described (5) (Chapter 2). Vesicular stomatitis virus glycoprotein (VSV-G) Env served as a positive control for CD4- and coreceptor-independent infection; No Env empty-pcDNA3.1 pseudotyped virions served as negative controls.

Pseudotype Infections

Infections were performed as described previously (5) (Chapter 2). Target 293T cells cultured in DMEM + 10% FBS + 1% Penicillin-Streptomycin + 1% L-Glutamine were transfected with 1µg CD4 and coreceptor using Fugene (Promega). For Maraviroc titrations, 293T cells were transfected with 1µg CD4 and 1µg CCR5 cloned from humans, rhesus macaques, and sooty mangabeys. Cells were washed 24 hours later and re-plated 2×10^5 cells per well in a 96 well plate. The next day, media was removed and replaced one hour pre-infection with 100µL media containing 1.5× vehicle DMSO or Maraviroc 0.1nM, 1nM, 10nM, 100nM, 1µM, 10µM, 100µM, 1mM. DNAse-treated Env pseudotype viruses were added 50uL/well for a final volume of 150µL and a final concentration of Maraviroc indicated. Cells were spin-inoculated and luciferase output was measured 72 hours post-infection in relative light units (RLU). Luciferase production in vehicle-treated controls was normalized to 100%. Best-fit sigmoidal curves and EC_{50} were calculated using GraphPad PRISM software (San Diego, CA, USA). For smCXCR6 transfections using lesser amounts of coreceptor plasmid DNA, cells were transfected with 1µg smCD4 plus 1µg total of smCXCR6 plus empty pcDNA3.1 vector. Cells transfected with CD4 and 1µg empty pcDNA3.1, and cells transfected with 2µg empty pcDNA3.1 served as negative controls. Cells were washed and re-plated as before. The following day, media was removed and replaced one hour pre-infection with 100µL media containing 1.5× CCR5 antagonist, 1.5× CXCL16 with vehicle DMSO, or 1.5× vehicle DMSO alone. DNAse-treated pseudotyped viruses were added 50uL/well

for a final volume of 150μL and a final concentration of 10μM Maraviroc, 10μM AD101, 1μM Aplaviroc, 6.7μM CMPD167, 10μM Vicriviroc, or 500ng/mL CXCL16 in a final concentration of 0.026% DMSO. Pseudoviruses were spin-inoculated and luciferase output was measured 72 hours post-infection.

HIV-1 Infections

Human PBMCs were isolated by apheresis by the Penn Human Immunology Core. PBMCs were rested for one day and then stimulated 3 days with 10μg/mL Phytohaemagglutinin (PHA) and 30Unit/mL Interleukin-2 (IL-2) in RPMI + 10% FBS + 1% Penicillin-Streptomycin + 1% L-Glutamine. Media was replaced one hour pre-infection with 10μg/mL IL-2 and 10μM Maraviroc or other CCR5 antagonist as indicated, 500ng/mL CXCL16 with DMSO, or the corresponding volume of vehicle DMSO alone (0.026%). Approximately 1.5×10^6 PBMCs in 1mL media were plated to 24-well plates. 5ng P24 Gag HIV-1 were added in a total of 100μL per well. Virus was spin-inoculated 2 hours at 1200×g and 25°C. One day later, cells were washed and re-plated in 1mL IL-2 media plus drug/chemokine (Day 0). 50μL supernatant was collected periodically for P24 Gag ELISA (Perkin-Elmer) performed by S. Bryan and F. Shaheen in the Penn Center for AIDS Research (CFAR) Virology Core.

qPCR

Infections for qPCR were performed in parallel with those for P24 Gag readouts. Cells were washed, lysed in DNA lysis buffer (100mM KCl, 0.1% NP-40, 20mM Tris [pH 8.4], 0.5 mg of proteinase K per ml), incubated at 55°C for 2 h, and boiled for 15 min as previously described (44). Primer and probe sets were designed by the Penn CFAR Virology Core to HIV-1 long terminal repeat (LTR) strong-stop (forward primer, 5′-GCT AGC TAG GAA ACC CAC TGC TTA-3′; reverse primer, 5′-GCT AGA GAT TTT CCA CAC TGA CT-3′; probe, FAM-5′-GCG AGT CAC ACA ACA GAC GGG CAC ACA CTA CTC GC-3′-DABCYL), Gag as previously described (44), and the cellular GAPDH gene (forward primer, 5′-GGT GGT CTC CTC TGA CTT CAA CA-3′;

reverse primer, 5′-CCA GCC ACA TAC CAG GAA ATG-3′; probe, FAM-5′-CGC AGC CTG GCA TTG CCC TCA ACG ACC ACG CTG CG-3′-DABCYL). PCR was performed on an ABI 7700 real-time PCR detection system with an initial incubation at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. For quantification, DNA from serial dilutions of ACH2 cells over the range of 10^1 to 10^5 was amplified in parallel. Data were analyzed using 7000 SDS Software (Applied Biosystems).

Flow Cytometry

For Maraviroc toxicity analysis, human PBMCs were stimulated for 3 days with PHA and IL-2 as above. Media was replaced with IL-2 and cells were plated 1×10^6 per well in a 6 well plate for 3 days of treatment with media alone, 100μM Maraviroc, 1mM Maraviroc, or the corresponding amounts of vehicle 0.26% DMSO, 2.6% DMSO. Cells were stained using Aqua LIVE/DEAD Fixable Dead Cell Stain Kit (Life Technologies #L34957) and analyzed using a BD LSR flow cytometer. For coreceptor expression analyses, human $CD4^+$ T cells were selectively purified following CD14, CD11b, CD16, HLA-DR, CD21 depletion by the Penn Human Immunology Core, and stimulated 3 days with PHA and IL-2. Where noted, media was replaced with IL-2 and 10μM Maraviroc, 500ng/mL CXCL16 with DMSO, or DMSO alone for 24 hours. 293T cells were transfected with 2μg total plasmid, washed, and read 48 hours post-transfection. Cells were independently stained with antibodies mouse αCCR5 (CD195) 3A9 PE (BD Biosciences), mouse αCXCR6 TG3 Alexa Fluor 647 (BioLegend), αCXCR4 (CD184) 12G5 PE (BD Biosciences), isotype mouse IgG_{2a} PE, isotype mouse IgG2bκ Alexa Fluor 647. Cells were imaged using a BD FACSCalibur flow cytometer. Flow cytometry analyses were performed using FlowJo software.

Data Analyses

Sigmoidal dose response curves were fitted to Maraviroc titration data and statistical analyses of EC_{50} were performed using GraphPad PRISM 6 software (La Jolla, CA, USA). All statistical

comparisons were made by a two-tailed unpaired t-Test with Welch's correction unless otherwise indicated. Significance was defined as $p<0.05$.

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) and the Tulane National Primate Research Center (TNPRC). Studies were also reviewed and approved by the University of Pennsylvania IACUC.

RESULTS

Small-molecule CCR5 antagonists fully block smCCR5- but not rmCCR5-mediated SIV pseudotype infection *in vitro*

To address the CCR5-dependence of SIV in nonhuman primate PBMCs, we developed a method to inhibit sooty mangabey smCCR5- and rhesus macaque rmCCR5-mediated viral entry. We initially tested the CCR5 antagonist Maraviroc, a clinically approved small-molecule compound that has been used extensively to block HIV-1 infection in humans. A prior study found that Maraviroc only blocks 80% of SIVmac 239 replication in rhesus macaque peripheral blood mononuclear cells (PBMCs) (21) whereas multiple pharmacological studies have found that Maraviroc completely blocks CCR5-tropic HIV-1 in human PBMCs (4). Therefore, we hypothesized that Maraviroc may differ in its ability to antagonize CCR5 from different primate species.

To determine the species-specific properties of Maraviroc inhibition, we determined the concentration of Maraviroc that blocks SIV Env pseudotype infection of 293T cells expressing CD4 and CCR5 from humans, rhesus macaques, or sooty mangabeys. Target 293T cells were transfected with human huCD4 and huCCR5, rhesus macaque rmCD4 and rmCCR5, or sooty mangabey smCD4 and smCCR5. The majority of published studies have used 10μM or 15μM Maraviroc to block HIV and SIV infection *in vitro*. In this experiment, cells were pre-treated with vehicle or concentrations of Maraviroc ranging from 100pM to 1mM. Cells were infected with HIV-1 and SIV Env pseudotypes of pNL-Luc-E^-R^+, a viral backbone that lacks Env and expresses luciferase in place of Nef. Infection was measured by luciferase production in relative light units (RLU) and data were normalized to 100% luciferase output in CD4- plus CCR5-transfected cells pre-treated with vehicle alone (No Drug). Data were fitted to a sigmoidal dose-response curve to estimate EC_{50}.

Maraviroc completely blocked CCR5-tropic HIV-1 JR-FL Env-mediated infection via huCCR5 at concentrations equal to or above 10μM (**Figure 3.1A**, 10μM corresponds to 10^4nM on the abscissa x-axis). Maraviroc 10μM also inhibited almost all SIV Env-mediated infection via huCCR5 (**Figure 3.1B**). Maraviroc was exceedingly effective in blocking smCCR5-mediated SIV pseudotype infection; 1μM Maraviroc inhibited nearly all smCCR5-mediated infection *in vitro* (**Figure 3.1C**). By contrast, Maraviroc was a relatively poor inhibitor of rmCCR5-medaited infection and did not completely block SIV pseudotype infection via rmCCR5 at concentrations below 100μM (**Figure 3.1D**). Between 16% and 27% (mean 21 ± 4.9% standard deviation SD) of rmCCR5-mediated SIV pseudotype infection remained in the presence of 10μM Maraviroc, which was significantly higher than the percentage of smCCR5-mediated SIV pseudotype infection (mean 3 ± 2.7% SD) in the presence of 10μM Maraviroc ($p<0.0005$). CCR5 antagonist Maraviroc is a poor inhibitor of rmCCR5-mediated SIV pseudotype infection *in vitro* relative to huCCR5- and smCCR5-mediated infection.

The ability of Maraviroc to inhibit SIV infection was primarily host-CCR5 dependent as there was little variation between viral Env at inhibitory concentrations of Maraviroc (**Table 3.1**). The SIV $\log_{10}EC_{50}$ concentrations were not significantly different between Env pseudotypes in huCD4 plus huCCR5 ($p=0.75$, NS) or rmCD4 plus rmCCR5 ($p=0.95$, NS) transfected cells based on the sigmoidal best fit. The SIV $\log_{10}EC_{50}$ were significantly different between Env pseudotypes in cells transfected with smCD4 plus smCCR5 ($p=0.012$), indicating that there may be some viral Env-specific Maraviroc blocking in transfected cells expressing sooty mangabey receptors. However, 10μM Maraviroc equally and fully blocked all SIV Env pseudotypes in smCD4- plus smCCR5-transfected cells regardless of variation between EC_{50} values.

Our data suggested that Maraviroc concentrations at or above 100μM block rmCCR5-mediated SIV infection *in vitro*. However, 100μM Maraviroc demonstrated off-target and likely toxic effects on coreceptor-independent VSV-G pseudotype positive control infections in 293T cells (data not

shown). Concentrations of Maraviroc above 100μM that appear to block SIV infection may actually induce target 293T cell death. Because we ultimately planned to use Maraviroc to block SIV infection in PBMCs, we measured the toxicity of 100μM and 1mM Maraviroc in readily available human PBMCs. Human PBMCs were stimulated with PHA and IL-2 and treated three days with 100μM or 1mM Maraviroc, or the corresponding amounts of 0.26% or 2.6% vehicle, DMSO. PBMCs were stained with Aqua Live/Dead stain, a reactive dye that permeates membranes of necrotic cells and reacts with free amines in the cytoplasm, resulting in intense fluorescent staining of dead cells. Stained cells were analyzed by flow cytometry.

At the lower 100μM concentration, neither Maraviroc nor the comparable amount of vehicle had a large effect on the percentage of Aqua-negative live cells relative to untreated controls and roughly 90% of cells remained intact (**Figure 3.2**). Higher concentrations of vehicle induced a modest 15% drop in the percentage of live cells relative to untreated controls. A high concentration of the Maraviroc compound itself caused a precipitous drop in the percentage of live cells. 1mM Maraviroc also caused visible changes in the number of intact cells (data not shown). These findings caution that concentrations of Maraviroc above 100μM that appear block SIV *in vitro* likely induce widespread cell death. In full abundance of caution because we had seen off-target effects of 100μM Maraviroc on VSV-G Env-mediated infection in 293T cells, we opted to use the standard 10μM concentration of Maraviroc for future experiments.

We next determined if other small-molecule CCR5 antagonists block smCCR5- and rmCCR5-mediated SIV infection. Specifically, we searched for a small molecule inhibitor that, unlike Maraviroc, could fully block SIV infection via rmCCR5. Target 293T cells were transfected with rmCD4 and rmCCR5, or smCD4 and smCCR5. We pre-treated cells with CCR5 antagonists at concentrations historically used to block HIV-1 infection *in vitro*: AD101 10μM, Aplaviroc 1μM, CMPD167 6.7μM, and Vicriviroc 10μM. Cells were infected with SIV Env pseudotypes of pNL-

Luc-E$^-$R$^+$ and luciferase output was expressed as the percentage of luciferase expression in antagonist-treated cells relative to vehicle-treated controls.

All CCR5 antagonists we examined failed to completely block rmCCR5-mediated SIV pseudotype infection (**Figure 3.3A**), while the majority of CCR5 antagonists efficiently blocked smCCR5-mediated SIV pseudotype infection (**Figure 3.3B**) *in vitro*. This finding was particularly surprising given that AD101 was previously shown to block SIVmac 251 replication in rhesus macaque PBMCs (1). Inhibition of rmCCR5-mediated SIVmac 239 and SIVmac 251 infection ranged from only 52% (Vicriviroc) to 85% (AD101). CMPD167 was a notably poor inhibitor of SIV infection through smCCR5. TAK779 also failed to completely block rmCCR5-mediated SIV infection *in vitro* (data not shown). None of the small-molecule CCR5 antagonists exhibited clearly superior rmCCR5 antagonism relative to Maraviroc. Rhesus macaque rmCCR5-mediated SIV pseudotype infection was not fully inhibited by small-molecule CCR5 antagonists.

Maraviroc lacks direct off-target effects on CXCR6- or GPR15-mediated infection

To verify the coreceptor specificity of Maraviroc, we determined if Maraviroc showed direct off-target effects on infection mediated by alternative coreceptors CXCR6 and GPR15 *in vitro*. Target 293T cells expressing sooty mangabey CD4 and coreceptors, or rhesus macaque CD4 and coreceptors, were pre-treated with vehicle or 10μM Maraviroc. Cells were infected with SIV pNL-Luc-E$^-$R$^+$ Env pseudotypes and infection was measured by luciferase output three days post-infection.

As we had observed previously, Maraviroc blocked SIV Env-mediated infection through smCCR5 and the majority of, but not all, infection through rmCCR5 (**Figure 3.4**). All SIVsmm and SIVmac Env pseudotypes infected target cells via smCCR5, smCXCR6, and smGPR15 (**Figure 3.4A**). These SIV pseudotypes also infected cells expressing rmCCR5 and rmGPR15 whereas rmCXCR6 was a weak alternative coreceptor of SIV (discussed further in Chapter 4) (**Figure**

3.4B). Importantly, with all but one of the SIV Env pseudotypes, Maraviroc did not cause a significant decrease in either CXCR6- or GPR15-mediated pseudotype infection relative to vehicle-treated controls.

To determine if Maraviroc had broad off-target effects on cells or post-entry infection steps, we examined the effect of Maraviroc on infection by a positive control VSV-G Env, which mediates infection of 293T cells independent of both CD4 and coreceptor. Maraviroc induced a slight decrease in VSV-G Env-mediated infection in 293T cells relative to vehicle-treated controls, but the difference did not reach significance (p=0.069, NS) (**Figure 3.4C**). The trend toward significance may indicate the toxic effects of Maraviroc we had observed earlier with high concentrations in human PBMCs. Therefore, again, we opted to use the published concentration of Maraviroc, 10μM, in future experiments. We concluded that Maraviroc was a specific inhibitor of CCR5-mediated infection and had no marked off target effects on infection mediated by alternative coreceptors CXCR6 or GPR15 *in vitro*.

Chemokine CXCL16 attenuates SIV pseudotype infection at low levels of smCXCR6 expression *in vitro*

We designed a method to block infection mediated by smCXCR6, the most robust alternative coreceptor of sooty mangabey SIVsmm *in vitro* (5). Because there are no available small-molecule CXCR6 antagonists, we determined if recombinant human chemokine CXCL16 inhibits smCXCR6-mediated SIV Env pseudotype infection *in vitro*. Prior experiments found that CXCL16 blocked very little smCXCR6-mediated SIV pseudotype infection in 293T cells (unpublished, data not shown). The alternative coreceptor smCXCR6 was likely expressed at very high levels in these transfected cells. Therefore, we titrated the expression of smCXCR6 in 293T cells by transfecting these cells with decreasing amounts of smCXCR6 plasmid to see if there was a limit where SIV Env pseudotypes exhibited robust infection and where chemokine CXCL16 significantly blocked that infection. Target 293T cells were transfected with 1μg of

smCD4 plasmid and smCXCR6 plasmid ranging from 1µg down to 0.01µg (10ng). Cells were pre-treated one hour with 500ng/mL recombinant human CXCL16 and infected with pNL-Luc-E^- R^+ Env pseudotypes of SIVsmm FFv and SIVsab 92018ivTF, a virus cloned from natural host Sabaeus African Green Monkeys that also uses alternative coreceptor CXCR6 (11).

The inhibitory effects of CXCL16 were moderate and did not vary between 1µg and 0.1µg of transfected smCXCR6 plasmid DNA (**Figure 3.5A**). At high levels of transfected smCXCR6 plasmid DNA and consequent high levels of smCXCR6 expression, CXCL16 blocked only 55% to 65% of smCXCR6-mediated SIV pseudotype infection. Importantly, when we transfected 293T cells with only 0.01µg of smCXCR6 plasmid DNA, SIV pseudotype infection remained robust and CXCL16 blocked 85% to 90% of smCXCR6-mediated SIV infection. Thus, CXCL16 attenuates smCXCR6-mediated SIV Env pseudotype infection best when smCXCR6 is expressed at low levels in 293T cells. Chemokine CXCL16 was not a complete inhibitor of smCXCR6-mediated pseudotype infection *in vitro*. A small amount of SIVsmm FFv and SIVsab 92018ivTF Env-mediated infection remained even in CXCL16-treated cells transfected with 0.01µg of smCXCR6 (**Figure 3.5B**); luciferase expression in CXCL16-treated cells transfected with 0.01µg of smCXCR6 was significantly above that in cells lacking a coreceptor ($p<0.002$) and above that in cells infected with a pseudotype negative control virus lacking Env ($p<0.003$).

Given recombinant human CXCL16 is an effective inhibitor of smCXCR6-mediated SIV infection only at low levels (0.01µg plasmid DNA) of smCXCR6 transfection *in vitro*, we compared CXCR6 expression in transfected 293T cells to more physiologically relevant expression in cultured $CD4^+$ T cells. We discovered that none of the commercially available flow cytometry-compatible antibodies to human CXCR6 (huCXCR6) bind nonhuman primate smCXCR6 (data not shown). Therefore, we performed this experiment using huCXCR6 plasmid DNA and human $CD4^+$ T cells. 293T cells were transfected with huCXCR6 plasmid DNA from 1µg down to 0.001µg (1ng). In

parallel, $CD4^+$ T cells were purified from human PBMCs and stimulated with PHA and IL-2. Cells were stained with anti-human CXCR6 antibody and expression was analyzed by flow cytometry.

Fewer than 2% of stimulated human CD4+ T cells expressed huCXCR6 and huCXCR6 was not detected in resting $CD4^+$ T cells (**Figure 3.6**). Notably, 293T cells transfected with 0.01µg of huCXCR6 had a frequency of CXCR6 positive cells (1.2%) below the frequency of expression on peripheral $CD4^+$ T cells. We did not directly compare median fluorescence intensity between groups because of the large cell size differential between 293T cells and $CD4^+$ T cells. Based roughly upon the frequency of target cells expressing CXCR6, it is conceivable that CXCL16 may inhibit a significant amount of CXCR6-mediated SIV infection in primary PBMCs.

Chemokine CXCL16 lacks direct off-target effects on CCR5- or GPR15-mediated infection

Before using CXCL16 to block CXCR6-mediated SIV infection in primary cells, we ensured that CXCL16 had not direct off-target effects on infection mediated by coreceptors other than CXCR6. To determine if CXCL16 has any direct off-target effects on SIV infection mediated by other CCR5 and GPR15 *in vitro*, target 293T cells were transfected with smCD4 and 0.01µg of smCCR5, smCXCR6, or smGPR15 plasmid DNA. Cells were pre-treated with recombinant human CXCL16 and infected with pNL-Luc-E^-R^+ SIV Env pseudotypes. Luciferase expression was measured three days post-infection.

Once again, we observed that recombinant human CXCL16 blocks over 80% of CXCR6-mediated SIV Env pseudotype infection relative to untreated controls at low levels of smCXCR6 expression. Crucially, CXCL16 had no significant effect on smCCR5-mediated or smGPR15-mediated SIV infection *in vitro* (**Figure 3.7A**). Chemokine CXCL16 also had no significant effect on VSV-G Env pseudotype infection (p=0.93, NS) (**Figure 3.7B**), indicating that the chemokine does not affect overall 293T cell viability.

Chemokine CXCL16 is an effector chemokine found at sites of inflammation and could potentially drive activation of T cells or enhance PBMC permissively to infection. Therefore, we further investigated the effects of chemokine CXCL16 on CCR5-tropic and CXCR4-tropic HIV-1 infection and replication in human PBMCs with the hypothesis that CXCL16 does not impact infection and replication of a CXCR6-independent virus *ex vivo*. Human PBMCs were stimulated with PHA and IL-2 and pre-treated with or without CXCL16, followed by infection with CCR5-tropic HIV-1 BaL or CXCR4-tropic HIV-1 NL4-3, HIV-1 3B, and HIV-1 TYBE. Human PBMCs were collected three days post-infection for quantitative real-time PCR (qPCR) measuring HIV-1 LTR and Gag viral DNA. We also collected and measured supernatant P24 Gag production over time. Data represent peak viral production at Day 8 post-infection.

Chemokine CXCL16 did not attenuate CCR5-tropic or CXCR4-tropic HIV-1 infection, indicating that CXCL16 has no negative off-target effects on CCR5- or CXCR4-mediated infection (**Figure 3.8**). Viral supernatant P24 Gag production at the peak of HIV-1 infection was slightly but consistently enhanced by CXCL16 (range 4%, HIV-1 NL4-3, to 37%, HIV-1 TYBE relative to untreated controls) (**Figure 3.8A**). This finding was consistent in a repeated experiment (data not shown). Chemokine CXCL16 enhanced early HIV-1 reverse transcription as the relative level of LTR strong-stop DNA was 14% (HIV-1 3B) to 44% (HIV-1 BaL) higher in cells treated with CXCL16 than in cells without CXCL16 (**Figure 3.8B**). However, CXCL16 did not consistently enhance HIV-1 Gag DNA expression relative to untreated controls (**Figure 3.8C**). Additional experiments are needed to determine the full effect of chemokine CXCL16 in primary PBMCs. These limited data provide preliminary evidence that CXCL16 may enhance infection in PBMCs. Therefore, any blocking effects of CXCL16 in primary PBMCs may slightly underestimate the true contribution of CXCR6 to SIV infection.

Maraviroc and CXCL16 lack off-target effects on coreceptor cell surface expression

Through Maraviroc and CXCL16 had no direct off-target effects on coreceptor function in transfected 293T cells, we ensured that neither the small-molecule antagonist nor the chemokine had marked off-target effects on coreceptor expression in primary cells. Because there is no antibody to nonhuman primate CXCR6, we again performed this experiment in human primary cells. Peripheral human $CD4^+$ T cells were stimulated with PHA and IL-2 and treated one day with vehicle, 10μM Maraviroc, or 500ng/mL CXCL16. One day later, cells were stained with antibodies to CCR5 and human CXCR6 and imaged by flow cytometry. Although CXCR4 is not a coreceptor of SIV, we also assayed the expression of CXCR4 as another marker of potential off-target effects of CXCL16 on coreceptor expression.

Overall, neither Maraviroc nor CXCL16 induced an obvious change in the expression of non-ligand coreceptors in primary human $CD4^+$ T cells (**Figure 3.9**, **Table 3.2**). As we expected from numerous prior studies, human $CD4^+$ T cells expressed high levels of CXCR4. Neither Maraviroc nor CXCL16 had a large effect on CXCR4 expression in peripheral $CD4^+$ T cells. The stimulated human $CD4^+$ T cells expressed very low levels of CCR5, perhaps reflecting poor activation, and it was difficult to determine if Maraviroc or CXCL16 impacted CCR5 expression. The median fluorescence intensity of CCR5 increased slightly following Maraviroc treatment, lending very limited support to prior evidence that Maraviroc stabilizes cell-surface expression of CCR5 and increases the percentage of $CD3^+CCR5^+$ PBMCs *in vivo* (20).

Many chemokines induce internalization and lower the surface expression of their natural receptors. While Maraviroc had no clear effect on CXCR6 expression, we observed that chemokine CXCL16 induced a drop in both the frequency of $CD4^+$ T cells expressing surface CXCR6 and the CXCR6 median fluorescence intensity (**Figure 3.9**, **Table 3.2**). Further experiments with additional PBMC donors and additional time points are needed to definitively measure the effect of Maraviroc and CXCL16 on chemokine receptor cell surface expression,

however these data suggest there is no severe off-target effect of these compounds on coreceptor expression.

FIGURES

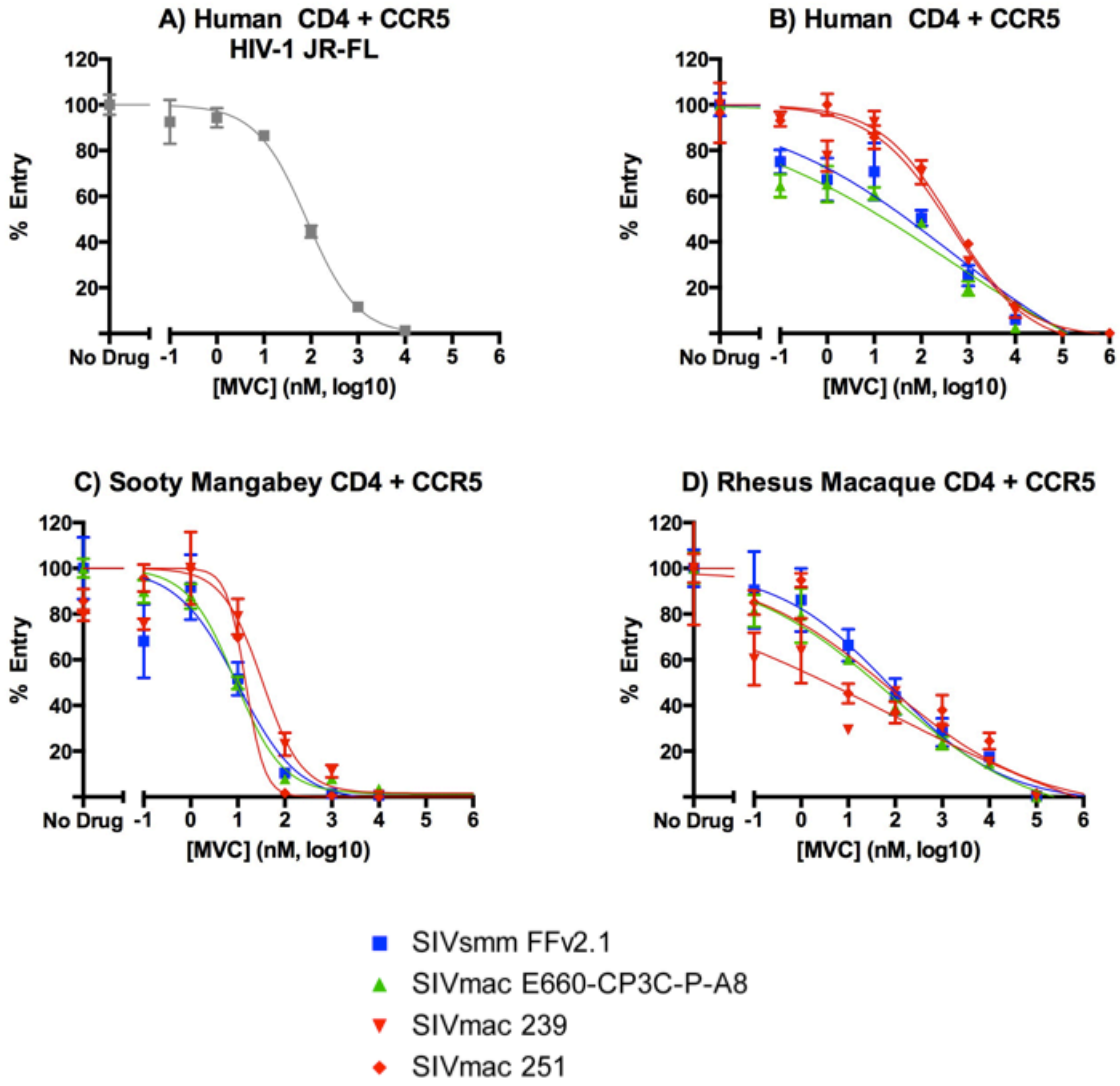

Figure 3.1. CCR5 antagonist Maraviroc does not entirely block rmCCR5-mediated SIV Env pseudotype infection in transfected target cells *in vitro*. Target 293T cells were transfected with **(A** and **B)** human CD4 and CCR5, **(C)** sooty mangabey CD4 and CCR5, or **(D)** rhesus macaque CD4 and CCR5. Cells were pre-treated 1 hour with vehicle (No Drug) or Maraviroc at final concentrations in $\log_{10}$ increments from 0.1nM to 1mM. Cells were infected with luciferase-expressing pNL-Luc-$E^{-}R^{+}$ Env pseudotypes of HIV-1 JR-FL (gray), SIVsmm FFv2.1 (blue), SIVsmm E660 CP3C (green), SIVmac 239 (red triangle), and SIVmac 251 (red diamond). Infection was measured by luciferase output 3 days post-infection and normalized to 100% entry in the presence of vehicle alone. Data were fitted to a sigmoidal dose response curve. (N=3, mean ± SD.)

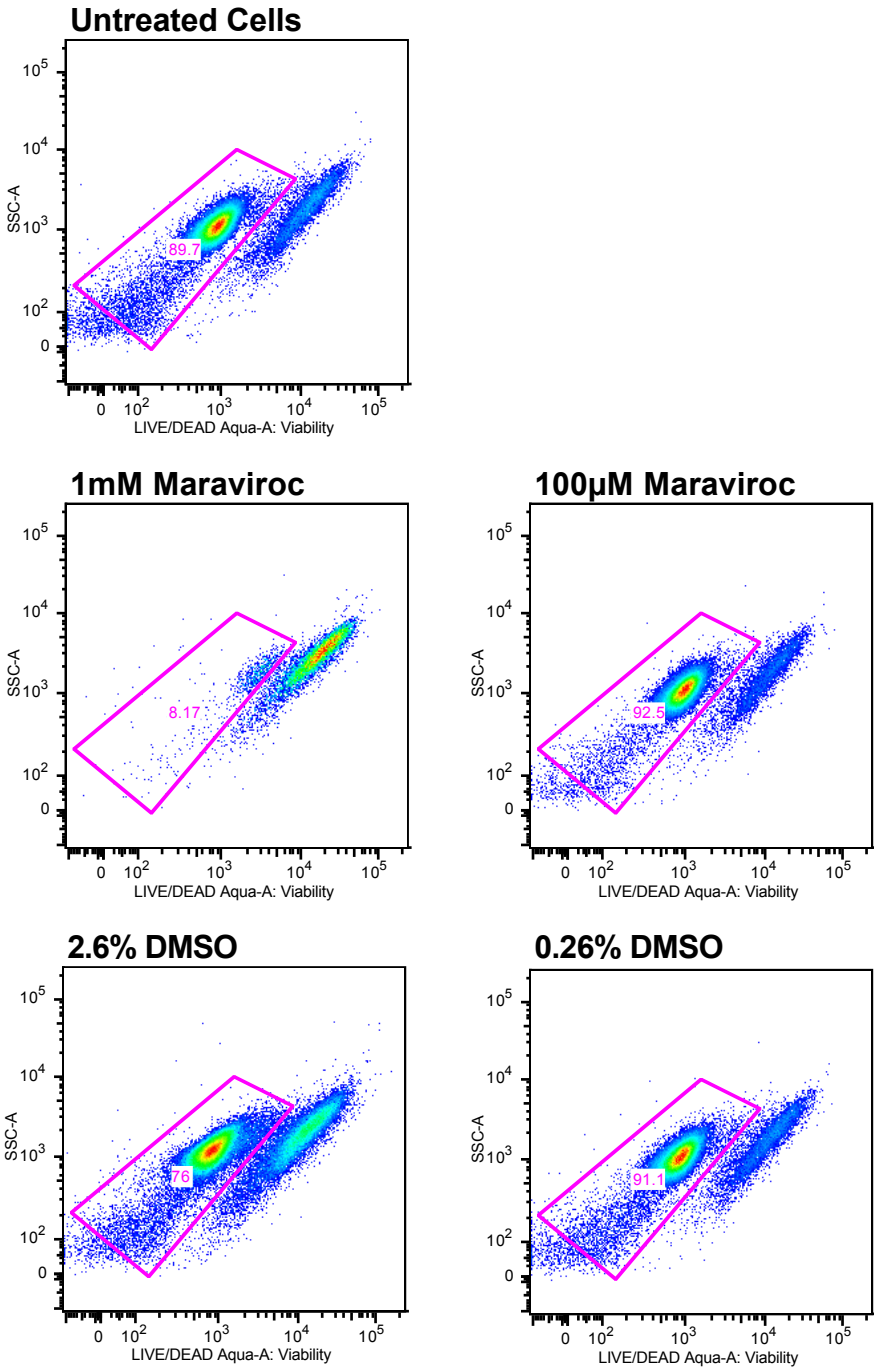

Figure 3.2. Maraviroc is toxic to human PBMCs at concentrations above 100µM. Primary human PBMCs were simulated 3 days with PHA and IL-2. Cells were then treated 3 days with IL-2 media alone (untreated), with 100µM Maraviroc, or with 1mM Maraviroc. Cells were also treated with the corresponding amounts of vehicle DMSO alone. PBMCs were stained with Aqua Live/Dead dye and analyzed by flow cytometry. Gates roughly represent Aqua-negative live cell populations as a fraction of the total cell population.

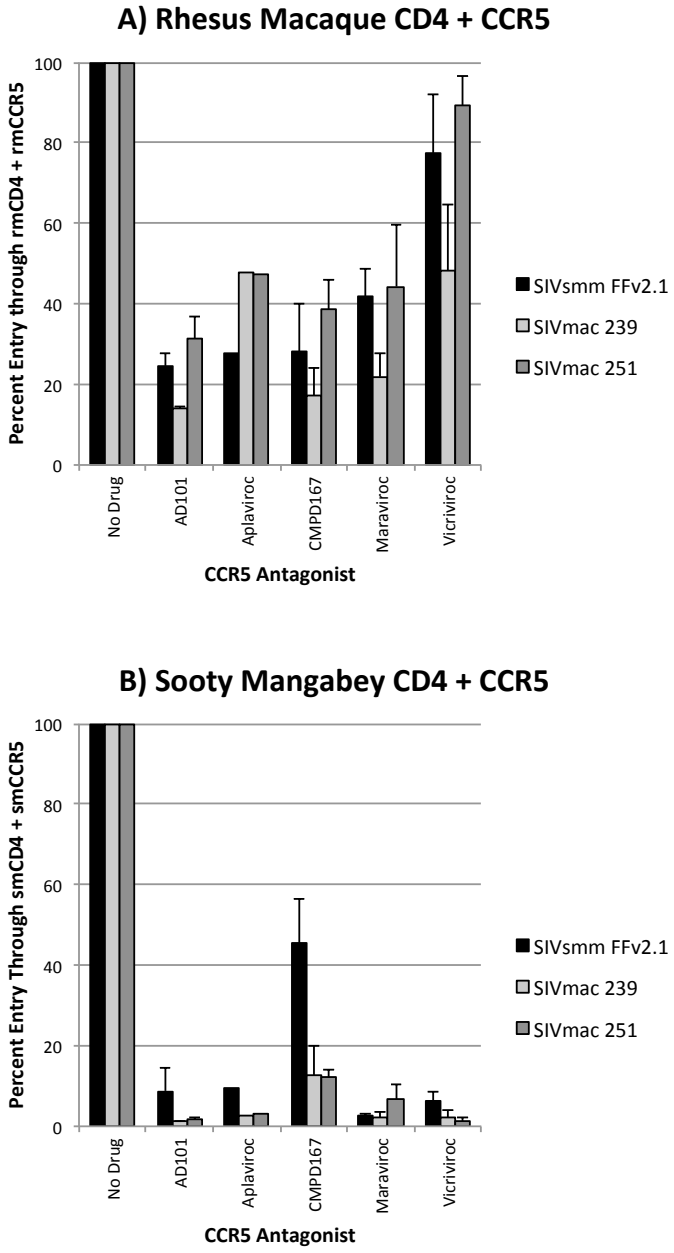

Figure 3.3. CCR5 antagonists do not completely block rmCCR5-mediated SIV Env pseudotype infection *in vitro*. Target 293T cells were transfected with **(A)** rhesus macaque rmCD4 and rmCCR5, or **(B)** sooty mangabey smCD4 and smCCR5. Cells were pre-treated with CCR5 antagonists AD101 (10μM), Aplaviroc (1μM), CMPD167 (6.7μM), Maraviroc (10μM), or Vicriviroc (10μM) and infected with pNL-Luc-E$^-$R$^+$ Env pseudotypes of SIVsmm FFv2.1 (black), SIVmac 239 (light gray), or SIVmac 251(dark gray). Infection was measured by luciferase output 3 days post-infection. Data for each viral Env is expressed as the percentage of infection in antagonist-treated cells relative to vehicle-treated cells (No Drug).

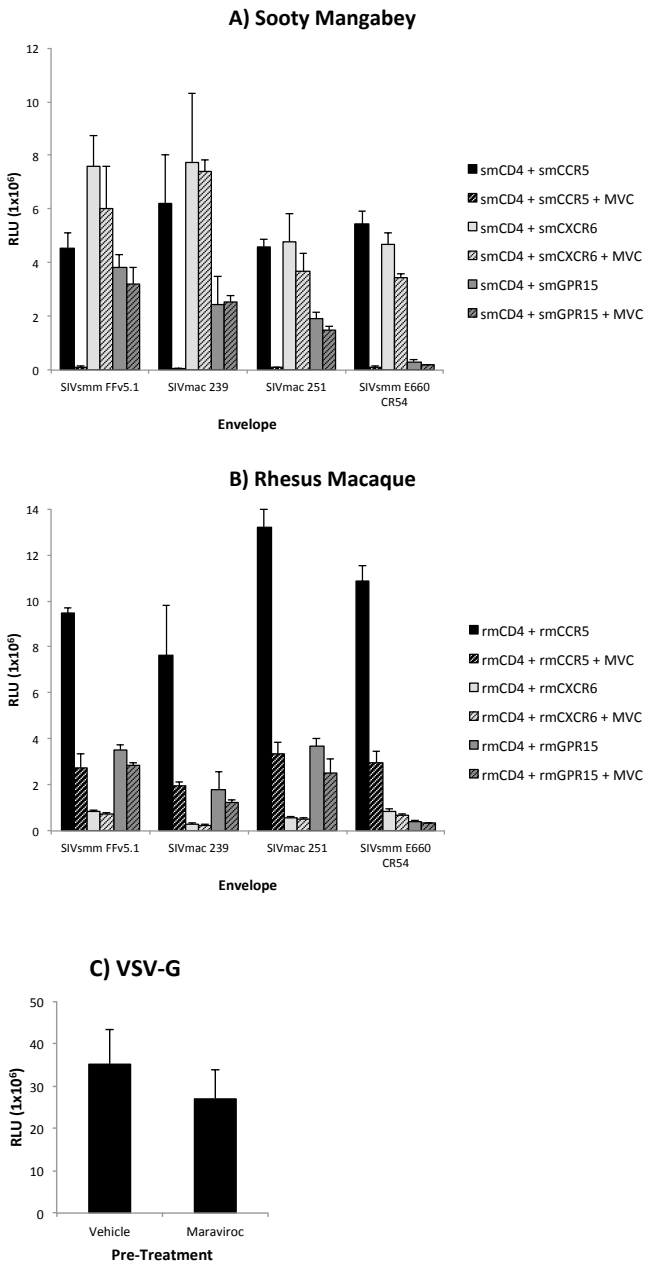

Figure 3.4. Maraviroc has no off-target effects on CXCR6- or GPR15-mediated SIV Env pseudotype infection *in vitro*. Target 293T cells were transfected with **(A)** sooty mangabey smCD4 and coreceptors, or **(B)** rhesus macaque rmCD4 and coreceptors. Cells were pre-treated one hour with 10μM Maraviroc (MVC) or vehicle and infected with luciferase-expressing pNL-Luc-E^-R^+ Env pseudotypes of SIVsmm FFv, SIVmac 239, SIVmac 251, and SIVsmm E660. Infection was measured by luciferase output three days post-infection in relative light units (RLU). (N=3, mean ± SD.) **(C)** 293T cells were pre-treated one hour with vehicle or 10μM Maraviroc and infected with positive control CD4- and coreceptor-independent VSV-G Env pseudotypes. Infection was measured by luciferase output. (N=18, mean ± SD, p=0.069.)

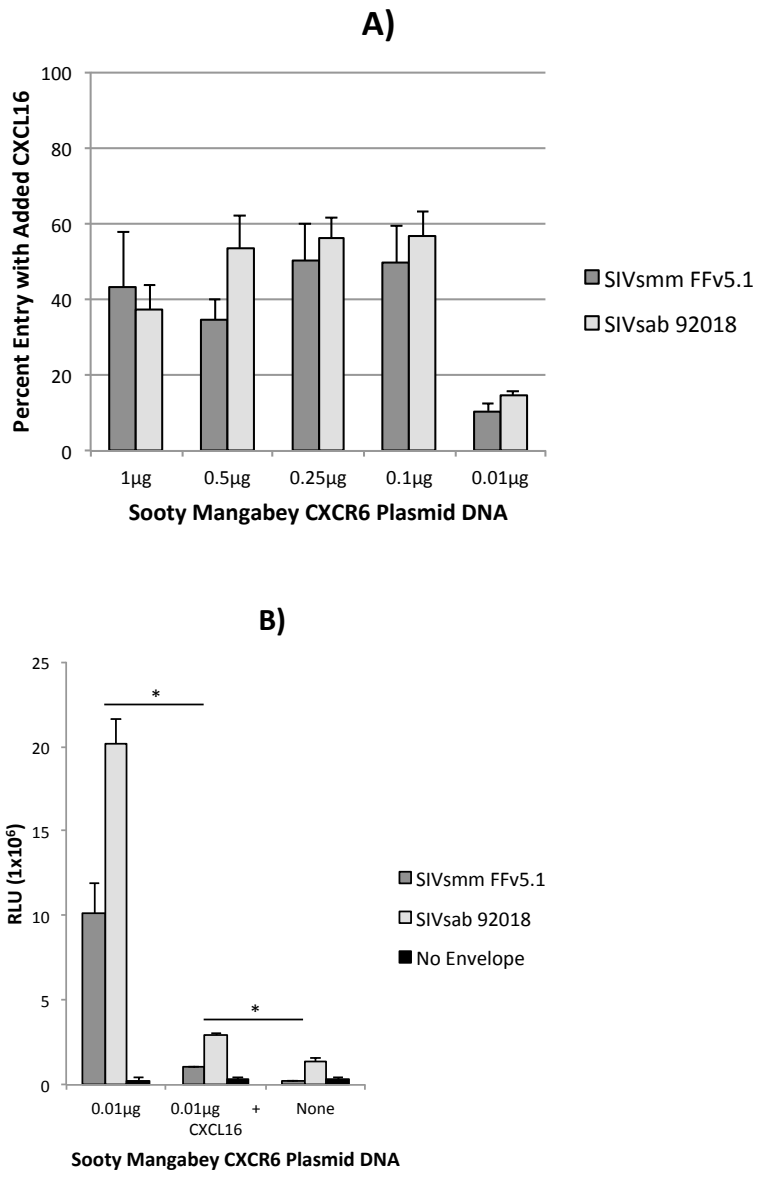

Figure 3.5. Chemokine CXCL16 attenuates SIV pseudotype infection at low levels of smCXCR6 expression *in vitro*. (A) Target 293T cells were transfected with 1ug of sooty mangabey smCD4 plasmid DNA and decreasing amounts of smCXCR6 plasmid DNA ranging from 1µg to 0.01µg. Cells were pre-treated with 500ng/mL recombinant human chemokine CXCL16 and infected with luciferase-expressing pNL-Luc-$E^{-}R^{+}$ Env pseudotypes of SIVsmm FFv (dark gray) and SIVsab (another natural host SIV known to use smCXCR6, light gray). Data is expressed as the percent of luciferase expression in CXCL16-treated cells relative to untreated cells. **(B)** Another representation of the same data set: Target 293T cells were transfected, pre-treated, and infected as in (A). Data is expressed as absolute luciferase production (RLU). Control cells transfected with smCD4 alone without coreceptor (None), and negative control infection with a pseudotype virus lacking Env (No Envelope, black) are included for comparison. (N=3, mean ± SD, * $p<0.05$ SIVsmm and SIVsab.)

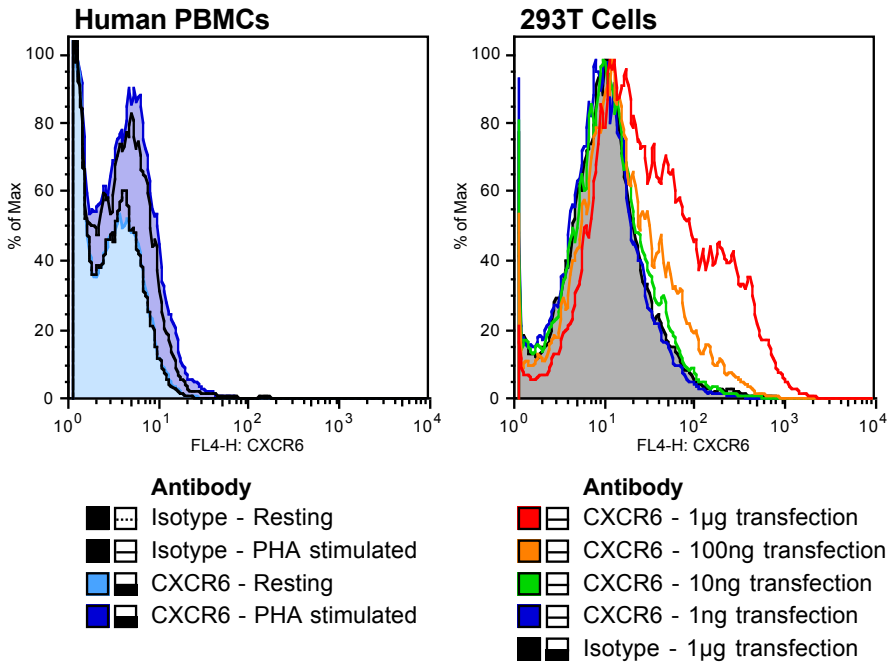

Figure 3.6. Expression of human CXCR6 on peripheral CD4+ T cells and transfected 293T cells. Human CD4+ T cells were purified from PBMCs and simulated with PHA/IL-2. 293T cells were transfected with decreasing amounts of human CXCR6 ranging from 1µg to 0.001µg of plasmid DNA. Cells were stained with an isotype control (Isotype), or stained with antibody to human CXCR6 and analyzed by flow cytometry.

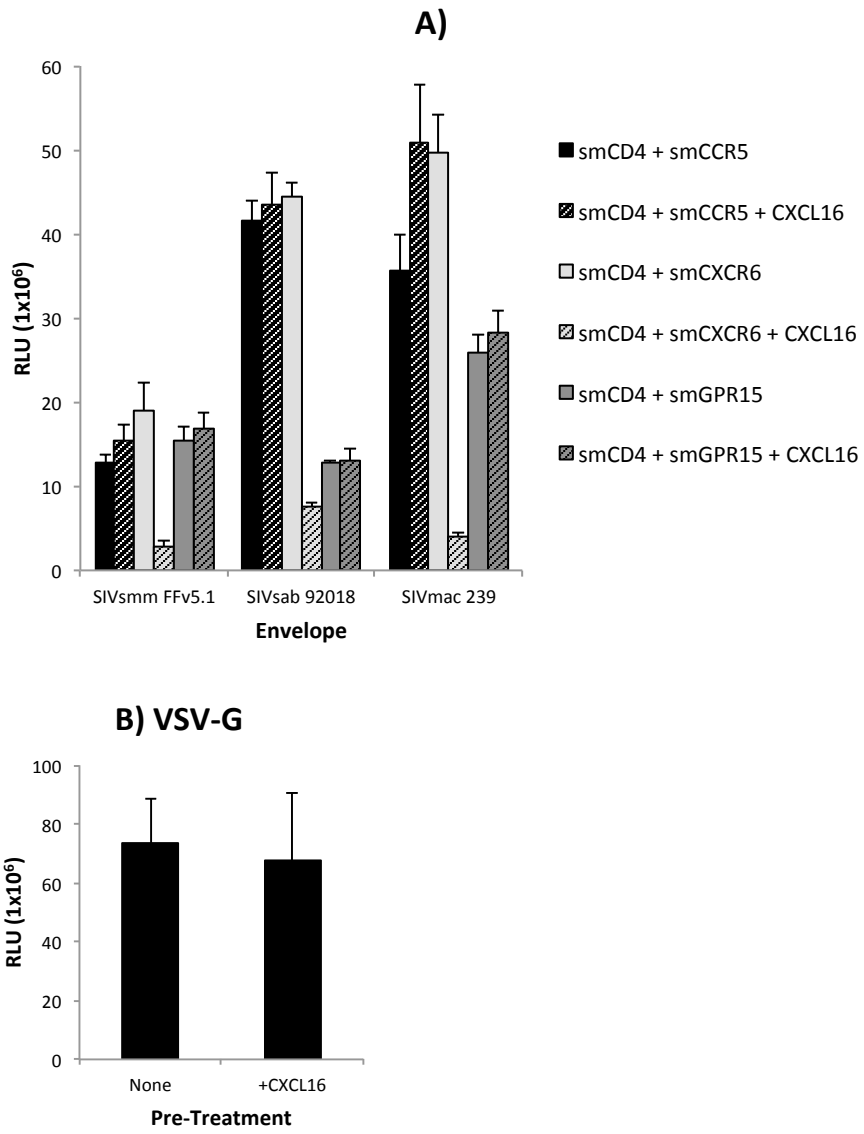

Figure 3.7. CXCL16 blocks CXCR6- but does not block CCR5- or GPR15-mediated SIV infection. (A) Target 293T cells were transfected with 1µg of smCD4 and 0.01µg of coreceptors smCCR5, smCXCR6 or smGPR15. Cells were pre-treated 1 hour with vehicle or 500ng/mL CXCL16 and infected with luciferase-expressing pNL-Luc-E$^-$R$^+$ Env pseudotypes of SIVsmm FFv, SIVmac and SIVsab. (N=3, mean ± SD.) **(B)** 293T cells were treated one hour with vehicle (None) or CXCL16 and infected with pNL-Luc-E$^-$R$^+$ Env pseudotypes of VSV-G. Infection was measured by luciferase output. (N=18, mean ± SD, p=0.94.)

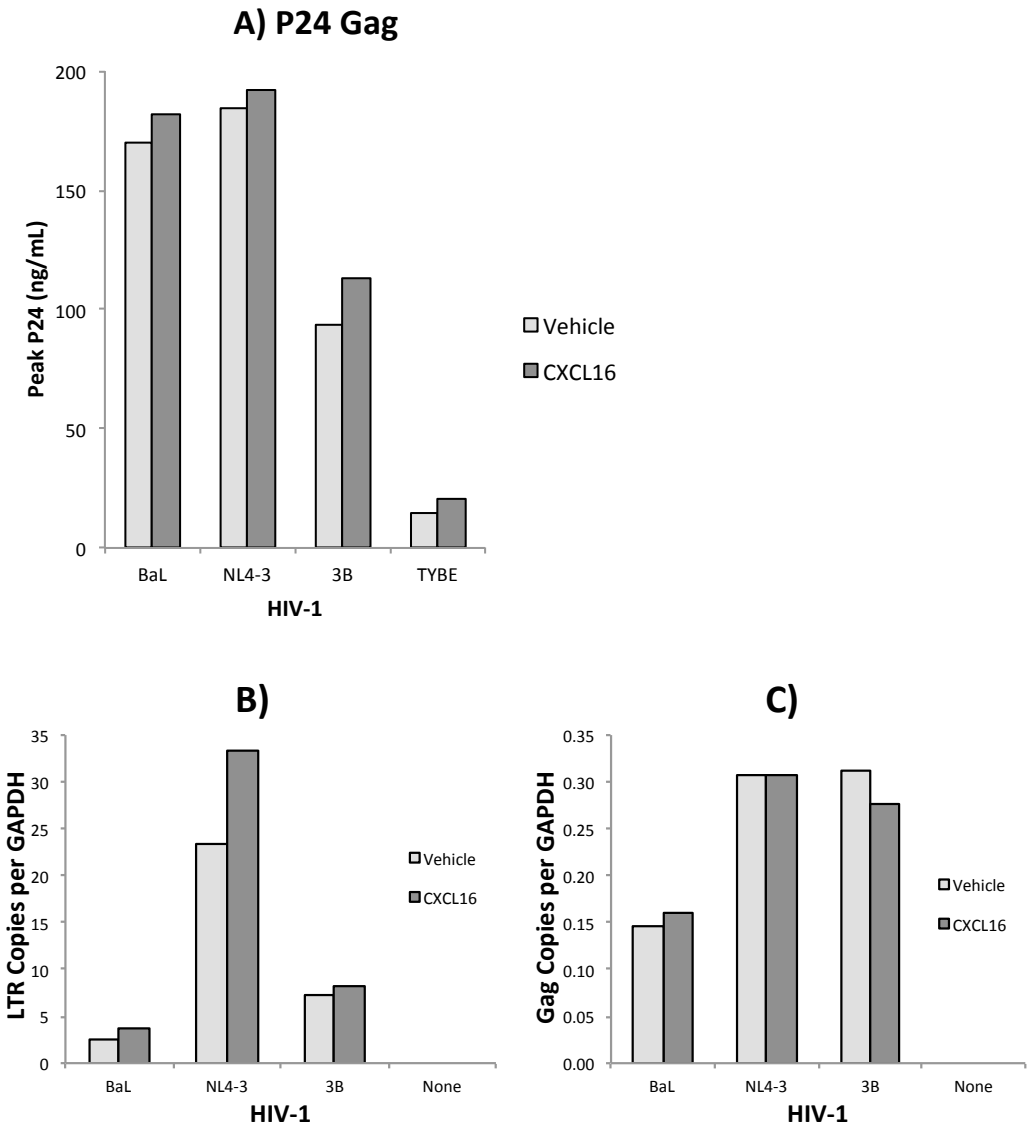

Figure 3.8. CXCL16 does not attenuate CCR5- or CXCR4-tropic HIV-1 infection and replication. Human PBMCs were stimulated with PHA and IL-2. Stimulated cells were pre-treated with or without 500ng/mL CXCL16. Cells were infected with replication-competent HIV-1 viruses: CCR5-tropic HIV-1 BaL and CXCR4-tropic HIV-1 NL4-3, HIV-1 3B, and HIV-1 TYBE. **(A)** Supernatant was collected at the peak of infection (Day 8) and viral P24 Gag was measured by ELISA. **(B,C)** Three days post-infection, PBMCs were lysed and assayed by qPCR for HIV-1 LTR strong-stop and HIV-1 Gag viral DNA copies relative to housekeeping gene GAPDH. Uninfected PBMCs (None) served as a negative control.

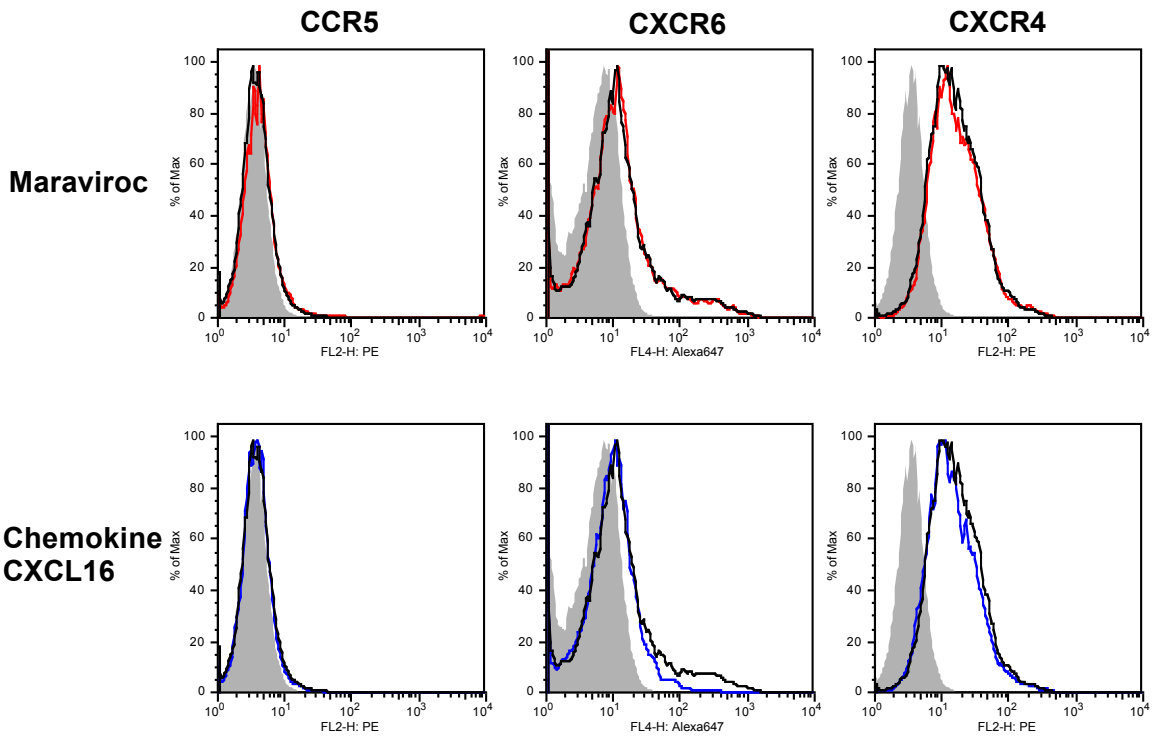

Figure 3.9. Maraviroc and CXCL16 do not affect expression of non-ligand alternative coreceptors. Human peripheral blood CD4+ T cells were purified and stimulated with PHA and IL-2. Following stimulation, cells were treated for one day with vehicle (black line), 10μM Maraviroc (red line), or 500ng/mL CXCL16 (blue line) and stained with antibodies to CCR5, CXCR6, and CXCR4. Cells were also stained with isotype controls (gray). Analysis was performed by flow cytometry.

Table 3.1. Maraviroc EC50 values in transfected 293T cells.

SIV Env[a]	EC50 (log_{10}, nM)		
	Human	*Rhesus Macaque*	*Sooty Mangabey*
SIVsmm FFv2.1	2.3	2.0	1.0
SIVmac 239	2.6	2.7	1.5
SIVmac 251	2.7	1.9	1.2
SIVsmm E660	2.1	1.7	0.9

[a] Luciferase-expressing pNL-Luc-E^-R^+ Env pseudotypes of SIVsmm FFv2.1, SIVmac 239, SIVmac 251, and SIVsmm E660 CP3C were used to infect Maraviroc-treated transfected cells expressing human CD4 and CCR5, rhesus macaque CD4 and CCR5, or sooty mangabey CD4 and CCR5. Nanomolar log_{10} EC50 were calculated from a best-fit sigmoidal dose-response curve.

Table 3.2. Maraviroc and CXCL16 do not affect expression of non-ligand alternative coreceptors.

Treatment[a]	Antibody[b]	Expression[c]	
		%	*MFI*
No Drug	CXCR4	53.6	26.4
Maraviroc		53.9	27.1
CXCL16		48.4	25.5
(Isotype)		--	18.3
No Drug	CCR5	2.14	19.3
Maraviroc		3.33	23.3
CXCL16		1.56	18.8
(Isotype)		--	18.3
No Drug	CXCR6	21.7	57.3
Maraviroc		21.4	55.2
CXCL16		11.3	34.0
(Isotype)		--	27.1

[a] Peripheral CD4+ T cells were stimulated with PHA and IL-2. Following stimulation, cells were treated for one day with vehicle DMSO (No Drug), 10μM Maraviroc (MVC), or 500ng/mL CXCL16.

[b] Cells were stained with antibodies to receptor CXCR4, CCR5, and CXCR6, or with isotype control antibodies.

[c] Data is expressed as the percentage of live cells expressing each coreceptor (%) relative to isotype control, and as the median fluorescence intensity (MFI) of each subset.

DISCUSSION

We designed and defined methods that can be used to block or attenuate CCR5-mediated and alternative coreceptor CXCR6-mediated SIV infection *in vitro*. Specifically, we will use these methods to determine the role of CCR5, CXCR6, and other alternative coreceptors in SIV infection of non-natural host rhesus macaque PBMCs and natural host sooty mangabey PBMCs (Chapter 4).

For the first time, we demonstrate that CXCL16 can be used to attenuate CXCR6-mediated SIV infection *in vitro*. Recombinant human CXCL16 significantly lowered SIV Env-mediated infection via smCXCR6 in cells transfected with small amounts of smCXCR6 plasmid DNA. The exact mechanism of CXCL16 inhibition of CXCR6-tropic SIV infection is not clear. We observed that CXCL16 induced a modest drop in CXCR6 expression on human peripheral $CD4^+$ T cells, which suggests homologous desensitization via receptor endocytosis as one mechanism preventing SIV infection. Notably, human CXCL16 only blocked smCXCR6-mediated 293T cell infection at low levels of smCXCR6 plasmid DNA transfection. We observed that 293T cells transfected with 0.01µg of human CXCR6 plasmid DNA and $CD4^+$ T cells stimulated with PHA and IL-2 display similar frequencies of $CXCR6^+$ cells. The comparison between a cell line and primary PBMCs is rough, but this may indicate that CXCL16 can attenuate a significant amount of CXCR6-mediated SIV infection in primary cells.

Further studies could define more potent inhibitors of smCXCR6-medaited SIV infection. It is possible that species-matched recombinant sooty mangabey CXCL16 would be a more potent inhibitor of smCXCR6-mediated SIV infection. We did not investigate the ability of human CXCL16 to block SIV infection via human CXCR6 and compare results to our data from smCXCR6. A prior study described a potent chemotactic CXCR6 antagonist formed by adding an N-terminal extension to CXCL16 (26). This CXCL16 construct may also be a more potent inhibitor of CXCR6-mediatead SIV infection than wild-type human CXCL16.

Maraviroc inhibition is host CCR5-dependent. Maraviroc was a potent antagonist of smCCR5 and inhibited smCCR5-mediated SIV infection in transfected 293T cells. By contrast, at concentrations tolerable to cultured cells, Maraviroc only inhibited 80% of SIV infection through rmCCR5 in transfected 293T cells. Therefore, we anticipate that Maraviroc may block more smCCR5-mediated SIV infection in sooty mangabey PBMCs than rmCCR5-mediatead SIV infection in rhesus macaque PBMCs.

Notably, viruses from sooty mangabeys and rhesus macaques were equally sensitive to Maraviroc; Maraviroc blocked smCCR5-mediated infection by sooty mangabey SIVsmm and rhesus macaque SIVmac 239, SIVmac 251, and SIVsmm Env pseudotypes. None of the SIV Env we studied appeared to be Maraviroc-resistant. All SIV Env pseudotypes we tested were fully sensitive to Maraviroc in cells expressing human CD4 and CCR5 or sooty mangabey CD4 and CCR5. Maraviroc-resistant HIV-1 have been observed before (19, 22, 25, 32, 34-36, 38, 42), however, this drug resistance typically evolves only after extensive passage in antagonist-treated culture or after long-term antagonist treatment *in vivo*.

Inhibiting CCR5-mediated entry in a transfected 293T cell assay *in vitro* is likely more challenging than blocking infection under more physiological conditions with primary viral isolates containing less Env in primary PBMCs with less CCR5 *ex vivo* (31). SIV Env was likely expressed at high levels on the surface of Env-pseudotyped virions relative to primary SIV isolates. Receptor CD4 and coreceptor CCR5 were clearly expressed at very high levels in transfected 293T cells. Previous studies found that the amount of CCR5 expressed on the cell surface has a major influence on the IC_{50} and efficacy of Maraviroc inhibition *in vitro* (9, 14, 43). The amount of Env on the surface of the virion may also affect Maraviroc inhibition. Nonetheless, our finding that Maraviroc blocks only 80% of rmCCR5-mediated SIV pseudotype infection in transfected 293T

cells closely recapitulates prior studies where Maraviroc blocked only 80% of provirus SIVmac 239 replication in rhesus macaque PBMCs (21).

Prior studies found that while amino acid sequence discrepancies between huCCR5 and rmCCR5 do not affect the binding of natural chemokine ligands of CCR5, these species-specific amino acid substitutions do affect the binding of small molecule antagonists. Previous data indicate that the relatively poor antagonism of Maraviroc against rmCCR5-mediated SIV infection is due to faster dissociation kinetics of Maraviroc and rmCCR5 than Maraviroc and huCCR5 (24). Presumably this pattern would also hold between rmCCR5 and smCCR5. We aligned the amino acid sequences of huCCR5, smCCR5, and rmCCR5 and found a single amino acid residue I198M unique to rmCCR5 that likely defines the poor interaction between Maraviroc and rmCCR5 relative to Maraviroc and smCCR5/huCCR5. Accordingly, a prior site-directed mutagenesis study found that an I198A substitution in the fifth transmembrane region of huCCR5 increased the dissociation constant K_D and decreased the binding efficiency of Maraviroc (8). I198V and I198L mutations in huCCR5 also impaired the ability of CCR5 antagonists AD101, SCH-C, and TAK779 to block SIV infection (1). Furthermore, introducing M198I in rmCCR5 restores the ability of CCR5 antagonist SCH-C to fully inhibit rmCCR5-mediated infection (1). Therefore, CCR5 amino acid residue 198 likely plays a critical role in the discrepant Maraviroc antagonism between smCCR5 and rmCCR5. Prior studies determined that amino acid residue I198 was less critical to Aplaviroc and Vicriviroc binding (16), however, we found that neither Aplaviroc nor Vicriviroc fully block SIV infection via rmCCR5. Perhaps attributes in addition to the CCR5 amino acid sequence mediate the relatively poor antagonism of Maraviroc against rmCCR5-medaited SIV infection.

Others have used CCR5 antagonists to decrease the plasma viral load in SIVmac-infected rhesus macaques *in vivo* (40) and to block infection by CCR5-tropic SHIV SF162 (20, 39, 41). In these studies, viral loads only slightly decreased and protection was only transient. These results could

be due to the poor activity of CCR5 antagonists against rmCCR5, but could also be explained by pharmacokinetics of these compounds in nonhuman primates, the level of CCR5 on cells at the challenge site, or perhaps alternative coreceptor use. Further studies are needed to define the ability of Maraviroc to block rmCCR5-mediated SIV infection in rhesus macaque primary cells (see Chapter 4).

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CHAPTER 4

RHESUS MACAQUE SIV INFECTION IS CCR5-DEPENDENT WHILE SOOTY MANGABEY SIV INFECTION IS PARTIALLY CCR5-INDEPENDENT AND CAN BE MEDIATED BY CXCR6

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ABSTRACT

Natural host sooty mangabeys infected with simian immunodeficiency virus (SIV) exhibit high plasma viral loads without widespread $CD4^+$ T cell loss. By contrast, non-natural host rhesus macaques experimentally infected with related SIV exhibit high plasma viral loads with subsequent $CD4^+$ T cell loss and progression to AIDS. Here, we identified host-dependent patterns of SIV coreceptor use that distinguish viral entry in sooty mangabeys from viral entry in rhesus macaques. We confirmed sooty mangabey CCR5, CXCR6, and GPR15 as coreceptors of SIV *in vitro*. Rhesus macaque CCR5 and GPR15 supported SIV infection *in vitro*, but rhesus macaque CXCR6 was a poor coreceptor of SIV relative to sooty mangabey CXCR6. Using CCR5 inhibitor Maraviroc, we determined that SIV was highly CCR5-dependent in rhesus macaque peripheral blood mononuclear cells (PBMCs), whereas SIV was partially CCR5-independent in sooty mangabey PBMCs. CXCL16, a chemokine ligand of CXCR6, partially attenuated SIV infection of sooty mangabey PBMCs, indicating that sooty mangabey CXCR6 was a functional alternative coreceptor of some SIV isolates in primary sooty mangabey cells. Importantly, SIV from sooty mangabeys and SIV from rhesus macaques exhibited similar patterns of alternative coreceptor use on equivalent coreceptors *in vitro*, and SIVmac 239 was highly CCR5-dependent in rhesus macaque PBMCs but largely CCR5-indepenent in sooty mangabey PBMCs. These data indicate that alternative coreceptor-mediated SIV infection was host-dependent. The expression patterns of CXCR6 and other alterative coreceptor(s) of SIV in sooty mangabey $CD4^+$ cell subsets may classify host target cells that can maintain high levels of SIV replication without leading to widespread $CD4^+$ T cell loss.

INTRODUCTION

Simian immunodeficiency virus (SIV) infections of primate natural hosts, including SIVsmm infection of sooty mangabeys, result in high viral loads without widespread $CD4^+$ T cell loss or progression to AIDS. By contrast, SIV infection of primate non-natural hosts, like SIVmac 239, SIVmac 251, and SIVsmm E660 infections of rhesus macaques, typically result in high viral loads, widespread loss of $CD4^+$ T cells, and rapid progression to AIDS. Rhesus macaques infected with SIVmac 239, SIVmac 251, and SIVsmm E660 are often used as animal models of human HIV-1 infection and pathogenesis.

Whereas SIVsmm viruses are endemic to wild sooty mangabeys in West Africa and remained so when animals were transferred to captive sites in the United States (2, 18, 20), pathogenic rhesus macaque SIV, including SIVmac 239, SIVmac 251, and SIVsmm E660, arose only after co-captive and experimental SIVsmm cross-species transmissions to Asian non-natural host rhesus macaques (Chapter 1, Introduction, Figure 1.8) (12). The three viruses SIVmac 239, SIVmac 251, and SIVsmm E660 are often used in rhesus macaque challenge studies as animal models ofHIV-1 infection and pathogenesis.

Note that, despite the confounding name, SIVsmm E660 is a virus used to infect rhesus macaques and induces pathogenesis in these non-natural hosts (12, 13). The laboratory isolate SIVsmm *E660* is distinct from SIVsmm primary isolates from natural infections that occur in sooty mangabeys.

The focus of the field has been on the question of how rhesus macaque SIV viruses can be boldly pathogenic in their rhesus macaque hosts, while very closely related parental sooty mangabey SIVsmm infections do not cause disease progression in their sooty mangabey hosts. There are many possible explanations as to why non-natural host rhesus macaques succumb to SIV infection whereas natural host sooty mangabeys do not progress to AIDS despite ongoing SIV

replication (1, 23, 34). In this study, we reveal differential coreceptor use by SIV in primary cells from natural host sooty mangabeys versus primary cells from non-natural host rhesus macaques as one possible mechanism explaining the dichotomy between disease outcomes in primate hosts.

Though all SIVsmm and SIVmac were previously presumed to be coreceptor CCR5-dependent, we discovered that alternative, non-CCR5 coreceptors support sooty mangabey SIVsmm infection *in vitro* and *in vivo* (6, 29) (Chapter 2). All sooty mangabey SIVsmm Envelope proteins (Env) we examined to date mediate infection of transfected target cells expressing sooty mangabey CD4 (smCD4) and sooty mangabey CCR5 (smCCR5). Crucially, alternative coreceptor sooty mangabey CXCR6 (smCXCR6) supports equally robust SIVsmm Env-mediated infection *in vitro* (6). Sooty mangabey smGPR15 and smGPR1 also support moderate to low SIVsmm Env-mediated infection.

Because cross-species transmissions of SIVsmm gave rise to rhesus macaque challenge viruses SIVmac 239, SIVmac 251, and SIVsmm E660, we hypothesized that these rhesus macaque SIV exhibit the general ability to use alternative coreceptors very similar to that of SIVsmm. SIVmac 239 and SIVmac 251 can use alternative coreceptors of human origin as these viruses replicate to varying degrees in $CCR5^{\Delta/\Delta}$ human PBMCs that lack functional CCR5 (3, 5, 14, 32, 39). Human CCR5, CXCR6, and GPR15 were found to support SIVmac 239 in cell lines *in vitro* (17). Thus, it appears that SIVmac 239 and other pathogenic rhesus macaque SIV in this viral family are generally CCR5-, CXCR6-, and GPR15-tropic *in vitro* much like sooty mangabey SIVsmm, not accounting for species-matched coreceptors.

Importantly, previous studies found that species-matched rhesus macaque CCR5 (rmCCR5) and rhesus macaque GPR15 (rmGPR15) are coreceptors of rhesus macaque SIV, but that rmCXCR6 is a poor coreceptor of SIVmac 239 and SIVmac 17E/F relative to human CXCR6 (26, 27). We

hypothesized that rmCXCR6 is also a poor coreceptor of other rhesus macaque SIV, including SIVmac 251 (which was co-derived with SIVmac 239 and SIVmac 17E/F) and independently-derived SIVsmm E660 (12) (Chapter 1, Introduction, Figure 1.8). In addition, we determined if rmCXCR6 is a poor coreceptor of SIV relative to smCXCR6. We investigated which coreceptors of both rhesus macaque and sooty mangabey origin support SIVsmm, SIVmac 239, SIVmac 251, and SIVsmm E660 Env-mediated infection. We discovered that SIVsmm and rhesus macaque SIV use the same coreceptors *in vitro*, and confirmed that rmCXCR6 is a poor coreceptor of all these SIV Env relative to smCXCR6.

Having defined the species-specific coreceptors that support sooty mangabey SIVsmm and rhesus macaque SIVmac 239, SIVmac 251, and SIVsmm E660 Env-mediated infection *in vitro*, we also sought to define the relative role of CCR5 and alternative coreceptors in infection of sooty mangabey and rhesus macaque peripheral blood mononuclear cells (PBMCs) *ex vivo*. SIV coreceptor use *in vitro* may not mimic SIV coreceptor use in primary cells *ex vivo,* or coreceptor use *in vivo*. This is especially relevant because there is no antibody to primate CXCR6 and we had not previously confirmed that CXCR6 is expressed on primate $CD4^+$ cells. We hypothesized that SIVsmm and rhesus macaque SIV exhibit distinct patterns of host-dependent coreceptor use in primary cells from sooty mangabeys and rhesus macaques. Implicit in this, we hypothesized that SIVsmm infection and replication in sooty mangabey PBMCs (smPBMCs) are highly dependent upon alternative coreceptors, including smCXCR6. We also hypothesized that SIVmac infection and replication in rhesus macaques are almost exclusively CCR5-dependent.

Prior evidence suggests that SIVsmm infection and replication in the majority of sooty mangabeys are highly CCR5-independent and alternative coreceptor-dependent. SIVsmm infection and robust viral loads within a small population of $CCR5^{\Delta/\Delta}$ sooty mangabeys offers the most striking proof of SIV alternative coreceptor use in sooty mangabeys (29). We also found that SIVsmm from both $CCR5^{wt/wt}$ and $CCR5^{\Delta/\Delta}$ sooty mangabeys use alternative coreceptors and, thus, the

virus likely retains the ability to use alternative coreceptors even in sooty mangabeys expressing functional CCR5 (6) (Chapter 2). Collectively, natural host sooty mangabeys express exceedingly low levels of wild-type CCR5 on peripheral and lymph node $CD4^+$ T cells (22, 24, 33, 35, 37). Because short-lived $CD4^+$ T cells support the majority of SIVsmm infection and replication *in vivo* (11), the low level of CCR5 on $CD4^+$ T cells from sooty mangabeys led us to hypothesize that alternative coreceptors support a portion of the exceedingly robust SIVsmm replication in sooty mangabeys.

In contrast to SIVsmm infection of sooty mangabeys, prior studies suggest SIVmac infection of rhesus macaques is highly CCR5-dependent. Peripheral and lymph-node $CD4^+$ T cells from rhesus macaques and other non-natural hosts express high levels of CCR5 relative to those observed in natural hosts (24), indicating that these hosts may easily support CCR5-mediated viral replication *in vivo*. When CCR5 antagonist CMPD167 was injected into chronically SIVmac 251-infected rhesus macaques, plasma viral loads decreased over 4-fold (36), indicating that CCR5 supports some SIVmac 251 infection and replication *in vivo*. High concentrations of CCR5 antagonist TAK-779 completely blocked SIVmac 239 and SIVmac 251 infection and replication in rhesus macaque PBMCs (39), demonstrating that rhesus macaque SIV are highly CCR5-dependent in primary rhesus macaque PBMCs (rmPBMCs) *ex vivo*. Although SIVmac 239 utilizes alternative coreceptor rmGPR15 *in vitro*, a single amino acid substitution in SIVmac 239 Env that specifically abrogated rmGPR15 use (but not rmCCR5 use) resulted in rhesus macaque plasma viral loads *in vivo* comparable to those achieved with a wild-type SIVmac 239 (27). Furthermore, when SIVmac 239 was used to infect human $CCR5^{\Delta/\Delta}$ PBMCs, viral protein products were not found in $CD4^+$ cells expressing GPR15 (17). These data suggest that rmGPR15 use is not a major replicative advantage to SIVmac 239 *in vivo* (27). Collectively, these data suggest SIV infection of rhesus macaques is highly dependent on coreceptor rmCCR5.

We defined the coreceptor use patterns of SIVsmm and SIVmac in sooty mangabey and rhesus macaque PBMCs side-by-side. Because CCR5 expression is exceedingly low in natural host sooty mangabeys (22, 24), alternative coreceptors may dictate the bulk of SIV tropism *in vivo*. Identifying divergent SIV coreceptor use patterns between host sooty mangabeys and host rhesus macaques will assist classification of dispensable natural host sooty mangabey immune target cells that maintain viral replication without precipitating widespread $CD4^+$ T cell loss. In addition, characterizing rhesus macaque SIVmac 239, SIVmac 251, and SIVsmm E660 as a primarily CCR5-topic viruses in rhesus macaques would lend credibility to rhesus macaque SIV infection as a model of human HIV-1 infection and pathogenesis (12), as HIV-1 is highly CCR5-dependent (especially in early stages of infection).

Herein, we define the coreceptors of sooty mangabey and rhesus macaque origin that support SIV infection in transfected cells *in vitro*. We also use selective inhibitors to determine if CCR5, CXCR6, and/or other coreceptors support SIVsmm and rhesus macaque SIV infection in sooty smPBMCs and rmPBMCs.

METHODS

Maraviroc and CXCL16

Maraviroc (Selzentry, Celsentri) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID (Cat #11580) and suspended at 20mg/mL in dimethyl sulfoxide (DMSO). Lyophilized recombinant human CXCL16 (R&D Systems #976-CX-025) was suspended at 25µg/mL in phosphate buffered saline and used within 2 days with ≤1 freeze/thaw cycle. Cells were treated pre-infection with vehicle DMSO alone, Maraviroc, CXCL16 plus DMSO, or Maraviroc plus CXCL16. Final concentrations at the time of infection were 10µM Maraviroc and/or 500ng/mL CXCL16 in less than 0.03% vehicle DMSO.

Viruses and viral Env

SIVsmm FFv 2.1 and SIVsmm FFv4.1 Env were cloned by C. Derdeyn and B. Li (Emory University) from a $CCR5^{wt/wt}$ Clade 1 SIVsmm-infected sooty mangabey (animal FFv, Yerkes National Primate Research Center nomenclature) using single genome amplification (SGA) (6, 19, 29, 31). SIVmac 239 Env was cloned from the full-length provirus SIVmac 239. SIVmac 251 Env RZu4-1.1 Day 11 was cloned by C. Derdeyn by SGA from a control (non-CD4-depleted) rhesus macaque infected with SIVmac 251 as previously described (10, 21). SIVsmm E660 CP3C-P-A8 and SIVsmm E660 CR54-PK-2A5 Env were cloned by SGA from acutely SIVsmm E660-infected rhesus macaques and were a gift from K. Barr and G. Shaw (University of Pennsylvania) (16, 30). The HIV-1 pNL-Luc-$E^{-}R^{+}$ backbone and Env pseudotype formation protocol were described previously (4, 6, 29) (Chapter 2). Replication-competent proviral clone SIVmac 239 was a gift from the laboratory of J. Hoxie (University of Pennsylvania). A replication-competent proviral SIVmac 251 Env clone was formed by N. Francella (University of Pennsylvania) by replacing SIVmac 239 Env with the SIVmac 251 RZu4-1.1 Day 11 Env, generating a proviral clone with a SIVmac 239 backbone and Env from a single SIVmac 251 virus (9, 10). These proviral clones were transfected into 293T cells using Fugene (Promega) and viral supernatant was harvested 3-4 days later and quantified by P27 Gag ELISA. SIVsmm E660

primary isolate was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from V. Hirsch and P. Johnson (Catalog #3288) (13). Primary isolates SIVsmm M935 and SIVsmm D215 were generated from SIVsmm-infected CCR5$^{wt/wt}$ sooty mangabeys M935 and D215 (Tulane National Primate Research Center nomenclature) and were a gift from P. Marx.

Cell lines and PBMCs

Adherent human embryonic kidney 293T cells were maintained in DMEM + 10% FBS + 1% Penicillin-Streptomycin + 1% L-Glutamine. Adherent canine thymocyte Cf2Th.Luc cells carry a stable HIV-1 LTR-driven luciferase gene activated by HIV-1 Tat (7) and were a gift from H. Barbian and F. Bibollet-Ruche in the laboratory of B. Hahn (University of Pennsylvania). These cells were maintained in DMEM + 3.5% FBS + 1% Penicillin-Streptomycin + 1% L-Glutamine. Uninfected sooty mangabey PBMCs from CCR5$^{wt/wt}$ animals FBa1, FRz, FUu, FYz, FIa1, and FIz were collected and Ficoll purified at the Yerkes National Primate Research Center with assistance from the laboratory of F. Villinger. Rhesus macaque PBMCs were a gift from B. Ferraro in the laboratory of D. Weiner (University of Pennsylvania). We PCR amplified and sequenced rmCXCR6 from seven rhesus macaques using primers previously described (6) (Chapter 2) and verified that 7 out of 7 rhesus macaques carried the rmCXCR6$^{R30/R30}$ genotype. Primate PBMCs were thawed and cultured in RPMI + 10% FBS + 1% Penicillin-Streptomycin + 1% L-Glutamine overnight prior to stimulation.

Pseudotype Infections

Rhesus macaque rmCCR5, rmCXCR6, and rmGPR15 were independently cloned from rhesus macaque PBMC genomic DNA using primers discussed previously (6) (Chapter 2). The sequences of these clones matched those published in GenBank. Two alleles of rmGPR15 encoding two proteins with a single amino acid difference were described previously (28, 38). All experiments were performed with the rmGPR15 M279 allele. Sooty mangabey smCCR5, smCXCR6, and smGPR15 were also cloned as previously described (6) (Chapter 2). Primate

CD4 plasmids were a gift from T. Vanderford (Emory University). Target 293T cell infections were performed as described previously (6) (Chapter 2). Briefly, cells were transfected with 1µg CD4 and 1µg coreceptor plasmid DNA using Fugene. Cells transfected with CD4 alone served as negative controls. Transfected cells were washed 24 hours post-transfection and re-plated 2×10^5 cells per well in a 96 well plate. The next day, DNAse-treated pNL-Luc-E^-R^+ Env pseudotype viruses were added 50uL/well. VSV-G pseudotyped viruses served as positive controls and Env-negative pseudotypes served as negative controls. Cells were spin-inoculated and luciferase output was measured 72 hours post-infection in relative light units (RLU) after 500mg integration. Luciferase production in CD4 + CCR5 cells was normalized to 100% by species such that:

> Percent entry = 100% × [(RLU in species CD4 + alternative coreceptor) ÷ (RLU in species CD4 + CCR5)]

Cf2Th.Luc cells were transfected with 1µg CD4 and 1µg coreceptor plasmid DNA using Fugene and were washed and re-plated 1×10^5 cells per well in a 96 well plate. Cells transfected with empty plasmid and with CD4 alone served as negative controls. Cf2Th.Luc cells were infected with 5ng P27 Gag SIVmac 239 provirus, 5ng P27 Gag SIVmac 251 Env provirus, 5ng SIVsmm E660 primary isolate, or a max volume 50µL SIVsmm M935 and D215 primary isolates (we had limited stock of primary isolates and did not know the P27 Gag concentration or $TCID_{50}$ before-hand). Uninfected cells served as negative controls for the relatively high baseline expression of luciferase in this cell line. Cells were spin-inoculated 2 hours at 1200×g and 25°C. Infection was measured 48 hours post-infection by luciferase production (Luciferase Assay System; Promega) with 500ms integration using a Luminoskan Ascent Microplate Luminometer (Thermo Scientific).

Infection and Replication Experiments

Rhesus macaque and sooty mangabey PBMCs were stimulated 3-5 days with 5µg/mL Concanavalin A (ConA) and 30Units/mL Interleukin-2 (IL-2). Media was replaced one hour pre-infection with IL-2 media containing vehicle, Maraviroc, CXCL16, or Maraviroc plus CXCL16. Cells were plated 1×10^6 cells/well in 1.5mL to 2mL total volume in a 24-well plate. Viral

supernatants were added as following: 5ng P27 SIVmac 239, 5ng P27 SIVmac 251, 5ng P27 SIVsmm E660, 100µL SIVsmm M935, 100µL SIVsmm D215. Cells were spin-inoculated 2 hours at 1200xg and 25°C. Media was collected one day post-infection (Day -1) followed by washing and replacing with IL-2 media containing fresh drug or chemokine (Day 0). 50µL of supernatant was collected every 2-3 days for P27 Gag ELISA performed by S. Bryan and F. Shaheen in the Penn Center for AIDS Research Virology Core.

Statistics

All statistical comparisons were made by a two-tailed unpaired t-Test using Welch's correction unless otherwise indicated. Significance was defined as $p<0.05$. Statistical analyses were performed using GraphPad PRISM 6 software (La Jolla, CA, USA).

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) and the Tulane National Primate Research Center (TNPRC). Studies were also reviewed and approved by the University of Pennsylvania IACUC.

RESULTS

The same coreceptors support SIVsmm and rhesus macaque SIV *in vitro*

Natural host SIVsmm Env use coreceptor smCCR5 but also exhibit robust infection through alternative coreceptor smCXCR6 and moderate entry through alternative coreceptor smGPR15 in transfected cells *in vitro* (6). We hypothesized that pathogenic SIV from rhesus macaques would exhibit a similar pattern of alternative coreceptor use with these sooty mangabey coreceptors *in vitro* because rhesus macaque SIV were developed from SIVsmm. We examined coreceptor use patterns of three SIV frequently used to induce pathogenesis in rhesus macaques: SIVmac 239, SIVmac 251, and SIVsmm E660. Importantly, SIVmac 239/251 and SIVsmm E660 viruses were derived from independent SIVsmm cross-species transmissions into rhesus macaques and these viruses represent diverse sub-species of pathogenic rhesus macaque SIV (12) (Chapter 1, Introduction, Figure 1.8). Infection with SIVmac 239, SIVmac 251, or SIVsmm E660 leads to AIDS in non-natural host rhesus macaques.

To determine if sooty mangabey SIVsmm and rhesus macaque SIVmac 239, SIVmac 251, and SIVsmm E660 exhibit similar use of sooty mangabey coreceptors, we transfected 293T cells with sooty mangabey CD4 (smCD4) in combination with smCCR5, smCXCR6, and smGPR15. Cells transfected with smCD4 alone served as a negative control. Transfected cells were infected with Env pseudotypes of pNL-Luc-$E^{-}R^{+}$ that express luciferase in place of Nef upon infection (4). Luciferase output was measured three days post-infection in relative light units (RLU). We used absolute luciferase RLU values to assign significance. Data are expressed as a percentage of entry through smCD4 and smCCR5 (**Figure 4.1A**).

We examined multiple Env from the SIVsmm/SIVmac viral lineage. Sooty mangabey SIVsmm FFv2.1 Env was previously cloned by single genome amplification (SGA) from a Clade 1 viral isolate collected from a $CCR5^{wt/wt}$ sooty mangabey (6, 29). The SIVmac 239 Env was cloned from the infectious molecular clone SIVmac 239. The SIVmac 251 Env was cloned by SGA from

a SIVmac 251-infected rhesus macaque as previously described (10, 21). Two SIVsmm E660 transmitter/founder Env clones, SIVsmm E660 CP3C and SIVsmm E660 CR54, were cloned by SGA from an acutely infected rhesus macaque challenged with SIVsmm E660 as previously described (15, 30).

SIVmac 239, SIVmac 251, and SIVsmm E660 Env exhibited a pattern of alternative coreceptor use *in vitro* similar to that of SIVsmm from sooty mangabeys. As we observed previously (6) (Chapter 2), alternative coreceptor smCXCR6 supported robust SIVsmm FFv2.1 Env-mediated infection at 90% of the level of infection mediated by smCCR5 (**Figure 4.1A**). Alternative coreceptor smGPR15 also supported moderate SIVsmm FFv2.1 Env-mediated infection *in vitro* at 65% of the level of infection through smCCR5 (**Figure 4.1A**).

Notably, smCXCR6 was a robust alternative coreceptor of rhesus macaque SIV Env. SIVsmm E660 CP3C, SIVsmm E660 CR54, and SIVmac 251 Env mediated infection through smCXCR6 significantly above infection through smCCR5 ($p=0.003$, $p=0.007$, $p=0.002$ respectively) (**Figure 4.1A**). SIVmac 239 infection through smCXCR6 occurred at 78% of the level of infection through smCCR5. Alternative coreceptor smGPR15 supported SIVmac 239, SIVmac 251, and SIVsmm E660 CP3C infection at levels from 13% (SIVsmm E660 CR54) to 110% (SIVmac 251) of infection through smCCR5 (p=NS). SIVsmm E660 CR54 exhibited very low infection through smGPR15 relative to smCCR5.

In summary, we found that smCXCR6 was a robust alternative coreceptor of both SIVsmm and rhesus macaque SIV *in vitro*. Viral Env from rhesus macaque viruses SIVmac 251 and SIVsmm E660 exhibited infection through smCXCR6 significantly above infection through smCCR5. Sooty mangabey smGPR15 was a strong coreceptor of SIVmac 239, SIVmac 251, and SIVsmm E660 CP3C Env; a moderate coreceptor of SIVsmm FFv2.1 Env; and a poor coreceptor of SIVsmm

E660 CR54 Env in transfected cells *in vitro*. Collectively, SIVsmm Env and rhesus macaque SIV Env exhibited similar patterns of sooty mangabey coreceptor use.

Rhesus macaque CXCR6 is a weak coreceptor of SIV *in vitro*

Viruses SIVmac 239, SIVmac 251, and SIVsmm E660 are frequently used to infect rhesus macaques, not sooty mangabeys. Thus, we next sought to answer a more relevant question: which coreceptors of rhesus macaque origin support SIVmac 239, SIVmac 251, and SIVsmm E660 infection *in vitro*? Previous studies found that rmCCR5 and rmGPR15 support SIVmac infection *in vitro*; crucially, these studies also observed that rhesus macaque rmCXCR6 was a poor coreceptor of SIVmac 239/251-related rhesus macaque SIV relative to human CXCR6 (26, 27). We were interested to determine if SIVsmm E660 CP3C and SIVsmm E660 CR54 Env, which represent a different sub-family of rhesus macaque SIV, also exhibit poor infection through rmCXCR6. We also determined if infection through non-natural host rmCXCR6 was low relative to infection through natural host smCXCR6.

To define the coreceptors of rhesus macaque origin that support SIVmac 239, SIVmac 251, and SIVsmm E660 Env-mediated infection *in vitro*, we cloned CD4 and putative coreceptors rmCCR5, rmCXCR6, and rmGPR15 from rhesus macaque genomic DNA using methods previously described (6) (Chapter 2). The DNA sequences of rmCD4, rmCCR5, rmCXCR6, and rmGPR15 matched rhesus macaque sequences published previously (8, 26, 38) (data not shown). Target 293T cells were transfected with rmCD4 and rmCCR5, rmCXCR6, or rmGPR15. Transfected cells were infected with pNL-Luc-$E^{-}R^{+}$ Env pseudotypes as before. Luciferase expression was measured three days post-infection, we used absolute luciferase RLU values to assign significance, and data are displayed as the percentage of entry through rmCD4 plus rmCCR5 (**Figure 4.1B**).

Absolute levels of luciferase expression following infection of 293T cells expressing rmCD4 plus rmCCR5 were comparable to levels of luciferase expression following infection of 293T cells expressing smCD4 plus smCCR5 (data not shown). Rhesus macaque rmCXCR6 was a poor coreceptor of SIVsmm FFv2.1 Env, as infection through rmCXCR6 was only 43% of that through rmCCR5 ($p=0.02$) (**Figure 4.1B**). Consistent with previous findings (26), rmCXCR6 was a remarkably poor coreceptor of SIVmac and SIVsmm E660 Env pseudotypes with rmCXCR6-mediated infection ranging from only 3% (SIVsmm E660 CR54) to 11% (SIVmac 251) of infection through rmCCR5 ($p\leq0.03$) (**Figure 4.1B**). Indeed, rmCXCR6-mediated SIVsmm E660 CP3C and SIVsmm E660 CR54 infections were not significantly above infection in control cells transfected with rmCD4 alone. Thus, relative to SIVmac 239/251 Env, SIVsmm E660 Env exhibited exceedingly poor infection through rmCXCR6. The inability of rmCXCR6 to support SIVsmm E660 infection was a particularly sharp contrast to smCXCR6, which supported SIVsmm E660 infection significantly above the levels of rmCCR5-mediated infection (compare **Figure 4.1A** and **Figure 4.1B**).

All SIV Env exhibited significantly lower levels of infection through rmCD4 plus rmCXCR6 than those through smCD4 plus smCXCR6 (SIVsmm FFv 2.1 $p=0.02$, SIVmac 239 $p=0.005$, SIVmac 251 $p=0.003$, SIVsmm E660 CP3C and SIVsmm E660 CR54 $p<0.001$). Therefore, we concluded that non-natural host rhesus macaque rmCXCR6 is a poor coreceptor of SIV relative to natural host sooty mangabey smCXCR6 *in vitro*. These data add to prior findings that rmCXCR6 is a weak coreceptor of SIV relative to human CXCR6 (26).

Rhesus macaque rmGPR15 supported SIVsmm FFv2.1 Env-mediated infection at levels 74% of that through rmCCR5 (**Figure 4.1B**). Rhesus macaque SIV Env also exhibited lower infection through rmGPR15 than rmCCR5. Rhesus macaque GPR15 supported rhesus macaque SIV infection at 15% (SIVsmm E660 CR54) to 78% (SIVmac 251) of infection through rmCCR5. Coreceptors rmGPR15 and smGPR15 supported similar absoluate levels of infection. Thus,

although rmCXCR6 is a poor coreceptor of SIV relative to smCXCR6, rmGPR15 and smGPR15 are comparable *in vitro*.

To confirm that rmCXCR6 is a weak coreceptor in other cell lines *in vitro*, and to define rmCXCR6 as a weak coreceptor of replication competent SIV, we performed a similar experiment using transfected canine cells *in vitro*. Canine thymus Cf2Th.Luc cells were previously engineered with a stable HIV-1 LTR-luciferase reporter; these cells express luciferase following infection by viruses carrying Tat (7). Target Cf2Th.Luc cells were transfected with CD4 and CCR5, CXCR6, and GPR15 derived from sooty mangabeys and rhesus macaques. We infected target Cf2Th.Luc cells with replication-competent provirus SIVmac 239, chimeric provirus SIVmac 239 with a SIVmac 251 Env (hereafter referred to as SIVmac 251 *Env chimera* to distinguish it from the SIVmac 251 swarm), and primary isolate SIVsmm E660. Luciferase output was measured two days post-infection and normalized to luciferase production in target cells with species-matched CD4 plus CCR5 as before.

We confirmed that rmCXCR6 is a poor coreceptor of replication competent SIV relative to smCXCR6 (**Figure 4.2**). Entry through smCD4 plus smCCR5 was comparable to entry through rmCD4 plus rmCCR5 (data not shown). Alternative coreceptor smCXCR6 supported robust infection by SIVmac 239, SIVmac 251 Env chimera, and SIVsmm E660 (**Figure 4.2A**). Notably, SIVsmm E660 infection through smCXCR6 was significantly higher than infection through smCCR5 ($p=0.003$) as we observed previously with individual SIVsmm E660 Env pseudotypes (Figure 4.1A). In contrast to smCXCR6, rmCXCR6 supported only 4% (SIVsmm E660) to 22% (SIVmac 251 Env chimera) of infection compared to infection through rmCCR5 (SIVmac 239 and SIVmac 251 Env chimera $p\leq0.02$) (**Figure 4.2B**). SIVmac 239 and SIVmac 251 Env chimera infection via smCD4 plus smCXCR6 was significantly (5-fold to 6-fold) higher than infection via rmCD4 plus rmCXCR6 ($p=0.003$, $p=0.03$ respectively). SIVsmm E660 infection was also much more robust in cells expressing smCD4 plus smCXCR6 than in cells expressing rmCD4 plus

rmCXCR6, although we did not perform enough replicates of SIVsmm E660 infection to asses significance.

Alternative coreceptors smGPR15 and rmGPR15 supported Cf2Th.Luc cell infection by replication competent SIVmac 239, SIVmac 251 Env chimera, and SIVsmm E660 (**Figure 4.2**). The pattern of rmGPR15 use varied amongst viruses. SIVmac 239 infection through rmGPR15 was 30% higher than through rmCCR5 ($p=0.052$, trend toward significance). SIVmac 251 Env chimera infection was 20% lower through rmGPR15 than rmCCR5. SIVsmm E660 also exhibited moderate use of alternative coreceptor GPR15 *in vitro*.

The Cf2Th.Luc assay also confirmed that individual SIV Env are indicative of coreceptor utilization patterns of the swarms from which they were isolated. Notably, replication competent SIVsmm E660 had a pattern of sooty mangabey coreceptor use similar to that of individual SIVsmm E660 CP3C and SIVsmm E660 CR54 Env, marked by robust infection through smCXCR6 above the level of infection through smCCR5 and moderate infection through smGPR15 (compare **Figure 4.1A** and **Figure 4.2A**). We also found that primary isolate SIVsmm D215 from an infected sooty mangabey exhibited a pattern of robust smCCR5, robust smCXCR6, and moderate smGPR15 coreceptor use trending toward that of the individual SIVsmm FFv2.1 Env *in vitro* (data not shown).

In summary, infections in transfected Cf2Th.Luc cells further demonstrated that rmCXCR6 was a very poor coreceptor of SIV Env *in vitro* relative to smCXCR6. Notably, rhesus macaque rmCXCR6 was a particularly poor coreceptor of rhesus macaque SIV typically used to infect non-natural hosts, including SIVmac 239, SIVmac 251, and SIVsmm E660. We also confirmed that alternative coreceptor rmGPR15 is a coreceptor of SIV *in vitro* and supports infection comparable to infection through smGPR15.

SIVmac in rmPBMCs is primarily CCR5-dependent, whereas SIVsmm in smPBMCs is partially CCR5-independent

We next set out to define SIV alternative coreceptor use in primary sooty mangabey and rhesus macaque PBMCs. A coreceptor that supports infection in transfected cells *in vitro* may not support infection and replication in PBMCs *ex vivo*. Transfected 293T and Cf2Th.Luc cells express artificially high levels of CD4 and coreceptor and are permissive to infection. It is possible that a functional coreceptor *in vitro* is not co-expressed with requisite receptor CD4 on the surface of primary cells *ex vivo*, or that cells expressing CD4 and coreceptor also co-express viral restriction factors, or that cells expressing CD4 and coreceptor lack expression of host genes required for productive infection. Therefore, it is critical to define the coreceptors that support SIV infection and replication in primary cells, as these are likely coreceptors that support SIV infection and replication *in vivo*. Initially, we defined the relative role of CCR5 in SIV infection and replication using the CCR5 antagonist Maraviroc, which specifically blocks CCR5-mediated but not CXCR6-medaited or GPR15-medaited SIV infection *in vitro* (Chapter 3, Figure 3.4).

Sooty mangabey smCXCR6 is a very robust alternative coreceptor of SIV *in vitro* and supports infection at levels similar to those supported by smCCR5 (6) (Chapter 2, Figure 4.1, Figure 4.2). Therefore, we hypothesized that SIVsmm infection and replication can occur independent of smCCR5 in smPBMCs. Because rmCXCR6 supports little to no SIV infection *in vitro* (26) (Figure 4.1, Figure 4.2) and because rmGPR15 appears to be dispensable both *ex vivo* and *in vivo* (17, 27), we also hypothesized that SIVmac infection and replication in rmPBMCs is highly CCR5-dependent. To address these hypotheses, we stimulated smPBMCs and rmPBMCs with ConA plus IL-2 and pre-treated cells one hour prior to infection with vehicle (DMSO) or 10μM Maraviroc. Sooty mangabey PBMCs from two uninfected $CCR5^{wt/wt}$ animals (FBa1 and FUu) were infected with SIVsmm primary isolates SIVsmm D215 and SIVsmm M935 derived from two infected $CCR5^{wt/wt}$ animals. Rhesus macaque PBMCs from three animals (Rm1, Rm2, and Rm3) were infected with SIVmac 239 and SIVmac 251 Env chimera. Culture supernatants were collected 24

hours post-infection (Day -1), after washing off the inocula (Day 0), and periodically over the course of infection. We performed SIV P27 Gag ELISA to measure SIV viral production and accumulation at each time point.

SIVsmm infection and replication in natural host smPBMCs occurred in the presence of CCR5 antagonist Maraviroc (**Figure 4.3A**). Both SIVsmm D215 and SIVsmm M935 exhibited robust infection in vehicle-treated smPBMCs and 14% (SIVsmm D215 in sooty mangabey FBa1) to 40% (SIVsmm M935 in sooty mangabey FUu) of infection remained in the presence of Maraviroc at the peak of viral production (mean 28.4 ± 11.4% standard deviation SD). There was no difference in sensitivity to Maraviroc between viruses SIVsmm D215 and SIVsmm M935 ($p=0.66$, NS, Mann Whitney U test) or between animals FBa1 and FUu ($p=0.33$, NS, Mann Whitney U test), but a much larger data set is needed to fully conclude that there is no variation between these parameters. Maraviroc is a potent inhibitor of smCCR5-mediated SIV Env pseudotype infection at concentrations greater than or equal to 1μM *in vitro* (Chapter 3, Figure 3.1C). Therefore, SIVsmm D215 and SIVsmm M935 infection and replication in smPBMCs treated with 10μM Maraviroc likely demonstrate that one or more alternative, non-CCR5 coreceptors support infection and replication in natural host smPBMCs.

In contrast to the partially CCR5-independent SIVsmm D215 and SIVsmm M935 infection of smPBMCs, SIVmac 239 and SIVmac 251 infection and replication in rmPBMCs were almost entirely dependent on CCR5 (**Figure 4.3B**). SIVmac 239 and SIVmac 251 Env chimera exhibited very robust infection of vehicle-treated rmPBMCs. In the presence of Maraviroc, SIVmac 239 and SIVmac 251 Env chimera were more than 90% blocked (mean 95.6 ± 3.9% SD). At the peak of infection, only 0.6% (SIVmac 251 Env chimera in rhesus macaque Rm3) to 10% (SIVmac 239 in rhesus macaque Rm1) of SIVmac 239 and SIVmac 251 replication remained in Maraviroc-treated rmPBMCs (mean 4.4 ± 3.9% SD). This result is especially remarkable given that Maraviroc is an incomplete inhibitor of rmCCR5-mediated SIV pseudotype infection in 293T cells *in vitro* (Chapter

3, Figure 3.1D); we predict that a full antagonist of rmCCR5 may further inhibit SIVmac infection in rmPBMCs. The marked attenuation of SIVmac infection and replication in the presence of Maraviroc indicated that SIVmac 239 and SIVmac 251 Env chimera infection of non-natural host rmPBMCs was highly CCR5-dependent.

rmPBMCs support CCR5-dependent SIV infection, whereas smPBMCs support CCR5- and CXCR6-mediated SIV infection

To further define coreceptors that support SIV infection and replication in sooty mangabeys and rhesus macaques, we pre-treated nonhuman primate PBMCs with CCR5 antagonist Maraviroc and/or recombinant human chemokine CXCL16. Chemokine CXCL16 is a natural ligand of CXCR6. We previously demonstrated that CXCL16 significantly blocks the majority of SIVsmm infection when smCXCR6 is expressed at low levels *in vitro* (Chapter 3, Figure 3.5). Additionally, we demonstrated that CXCL16 has no direct off-target effects on SIV infection mediated by smCCR5 or smGPR15 *in vitro* (Chapter 3, Figure 3.7). In PBMCs, infections that are CCR5-dependent will be almost entirely blocked by Maraviroc (see Figure 4.3B); infections that are CCR5- and alternative-coreceptor dependent will not be entirely blocked by Maraviroc and residual infection and replication will remain (see Figure 4.3A); infections that are highly dependent on CXCR6 may be significantly blocked by CXCL16 alone; and notably, infections that are significantly blocked by the combination of Maraviroc plus CXCL16 relative to Maraviroc alone also indicate that some SIV infection is supported by CXCR6.

ConA and IL-2 stimulated rmPBMCs and smPBMCs were pre-treated with vehicle, 10μM Maraviroc, 500ng/mL CXCL16, or a combination of both Maraviroc and CXCL16. Rhesus macaque rmPBMCs from one animal (Rm4) were infected with SIVmac 239 and SIVmac 251 Env chimera. Sooty mangabey smPBMCs from five $CCR5^{wt/wt}$ animals (FRz, FUu, FYz, FIa1, FIz) were infected with SIVsmm M935, SIVsmm D215, SIVsmm E660, and SIVmac 239. Supernatant P27 Gag was measured by ELISA with peak viral production occurring near Day 8 post-infection.

As we observed previously, SIVmac 239 and SIVmac 251 Env chimera infections in rmPBMCs were almost completely CCR5-dependent. Maraviroc blocked 85% to 90% of peak SIVmac P27 Gag production in rmPBMCs from animal Rm4 (**Figure 4.4, Table 4.1**). When data for Rm4 were combined with the above data from Rm1, Rm2, and Rm3 (Figure 4.3B), we found that SIVmac 239 (mean 95 ± 4.2% SD) and SIVmac 251 (mean 92 ± 6.5% SD) were almost entirely blocked by Maraviroc in rmPBMCs (**Figure 4.4A** and **Figure 4.4B** respectively). Chemokine CXCL16 did not block SIVmac 239 infection of Rm4 rmPBMCs. Unexpectedly, chemokine CXCL16 did block 42% of SIVmac 251 Env chimera infection of Rm4 rmPBMCs. This could indicate that rmCXCR6 supports some SIVmac 251 infection in rmPBMCs despite being a poor coreceptor of SIVmac 251 Env pseudotypes *in vitro*. However, the blocking of SIVmac 251 infection in Rm4 rmPBMCs was not enhanced by the combination of Maraviroc and CXCL16 relative to Maraviroc alone, indicating that rmCXCR6 did not support SIVmac 251 Env chimera infection on top of rmCCR5-mediated infection. Further experiments are needed to address whether or not rmCXCR6 supports any SIV infection and replication in rmPBMCs. However, given the ability of Maraviroc to block over 90% of infection in these rmPBMCs, it is apparent that rmCCR5 is the dominant coreceptor in rmPBMCs.

As we observed previously, sooty mangabey viruses SIVsmm D215 and SIVsmm M935 were partially CCR5-independent in smPBMCs (**Figure 4.5A** and **Figure 4.5B**, **Table 4.2**). At the peak of infection, Maraviroc blocked between 21% and 61% (mean 43.7 ± 14.8% SD) of peak SIVsmm D215 and SIVsmm M935 viral production relative to vehicle treated controls ($p \leq 0.01$ for both viruses). Interestingly, pathogenic rhesus macaque virus SIVsmm E660 was also partially CCR5-independent in smPBMCs (**Figure 4.5C**). Maraviroc failed to block any SIVsmm E660 viral production in smPBMCs from animals FIa1 or FIz and blocked only 23% (FRz) to 32% (FYz) of SIVsmm E660 viral production in smPBMCs from remaining animals (**Figure 4.5C, Table 4.2**). Furthermore, SIVsmm E660 was significantly less sensitive to Maraviroc than either SIVsmm

D215 (p=0.046) or SIVsmm M935 (p=0.02). This recapitulates the finding that individual SIVsmm E660 CP3C and SIVsmm E660 CR54 Env display significantly higher infection in transfected 293T cells expressing smCD4 plus smCXCR6 than in transfected 293T cells expressing smCD4 plus smCCR5 *in vitro* (see Figure 4.1A), as well as the finding that SIVsmm E660 primary isolate infects Cf2Th.Luc cells expressing smCD4 and smCXCR6 at levels significantly above infection in Cf2Th.Luc cells expressing smCD4 and smCCR5 (see Figure 4.2A).

Crucially, we observed the first direct evidence that smCXCR6 is expressed on smPBMCs and can support SIV infection in smPBMCs. Both SIVsmm D215 and SIVsmm E660 exhibited use of alternative coreceptor smCXCR6 in primary smPBMCs. Chemokine CXCL16 blocked a significant percentage of SIVsmm D215 infection relative to vehicle-treated controls (p=0.01) and the combination of Maraviroc and chemokine CXCL16 significantly lowered SIVsmm D215 infection relative to infection in smPBMCs treated with Maraviroc alone (p=0.02) (**Figure 4.5A**). Chemokine CXCL16 alone had no significant effect on SIVsmm E660 infection. However, SIVsmm E660 infection was significantly attenuated by the combination of Maraviroc and CXCL16 relative to Maraviroc alone (p = 0.02) (**Figure 4.5C**). The combination of Maraviroc and CXCL16 did not entirely block either SIVsmm D215 or SIVsmm E660 infection. Therefore, we concluded that SIVsmm D215 and SIVsmm E660 use alternative coreceptor smCXCR6 in addition to smCCR5, and may exhibit infection through additional non-CCR5 alternative coreceptors as well.

SIVsmm M935 did not exhibit clear use of smCXCR6. Chemokine CXCL16 did not block a significant amount of SIVsmm M935 infection relative to vehicle-treated controls (p=0.17, NS) and the combination of CXCL16 and Maraviroc did not enhance blocking of SIVsmm M935 infection relative to Maraviroc alone (**Figure 4.5B**). (This was true even when SIVsmm M935 infection of Fla1 smPBMCs pre-treated with both Maraviroc and CXCL16 was temporarily discarded as an outlier.) Thus, it appears that not all SIV exhibit clear use of smCXCR6 in smPBMCs. The

combination of Maraviroc and CXCL16 blocked only 32% of SIVsmm M935 infection in smPBMCs. On average, there was more residual SIVsmm M935 infection than residual SIVsmm D215 or SIVsmm E660 in the presence of Maraviroc plus CXCL16, although the trend did not reach statistical significance ($p \geq 0.19$, NS relative to both SIVsmm D215 and SIVsmm E660). It is possible that alternative coreceptors other than smCXCR6 support the majority of SIVsmm M935 CCR5-independent infection in smPBMCs.

To specifically investigate the host role in CCR5-dependent versus CCR5-independent SIV infection, we measured SIVmac 239 infection and replication in smPBMCs from two sooty mangabeys (FIa1, FIz) and directly compared the results to SIVmac 239 infection and replication in rmPBMCs. Strikingly, Maraviroc only blocked 4% and 15% of SIVmac 239 viral production in FIa1 and FIz smPBMCs respectively (**Figure 4.5D** and **Table 4.2**). By contrast, we previously found that Maraviroc blocks over 90% of SIVmac 239 infection in rmPBMCs (see Figure 4.4A, Table 4.1). Therefore, SIVmac 239 is primarily CCR5-independent in smPBMCs whereas SIVmac 239 is primarily CCR5-dependent in rmPBMCs.

The role of smCXCR6 in SIVmac 239 infection of smPBMCs was not clear given the small sample size but we saw a trend in support of smCXCR6-mediated infection in both sets of smPBMCs. Chemokine CXCL16 blocked 68% of SIVmac 239 viral production in smPBMCs from sooty mangabey FIz. Although neither Maraviroc nor CXCL16 alone inhibited SIVmac 239 infection in smPBMCs from animal FIa1, the effect of Maraviroc and CXCL16 together was clearly additive and the combination of both drugs blocked 75% of SIVmac 239 viral production in these smPBMCs. Additional experiments are needed to address the role of smCXCR6 in SIVmac 239 infection of smPBMCs. However, it is clear that SIVmac 239 is partially to mostly CCR5-independent in natural host sooty mangabey smPBMCs and primarily CCR5-dependent in non-natural host rhesus macaque rmPBMCs. Furthermore, these findings clearly demonstrate that alternative coreceptor use in PBMCs *ex vivo* is host-dependent.

In summary, our data provided the first evidence that smCXCR6 supports partially CCR5-independent SIV infection and replication in smPBMCs. SIVsmm D215 and SIVsmm E660 clearly utilized alternative coreceptor smCXCR6 for some portion of infection and replication in sooty mangabey PBMCs. Other alternative coreceptors, such as smGPR15, may have also supported SIV infection and replication in smPBMCs. Ultimately, the relative CCR5-dependence of SIV in sooty mangabey smPBMCs and rhesus macaque rmPBMCs is dictated by the host cells, not the SIVsmm/SIVmac viruses that are typically used to infect those cells.

FIGURES

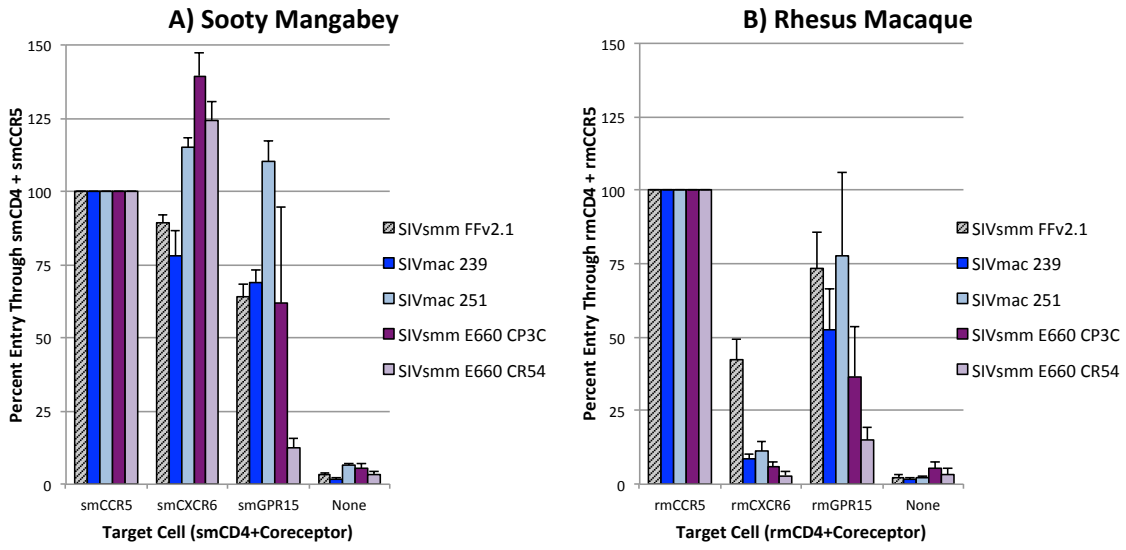

Figure 4.1. Species-specific coreceptor use by SIV Env pseudotypes *in vitro*. Target 293T cells were transfected with **(A)** sooty mangabey smCD4 and sooty mangabey smCCR5/CXCR6/GPR15, or with **(B)** rhesus macaque rmCD4 and rhesus macaque rmCCR5/CXCR6/GPR15. Target cells transfected with CD4 alone (None) served as negative controls. Cells were infected with luciferase-expressing Env-pseudotypes. Natural host sooty mangabey SIVsmm FFv2.1 Env was cloned from an infected sooty mangabey that did not progress to AIDS (gray, hashed bars). Remaining SIVmac 239, SIVmac 251, SIVsmm E660 CP3C, and SIVsmm CR54 Env were derived from viruses that are typically used to infect and induce pathogenesis in rhesus macaques (blue and violet, solid bars). Infection was measured 3 days post-infection by luciferase expression and data were normalized to 100% entry through species-matched CD4 and CCR5. (N=3, mean ± SD.)

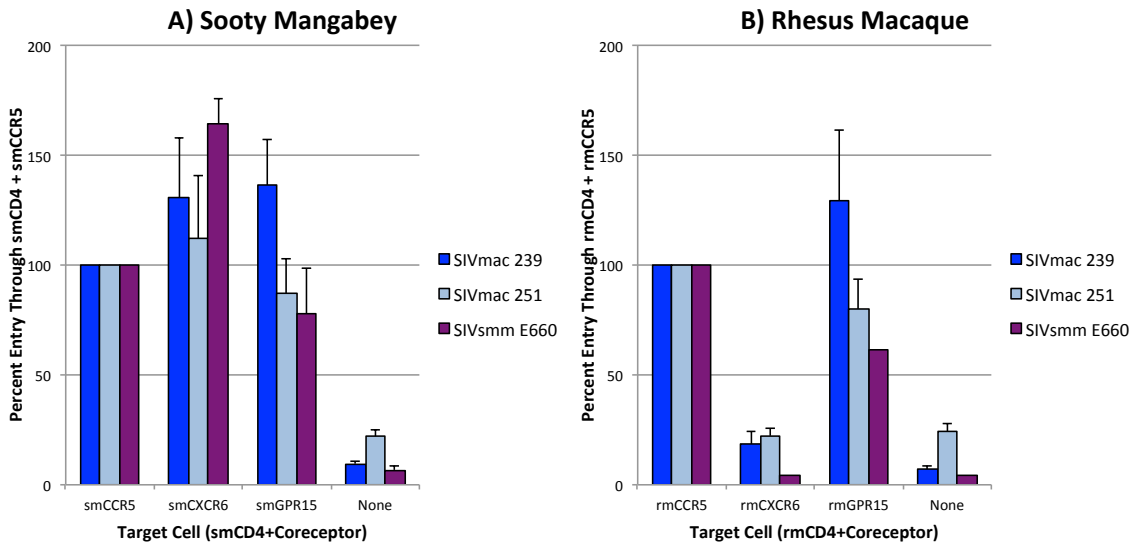

Figure 4.2. Rhesus macaque CXCR6 is a weak coreceptor of replication-competent SIVmac and SIVsmm E660 *in vitro*. Target Cf2Th.Luc cells were transfected with **(A)** smCD4 and smCCR5/CXCR6/GPR15, or with **(B)** rmCD4 with and rmCCR5/CXCR6/GPR15. Cells transfected with CD4 alone served as negative controls. Target cells were infected with viruses produced from infectious molecular clone SIVmac 239, and a chimeric infectious molecular clone of SIVmac 239 carrying SIVmac 251 Env. Target cells were also infected with SIVsmm E660 primary isolate. Infection was measured 3 days post-infection via luciferase expression and data were normalized to 100% entry through species-matched CD4 and CCR5. (N=3 for all viruses in (A), N=3 for SIVmac 239 and SIVmac 251 in (B), N=1 for SIVsmm E660 in (B), mean ± SD where applicable.)

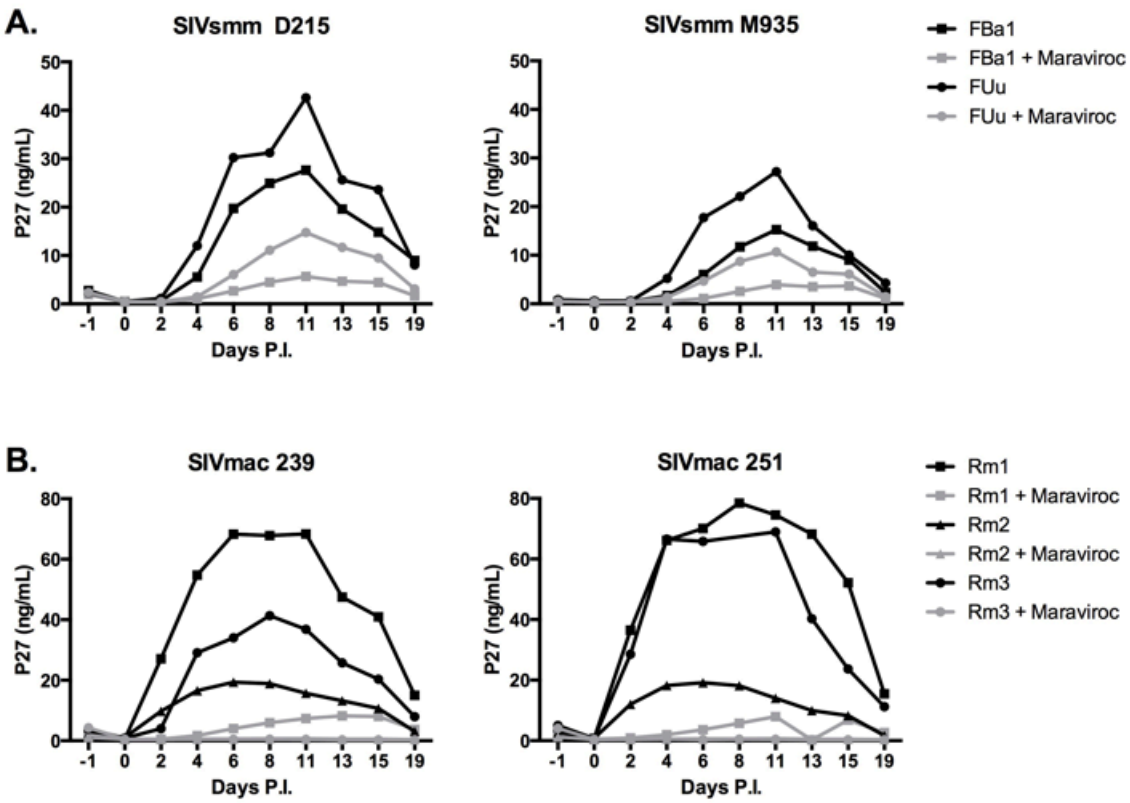

Figure 4.3. SIVmac infection and replication are highly dependent on CCR5 in rhesus macaque PBMCs, whereas SIVsmm infection and replication are partially CCR5-independent in sooty mangabey PBMCs. PBMCs from **(A)** two sooty mangabeys (FBa1, FUu) and **(B)** three rhesus macaques (Rm1, Rm2, Rm3) were stimulated with ConA/IL-2 and pre-treated with vehicle (No Drug) or 10μM Maraviroc. Sooty mangabey PBMCs were then infected with primary isolates SIVsmm D215 and SIVsmm M935. Rhesus macaque PBMCs were infected with SIVmac 239 and a SIVmac 251 Env chimera. Pre-wash supernatant was collected 24 hours post-infection (P.I.) (Day -1), cells were washed with fresh media (Day 0), and viral production was measured by supernatant P27 Gag ELISA at the time points indicated.

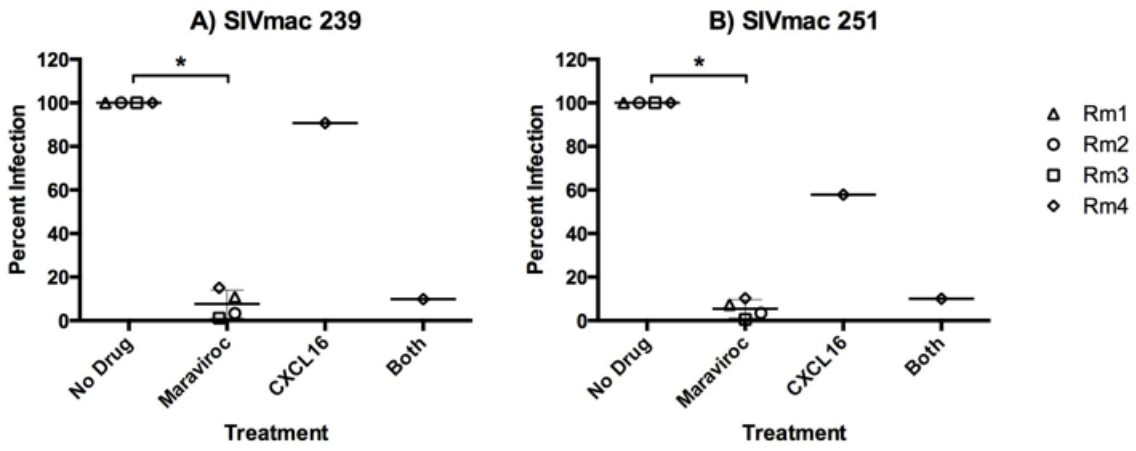

Figure 4.4. SIVmac infection and replication in rhesus macaque PBMCs is CCR5-dependent. Rhesus macaque PBMCs from four animals (Rm1, Rm2, Rm3, Rm4) were stimulated with ConA/IL-2 and pre-treated with vehicle (No Drug) or 10μM Maraviroc. PBMCs from rhesus macaque Rm4 were also pre-treated with 500ng/mL CXCL16 or both Maraviroc and CXCL16. Cells were infected with **(A)** SIVmac 239 or **(B)** SIVmac 251 Env chimera. Culture supernatants were collected at multiple time points. Data represent Day 8 peak supernatant P27 Gag production as measured by ELISA. (Mean ± SD, *$p<0.05$).

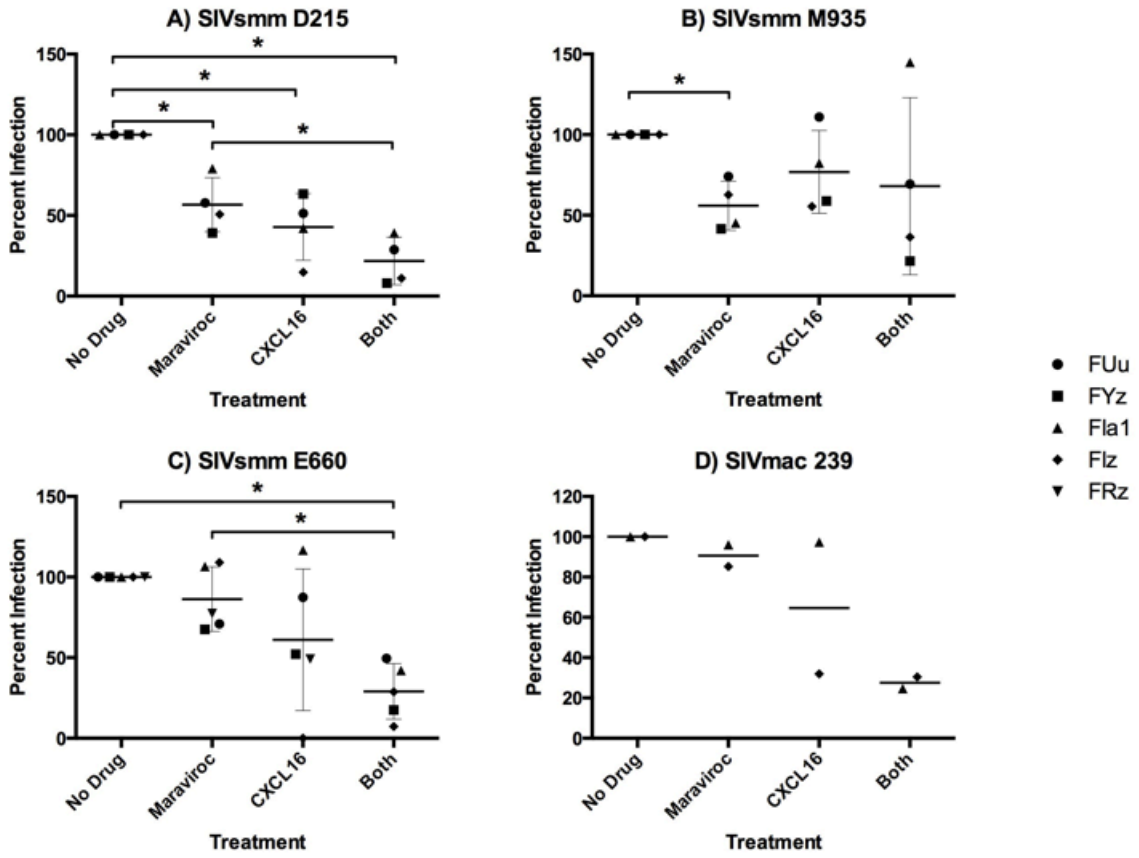

Figure 4.5. Alternative coreceptors, including smCXCR6, support SIV infection and replication in smPBMCs. Sooty mangabey PBMCs from five animals (FRz, FUu, FYz, Fla1, Flz) were stimulated with ConA/IL-2 and pre-treated one hour with vehicle, 10μM Maraviroc, 500ng/mL CXCL16, or both Maraviroc and CXCL16 as shown. Cells were infected with sooty mangabey primary isolates **(A)** SIVsmm D215 and **(B)** SIVsmm M935, with primary isolate **(C)** SIVsmm E660, or with provirus **(D)** SIVmac 239. Culture supernatants were collected at multiple time points post-infection. Data represent Day 8 peak infection supernatant P27 Gag production as measured by ELISA. (Mean ± SD, *p<0.05).

Table 4.1. Maraviroc and CXCL16 blocking in primary rhesus macaque PBMCs

Virus	Treatment[a]	Blocked (%)[b]				Average[c]
		Rm1	*Rm2*	*Rm3*	*Rm4*	
SIVmac 239	Maraviroc	89	97	99	85	95 ± 4.2
	CXCL16	ND	ND	ND	9	--
	Both	ND	ND	ND	90	--
SIVmac 251	Maraviroc	93	97	99	90	92 ± 6.5
	CXCL16	ND	ND	ND	42	--
	Both	ND	ND	ND	90	--

[a] Cells were treated one hour pre-infection and continuously thereafter with 10μM Maraviroc, 500ng/mL CXCL16, or a combination of 10μM Maraviroc and 500ng/mL CXCL16.

[b] Percentage of entry blocked by the addition of drug/chemokine relative to controls treated with vehicle alone. [100% - 100 × (P27 Gag in treated) ÷ (P27 Gag in vehicle control)]. Maraivorc-blocking data from Figure 3 (Rm1, Rm2, Rm3) are also combined into this data set.

[c] Average ± standard deviation by row.

Table 4.2. Maraviroc and CXCL16 blocking in primary sooty mangabey PBMCs.

Virus	Treatment[a]	Blocked (%)[b]					Average[c]
		FRz	*FUu*	*FYz*	*Fla1*	*Flz*	
SIVsmm M935	Maraviroc	ND	26	58	55	37	44 ± 15.2
	CXCL16	ND	0	41	18	45	26 ± 21.0
	Both	ND	31	78	0	64	43 ± 35.0
SIVsmm D215	Maraviroc	ND	42	61	21	49	43 ± 16.8
	CXCL16	ND	49	37	58	85	57 ± 20.7
	Both	ND	71	92	61	89	78 ± 14.7
SIVsmm E660	Maraviroc	23	29	32	0	0	17 ± 15.7
	CXCL16	51	13	48	0	100	42 ± 39.1
	Both	71	50	82	58	93	71 ± 17.3
SIVmac 239	Maraviroc	ND	ND	ND	4	15	9
	CXCL16	ND	ND	ND	3	68	35
	Both	ND	ND	ND	75	70	72

[a] Cells were treated one hour pre-infection and continuously thereafter with10μM Maraviroc, 500ng/mL CXCL16, or a combination of 10μM Maraviroc and 500ng/mL CXCL16.

[b] Percentage of entry blocked by the addition of drug/chemokine relative to controls treated with vehicle alone. ND represents infection not done given a limited number of cells or limited viral stock. [100% - 100 × (P27 Gag in treated) ÷ (P27 Gag in vehicle control)]. All negative values representing infections above vehicle controls were converted to zero for this table but not for in-text statistics or graphical analyses.

[c] Average ± standard deviation by row.

DISCUSSION

We determined that SIVmac infection and replication in rhesus macaque rmPBMCs is highly CCR5-dependent whereas SIV infection and replication in sooty mangabey smPBMCs is partially CCR5-independent. Averaging all experiments, Maraviroc blocked 93.5% (± 5.2% SD) of SIVmac 239 and SIVmac 251 peak viral production in rhesus macaque rmPBMCs. The CCR5-dependence in this study is consistent with that observed previously where high concentrations of CCR5 antagonist TAK779 blocked SIVmac 239 and SIVmac 251 infection and replication in rhesus macaque PBMCs from multiple donors (39). These data indicate that rmCCR5 is the primary coreceptor of these pathogenic SIVmac in these non-natural host primary cells. By contrast, Maraviroc blocked only 44% (± 14.8% SD) of primary sooty mangabey SIVsmm D215 and SIVsmm M935 viral production smPBMCs. Therefore, coreceptors other than smCCR5 support robust natural SIVsmm infection in these natural host primary cells.

The relative CCR5-dependence of SIV infection in rhesus macaque and sooty mangabey PBMCs *ex vivo* is dictated primarily by the primate host cells and not by the virus itself. Of note, the only virus-dependent factor that dictated coreceptor use in each species *in vitro* was the general ability to use CCR5/CXCR6/GPR15, and this ability was conserved between all viruses from the SIVsmm/SIVmac lineage we studied. Pathogenic rhesus macaque SIV were capable of being highly CCR5-independent. Maraviroc blocked only 17% (±15.7% SD) of SIVsmm E660 infection of smPBMCs and this inhibition did not reach statistical significance. Furthermore, the blocking effect of Maraviroc against SIVsmm E660 was significantly less than that against sooty mangabey viruses SIVsmm D215 and SIVsmm M935. Clearly, the SIVsmm E660 virus from rhesus macaques is capable of using sooty mangabey alternative coreceptors for infection and replication in smPBMCs. SIVmac 239, a virus also used to induce pathogenesis in rhesus macaques, showed a trend toward alternative coreceptor use in smPBMCs much like SIVsmm E660. Maraviroc blocked only 10% (range 4% to 15%) of SIVmac 239 infection and replication in smPBMCs, indicating that SIVmac 239 utilizes non-CCR5 alternative coreceptors for infection

and replication in smPBMCs. The CCR5-independent SIVmac 239 infection in smPBMCs is in stark contrast to CCR5-dependent SIVmac 239 infection rmPBMCs where Maraviroc blocked 92% (±6.4% standard deviation) of SIVmac 239 infection.

Host-dependent coreceptor use was also apparent from SIV Env pseudotype infections of CD4-plus coreceptor-transfected 293T cells *in vitro*. Sooty mangabey smCCR5, smCXCR6, and smGPR15 supported pseudotype infection mediated by SIVsmm Env and rhesus macaque SIVmac 239, SIVmac 251, and SIVsmm E660 Env. SIVsmm E660 and SIVmac 251 Env actually exhibited significantly more infection in cells expressing alterative coreceptor smCXCR6 than in cells expressing smCCR5. Rhesus macaque rmCXCR6 was a remarkably poor coreceptor of SIV relative to smCXCR6. The same panel of SIV Env pseudotypes utilized rmCCR5 and rmGPR15, but showed little infection of cells expressing rmCXCR6. Strikingly, SIVsmm E660 Env that had very robust infection through smCXCR6 did not utilize rmCXCR6 at all. For all Env pseudotypes, infection in cells expressing rmCD4 plus rmCXCR6 was significantly lower than infection in cells rmCD4 plus rmCCR5 as well as infection in cells expressing smCD4 plus smCXCR6. Sooty mangabey primary PBMCs likely express robust alternative coreceptor smCXCR6 whereas rhesus macaque primary PBMCs likely express weak rmCXCR6, which does not mediate robust SIV infection.

We identified sooty mangabey smCXCR6 as an alternative coreceptor of SIVsmm infection and replication in primary smPBMCs. Chemokine CXCL16 significantly blocked SIVsmm D215 viral production in smPBMCs relative to vehicle-treated controls, indicating that smCXCR6 supports SIVsmm D215 infection *ex vivo*. If observing SIV that use both smCCR5 and smCXCR6 efficiently, pre-treatment with Maraviroc alone or CXCL16 alone may have little effect because the virus can use the alternate uninhibited coreceptor. In such cases, the combination of Maraviroc and CXCL16 may be needed to generate an effect. Accordingly, when CXCL16 was used in combination with Maraviroc, SIVsmm E660 viral production was significantly lower than in

cells treated with Maraviroc alone. Like SIVsmm D215, SIVsmm E660 can use smCXCR6 to infect primary smPBMCs. The combination of Maraviroc and CXCL16 did not fully block SIV infection in smPBMCs, making it impossible to rule out alternative coreceptors in addition to smCXCR6. These experiments indicate that smCXCR6 supports SIVsmm D215 and SIVsmm E660 infection in addition to smCCR5, and perhaps in addition to other alternative coreceptors. No one has defined the expression of smCXCR6 in smPBMCs, so these experiments also secondarily confirmed, by virtue of smCXCR6-mediated infection, that smCXCR6 is expressed on the surface of smPBMCs.

The discovery that Maraviroc blocks a portion of SIVsmm infection and replication in smPBMCs was somewhat surprising. Maraviroc blocked an average of 44% (± 14.8% SD) of primary sooty mangabey SIVsmm D215 and SIVsmm M935 infection and replication in smPBMCs *ex vivo*. Previous experiments found that Maraviroc had no effect on SIVsmm infection and replication in smPBMCs (29). While contradictory, our findings are consistent with the observed relationship between SIVsmm CCR5 genotype and plasma viral loads *in vivo*. Sooty mangabeys with a $CCR5^{wt/wt.}$ genotype display higher viral loads (mean $\log_{10}$ 4.83 ± 0.10 SEM) than sooty mangabeys with a $CCR5^{\Delta/\Delta}$ genotype that lack functional CCR5 expression (mean $\log_{10}$ 4.65 ± 0.10 SEM) (29). Thus, sooty mangabeys lacking functional CCR5 have 34% lower plasma viral loads than animals with functional CCR5. This number is remarkably consistent with our observation here: Maraviroc blocked 44% of SIVsmm infection and replication in smPBMCs. Therefore, it is far from inconceivable that Maraviroc blocks some SIVsmm infection *ex vivo* or that smCCR5 supports some SIVsmm infection and replication in smPBMCs.

While, of the coreceptors tested, sooty mangabey smCXCR6 is the most robust alternative coreceptor of SIV Env pseudotypes in transfected cells *in vitro* (Chapter 2, Figure 4.1A, Figure 4.2A), it may not be the only alternative coreceptor of SIV in smPBMCs. Chemokine CXCL16 treatment had no significant effect on sooty mangabey SIVsmm M935 infection and replication in

smPBMCs either by itself or in combination with Maraviroc, indicating that SIVsmm M935 may rely on other alternative coreceptors for CCR5-independent infection and replication. In addition, neither SIVsmm D215 nor SIVsmm E660 were fully inhibited by the combination of CXCL16 and Maraviroc, again implying that additional alternative coreceptors could play a role in SIVsmm infection of smPBMCs. We were not able to pharmacologically block smGPR15-mediated infection in smPBMCs; there is no known small-molecule inhibitor or chemokine ligand for GPR15. Experiments directly addressing the role of smGPR15 are needed. Sooty mangabey smGPR15 supports SIV Env pseudotype infection *in vitro* and may support some SIV replication in smPBMCs *ex vivo*.

Some SIVsmm infection in the presence of CXCL16 is also likely due to incomplete inhibition of smCXCR6-mediated infection. Chemokine CXCL16 is not a complete inhibitor of smCXCR6-mediated infection and CXCL16 significantly lowers smCXCR6-mediated infection only when used in transfected cells expressing low levels of smCXCR6 (Chapter 3, Figure 3.5). The level of CXCR6 expressed in these transfected cells may approximate the level of CXCR6 expressed in primary $CD4^+$ T cells (Chapter 3, Figure 3.6), but we were not able to confirm that directly with smCXCR6 and sooty mangabey primary $CD4^+$ target cells. Despite significantly lowering smCXCR6-mediated SIV Env pseudotype infection in smCD4- plus smCXCR6 transfected cells relative to untreated controls *in vitro*, a significant amount of infection occurs above background negative controls (Chapter 3, Figure 3.5B). A more potent small-molecule inhibitor of CXCR6-mediated infection, sooty mangabey species-specific CXCL16, or an efficient way to knockdown smCXCR6 expression in PBMCs would be helpful in assigning the relative role of smCXCR6 and other alternative coreceptors in SIV infection of smPBMCs.

The relative inability of rmCXCR6 to support SIV infection *in vitro* and perhaps *ex vivo* can likely be attributed to amino acid 31 in the N-terminal domain of CXCR6 (26). Sooty mangabey smCXCR6 carries amino acid serine S31 whereas rhesus macaque rmCXCR6 carries arginine

R31. Importantly, reversion of rmCXCR6 R31 to S31 restores the function of rmCXCR6 *in vitro* (26), indicating that this residue alone could be responsible for the poor coreceptor activity of rmCXCR6. The rhesus macaque rmCXCR6 R31 is the only allele of rmCXCR6 identified to date. We sequenced rmCXCR6 from seven rhesus macaques, including Rm1 through Rm4 used in this study, and only found the R31 allele of rmCXCR6 (data not shown). Additionally, in a survey of 36 rhesus macaques at the Wisconsin National Primate Research Center, only the rmCXCR6 R31 allele was identified (38). We did not sequence smCXCR6 from all sooty mangabeys in this study, but to date no one has described allelic variation in smCXCR6 (6, 26). It is not known how the single amino acid substitution S31R between sooty mangabeys and rhesus macaques affects SIV Env binding, CXCR6 signaling, or other attributes necessary for SIV entry and infection. It is conceivable this S31R charge difference proximal to the first trans-membrane domain of CXCR6 may affect folding and membrane trafficking of the receptor. Prior studies found that transfected cells express AU1-tagged rmCXCR6 and human CXCR6 at similar levels, but perhaps the AU1 tag itself could stabilize surface expression. In humans, an E3Q substitution and charge change in the N-terminal domain of human CXCR6 results in intracellular accumulation of CXCR6 and defective trafficking due to the loss of negative charge (25).

Rhesus macaque rmGPR15 supported moderate SIVmac 239 and SIVmac 251 infection *in vitro*, but did not support obvious SIVmac 239 or SIVmac 251 infection in rmPBMCs *ex vivo,* as CCR5 antagonist Maraviroc blocked almost all SIVmac infection in PBMCs. Prior evidence suggests GPR15 may be essential to SIV replication *in vitro* in cells lines lacking CCR5 because SIVmac 239 and SIVmac 251 replicate well in human CEMx174 cells that express high levels of human GPR15 but do not express CCR5 (3, 17). It is not clear why SIVmac 239 and SIVmac 251 did not utilize rmGPR15 in Maraviroc-treated rmPBMCs, but Maraviroc alone blocked the vast majority of SIVmac 239 and SIVmac 251 peak infection as well as infection over a period up to 19 days in rmPBMCs. SIVmac 239 and SIVmac 251 did replicate at low levels in rmPBMCs from one animal starting at Day 6 post-infection, perhaps indicating some adaptation to use of rmGPR15 in

a single set of rmPBMCs. However, it is also possible the SIVmac adapted to use Maraviroc-bound receptor or that Maraviroc activity decayed over time in culture. Perhaps rmGPR15 is not expressed at high enough levels on peripheral $CD4^+$ target rmPBMCs to support significant SIV infection and replication, but a comprehensive study of rmGPR15 expression in rmPBMCs and $CD4^+$ cell subsets has not been done. Previous experiments demonstrate that SIVmac 239 that can not infect cells via rmGPR15 *in vitro* do infect and replicate to the same degree as wild-type SIVmac 239 in rhesus macaques *in vivo* (27). A prior study also found that SIVmac 239 does not infect $CD4^+GPR15^+$ human PBMCs, even in cells from a $CCR5^{\Delta/\Delta}$ donor (17). Together with our findings, it appears that rmGPR15 is not a robust coreceptor of SIVmac 239 and SIVmac 251 infection and replication in rmPBMCs.

We observed variation between individual animal hosts following infection of PBMCs. For example, Maraviroc had no effect on SIVsmm E660 infection in smPBMCs from FIa1 and FIz, whereas Maraviroc blocked 23% to 32% of SIVsmm E660 infection in smPBMCs from other sooty mangabeys. In smPBMCs from FIa1, neither Maraviroc nor CXCL16 had an effect on SIVsmm E660 or SIVmac 239 infection, whereas both Maraviroc and CXCL16 individually blocked some SIVsmm M935 and SIVsmm D215 infection. This variation could reflect differences in alternative coreceptor expression between sooty mangabey hosts. The variation in Maraviroc, CXCL16, and combined blocking between sooty mangabeys in this study was neither consistent nor significant (p=NS, ANOVA). The measure of P27 Gag production and accumulation over time may be one source of experimental variation. In the future, we may repeat experiments using an assay to measure the role of individual coreceptors closer to the point of SIV entry, or after a single round of viral replication, to control for confounding data that may arise by measuring combined viral production and accumulation/decay in supernatant over several days. We attempted to measure SIV Gag and SIV LTR strong-stop DNA in infected PBMCs using an existing qPCR protocol (unpublished), but the data were inconsistent and the protocol needs to be refined.

Future experiments of rmPBMCs infected with SIVsmm E660 and SIVsmm primary isolates will further demonstrate that SIV infection and replication in rhesus macaque rmPBMCs are highly CCR5-dependent, and demonstrate that SIVsmm/SIVmac alternative coreceptor use is host-dependent, not virus-dependent. In this experiment, rmPBMC infections were performed with clonal viruses whereas most smPBMC infections were performed with viral swarms. In order to address this, we also plan to infect rmPBMCs with SIVmac 251 swarm, and to infect smPBMCs with clonal Env chimeras of SIVsmm. Additional experiments are needed to measure SIVsmm infection and replication in $CCR5^{\Delta/\Delta}$ smPBMCs treated with CXCL16 to further define the contributions of smCXCR6 and other alternative coreceptors in smPBMCs.

In summary, we determined that SIVsmm infection is partially CCR5-independent and that alternative coreceptor smCXCR6 can support SIVsmm infection in smPBMCs from natural host sooty mangabeys. By contrast, SIVmac infection and replication in non-natural host rmPBMCs is highly dependent upon coreceptor rmCCR5. This substantiates SIVmac infection of rhesus macaques as a model of HIV-1 infection of humans because both infections are highly CCR5-restricted in their cognate hosts. However, the mechanisms underlying CCR5 restriction in rhesus macaques and humans are is quite different. CCR5-restriction in rhesus macaque SIVmac infection is host-dependent whereas CCR5-restriction in human HIV-1 infection is virus-dependent. Strikingly, these two different mechanisms leading to CCR5-restricted infection lead to similar disease outcomes including $CD4^+$ T cell loss and progression to AIDS in both rhesus macaque and human hosts. Notably, both of these hosts express high levels of CCR5 on $CD4^+$ T cells, suggesting that CCR5-restriction infection can occur easily.

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CHAPTER 5

DISCUSSION AND IMPLICATIONS

Nonhuman primate models greatly enhance our understanding of HIV-1 pathogenesis. Natural hosts of simian immunodeficiency virus (SIV) do not progress to AIDS following infection while more recent non-natural hosts of SIV do progress to AIDS. Understanding mechanisms that underlie this dichotomy between infection of natural and non-natural hosts may lead to a better understanding of mechanisms of disease progression and AIDS in HIV-1-infected patients. There are many features of natural host SIV infections that possibly underlie the lack of disease progression in these primates (see Chapter 1, Introduction, Table 1.1). These features include, but are not limited to, intact mucosal immunity (36, 65, 75), lack of chronic immune activation (63, 68, 84), tempered innate and adaptive immune responses in the chronic phase of infection (6, 24, 45), maintenance of primary lymphoid architecture (68), and sparing of critical cell subsets from direct infection (9, 15, 72).

Natural host sooty mangabeys (*Cercocebus atys*) and non-natural host rhesus macaques (*Macaca mulatta*) exhibit divergent responses to SIV infection. SIVsmm-infected sooty mangabeys exhibit high viral loads without widespread $CD4^+$ T cell loss or progression to AIDS. By contrast, SIV-infected rhesus macaques exhibit high viral loads with widespread $CD4^+$ T cell loss and rapid progression to AIDS. Strikingly, even sooty mangabeys and rhesus macaques concomitantly challenged with the same primary isolate of SIVsmm display these very different outcomes following infection (83). These data clearly indicate that host factors, not properties of the virus alone, mediate discrepant outcomes in sooty mangabeys and rhesus macaques.

In this thesis, I identified SIV alternative coreceptor use as a host-dependent factor that distinguishes natural host sooty mangabey SIV infection from non-natural host rhesus macaque SIV infection *ex vivo*. I discovered SIV infection of sooty mangabey peripheral blood mononuclear cells (smPBMCs) is partially CCR5-independent. Alternative, non-CCR5 coreceptor sooty mangabey CXCR6 (smCXCR6) supported robust SIV infection in transfected cells *in vitro* and also supported some SIV infection in primary PBMCs *ex vivo*. Other alternative coreceptors,

notably smGPR15, supported moderate SIV infection *in vitro* and may support SIV infection in sooty mangabey smPBMCs *ex vivo*. In stark contrast to SIV infection of sooty mangabey smPBMCs, I discovered that SIV infection of rhesus macaque PBMCs (rmPBMCs) was almost entirely CCR5-dependent. Rhesus macaque CXCR6 (rmCXCR6) was an exceedingly weak coreceptor of SIV in transfected cells *in vitro*. Rhesus macaque rmGPR15 supported SIV infection *in vitro*, but did not support robust SIV infection of rmPBMCs *ex vivo* as rmCCR5 was by far the dominant coreceptor in these cells.

The relative CCR5-dependence of SIV infection was host-dependent. SIV isolates from nonpathogenic infection of sooty mangabeys and pathogenic SIV from rhesus macaques exhibited similar patterns of CCR5 and alternative coreceptor use *in vitro*. Additionally, a pathogenic rhesus macaque virus, SIVmac 239, was CCR5-dependent in rmPBMCs but largely CCR5-independent in smPBMCs. Thus, SIV alternative coreceptor use in sooty mangabey infection is a host-determinant that distinguishes partially CCR5-independent sooty mangabey SIV infections from highly CCR5-dependent rhesus macaque SIV infections.

Conserved patterns of coreceptor use in other hosts of SIV

In this thesis, I examined SIV infection in a single natural host primate species and a single non-natural host primate species. Defining alternative coreceptor-mediated SIV infection as a feature distinguishing additional natural host species from CCR5-dependent SIV infection in additional non-natural host species will provide further evidence of CCR5-independent coreceptor use as a possible determinant of $CD4^+$ T cell homeostasis in the presence of high-level viral replication. We hypothesize that alternative coreceptor-mediated SIV infection is a conserved feature in natural hosts whereas CCR5-restricted SIV infection is a conserved feature in non-natural hosts and human hosts that progress to AIDS.

There is evidence of CCR5-independent and alternative coreceptor-dependent SIV infection in other natural hosts. African primate red-capped mangabeys (*Cercocebus torquatus*) are presumed not to progress to AIDS following endemic SIVrcm infection (5, 33). SIVrcm infected $CD4^+$ cell lines engineered to express human CCR2 or human CXCR6 but not human CCR5 *in vitro* (5, 16). After SIVrcm was passaged in macaque cells, SIVrcm gained the ability to infect $CD4^+$ cell lines expressing human CCR4 but still lacked the ability to infect $CD4^+$ cell lines expressing CCR5 (32). Similar experiments were not performed with coreceptors of red-capped mangabey origin and further studies of SIVrcm infection and replication in primary cells *ex vivo* are needed. However, these data suggest that SIVrcm infection in red-capped mangabeys may be entirely CCR5-independent and alternative coreceptor-dependent.

SIVagm infections of African green monkeys (*Chlorocebus*) also exhibit evidence of CCR5-independence and alternative coreceptor use. African green monkeys are clearly natural hosts of SIVagm and infection results in high viral loads without chronic immune activation, $CD4^+$ T cell loss, or progression to AIDS (44, 55, 63, 69, 70, 73). Prior experiments found that SIVagm infected $CD4^+$ transfected cell lines via alternative coreceptors CCR5, CXCR6, and GPR15 of human origin (35, 55, 56) and that Maraviroc blocked only a fraction of SIVagm infection in TZM-bl cells (27, 35). We recently observed that African green monkey sub-species sabaeus CXCR6 (agmCXCR6) was a robust alternative coreceptor of a sabaeus SIVagm.sab Env in transfected 293T cells (**Figure 5.1**, unpublished, data courtesy of K. Sheehan-Wetzel). Alternative coreceptors sabGPR15 and sabGPR1 also supported moderate to low SIVagm Env-mediated infection *in vitro*. Thus, the overall pattern of alternative coreceptor use by SIVagm appears similar to that of SIVsmm/SIVmac *in vitro.* Additional *in vitro* experiments with a diverse panel of primary sabaeus SIVagm Env are needed; unfortunately, many available SIVagm isolates were passaged extensively in human cell lines and may no longer resemble coreceptor use by wild SIVagm viruses (1, 35, 47). Further studies of SIVagm infection and replication in primary cells treated with coreceptor antagonists are also needed.

Human HIV-1 infection is clearly pathogenic. In the majority of untreated cases, HIV-1 leads to $CD4^+$ T cell loss and progress to AIDS marked by increased susceptibility to opportunistic infections. The HIV-1 virus itself is CCR5-tropic and, like SIVsmm/SIVmac infection of rhesus macaques, human HIV-1 infection is highly CCR5-dependent both at the point of infection and throughout early stages of infection. In a subset of cases, HIV-1 can acquire the use of CXCR4. However, CXCR4 use is clearly not required for pathogenesis and progression to AIDS.

SIVcpz-infected chimpanzees exhibit signs of pathogenesis including loss of $CD4^+$ T cells in the spleen and lymph node, as well as an increased death hazard relative to uninfected chimpanzees (48). Pathogenic SIVcpz infection of chimpanzees (*Pan troglodytes*) may also be CCR5-dependent. Preliminary data suggest SIVcpz Env display robust infection through chimpanzee CCR5 and little to no infection through CXCR6, GPR15, or other putative coreceptors *in vitro* (K. Sheehan-Wetzel, unpublished, data not shown). Further experiments will be needed to fully define SIVcpz coreceptor utilization *in vitro* and *ex vivo*. SIVcpz is of special interest because the two SIV from which it arose are presumed to be non-pathogenic in their respective African hosts (3, 19, 33), whereas transmission of SIVcpz to humans led to the pathogenic HIV-1 Group M pandemic (80) (see Chapter 1, Introduction, Figure 1.1). It not known if African greater spot-nosed monkey (*Cercopithecus nictitans*) SIVgsn Env, the precursor to SIVcpz Env, use CCR5 or alternative coreceptors for infection and replication *in vitro* or *ex vivo*. We hypothesize that SIVgsn infection is at least partially CCR5-independent. Further evidence that greater spot-nosed monkeys do not progress to AIDS as well as identification of SIVgsn alternative coreceptors may define a bottleneck leading to CCR5-tropism, CCR5-dependence, and pathogenesis during transmission of SIVgsn to chimpanzees.

It is intriguing that multiple convergent mechanisms may lead to CCR5-dependence in non-natural hosts and CCR5-independence in natural hosts of SIV and HIV-1. Notably, the

mechanisms leading to highly CCR5-dependent human HIV-1 infection and highly CCR5-dependent rhesus macaque SIV infection are very different. Whereas host factors dictate CCR5-dependence of multi-tropic SIV in rhesus macaques, the CCR5-tropic HIV-1 virus itself dictates CCR5-dependence in humans. Similarly, divergent mechanisms may lead to CCR5-independence in hosts that do not progress to AIDS. SIVrcm CCR5-independence is likely mediated by the SIVrcm virus, which appears not use CCR5 at all, whereas SIVsmm and SIVagm CCR5-independence are a function of the multi-tropic SIV (26, 35, 79), the exceedingly low expression of CCR5 on $CD4^+$ T cells (72, 74), and the expression of functional CXCR6 in sooty mangabeys and African green monkeys.

Lack of CXCR6-mediated SIV infection may be a common feature in non-natural host primates

The relative CCR5-dependence of SIV infection of other non-natural hosts has not been studied. I found that rhesus macaque rmCXCR6 was a very poor coreceptor of SIV *in vitro* relative to rmCCR5 (as had been shown previously (77)) and relative to smCXCR6. A lack of rmCXCR6-mediated infection in rmPBMCs could explain why SIV infection and replication are almost entirely rmCCR5-dependent in rmPBMCs. The poor SIV coreceptor activity of rmCXCR6 relative to human CXCR6 *in vitro* was previously attributed to a S31R amino acid substitution in the N-terminal domain of rmCXCR6 (**Figure 5.2**) (77). Human CXCR6 and sooty mangabey CXCR6 carry S31 and both support robust CXCR6-tropic SIV infection *in vitro*. Rhesus macaque CXCR6 carries R31 and supports negligible SIV infection *in vitro* (77) (Chapter 4). Importantly, previous studies observed that the reversion of R31 to S31 in rhesus macaque CXCR6 restored its robust function as a coreceptor of SIV *in vitro*, indicating that this residue is solely responsible for the poor coreceptor activity of wild-type rmCXCR6 (77). We can infer some attributes of SIVsmm/SIVmac infection of other non-natural host primate models of AIDS based on CXCR6 alleles.

Asian pig-tailed macaques (*Macaca nemestrina*) exhibit high levels of baseline immune activation and rapidly succumb to AIDS following infection with SIV isolated from natural hosts (4, 32, 43). SIVmac 239 infection of pig-tailed macaques also leads to $CD4^+$ T cell loss and exceedingly rapid progression to AIDS (53). Asian cynomolgus macaques (*Macaca fascicularis*) also succumb to AIDS following infection with viruses from the SIVsmm/SIVmac lineage (58, 89). The role of CCR5 in pig-tailed macaque and cynomolgus macaque SIV infection has not been defined. However, like rhesus macaques, both pig-tailed macaques and cynomolgus macaques carry the R31 amino acid in CXCR6 and it is likely that both pig-tailed macaque CXCR6 and cynomolgus macaque CXCR6 are poor coreceptors of SIV from the SIVmac/SIVsmm lineage. Further studies are needed to define the allelic frequency leading to the R31 CXCR6 protein, the prevalence of CXCR6 genotypes, and CXCR6 function in a large number of non-natural host primates that exhibit $CD4^+$ T cell loss and progression to AIDS.

The conserved nature of amino acid R31 between non-natural host experimental primate models of pathogenic infection is intriguing. Notably, the functional S31 CXCR6 is conserved between natural host sooty mangabeys, natural host African green monkeys, and presumed natural host red-capped mangabeys. This pattern in CXCR6 amino acid sequences provides further evidence that alternative coreceptor CXCR6 is an important host factor that may dictate differential outcomes of infection in natural and non-natural hosts.

Model: Alternative coreceptor expression limited to expendable cells maintains SIV replication without leading to $CD4^+$ T cell loss in natural hosts

Importantly, not all $CD4^+$ T cell loss in pathogenic HIV-1 and SIV infection is due to direct viral infection and cytotoxicity (2, 29, 37, 38, 85). Direct infection of $CD4^+$ T cell subsets critical to maintaining immune homeostasis is one likely mechanism of chronic immune activation, leading to bystander $CD4^+$ T cell exhaustion and precipitous widespread $CD4^+$ T cell loss. In this model, critical $CD4^+$ T cell subsets are defined as permissive cells that maintain healthy immune

homeostasis by replenishing $CD4^+$ T cells lost to infection and chronic immune activation. Critical cells can also be defined as cells that maintain healthy immune tissue architecture. Expendable $CD4^+$ T cell subsets are defined as permissive terminally differentiated cells that are frequently replaced by proliferation of critical $CD4^+$ T cells. Both the target cells spared from infection and those supporting replication are critical to understanding the nature of infection and pathogenesis (39) (**Figure 5.3**).

Non-natural hosts of SIV and human hosts of HIV-1 express high levels of CCR5 on $CD4^+$ T cells (74). CCR5 is expressed at high levels on critical and expendable $CD4^+$ T cell subsets in non-natural hosts and in human hosts that progress to AIDS. In this model, CCR5-dependent SIV and CCR5-dependent HIV-1 infection occurs in both critical and expendable $CD4^+$ T cell subsets. Cells lost to direct infection and chronic immune activation would not be replenished due to the loss of critical replenishing cells. Healthy immune architecture would be lost, precipitating chronic immune activation, widespread CD4+ T cell loss and disease progression.

In contrast to non-natural hosts and humans, natural hosts exhibit broadly low levels of CCR5 on $CD4^+$ T cells (74). Alternative coreceptors of SIV likely mediate the bulk of viral replication in this environment of limited CCR5 expression. In this model, alternative coreceptors, such as CXCR6, are expressed at high levels on expendable $CD4^+$ T cell subsets while they are expressed at low to negligible levels on critical $CD4^+$ T cell subsets. SIV infection of expendable $CD4^+$ T cell subsets maintains high levels of viral replication made evident by the high plasma viral loads in natural hosts. In parallel, critical $CD4^+$ T cell subsets protected from direct SIV infection replenish expendable $CD4^+$ T cells lost to infection. Thus, SIV-infected natural hosts exhibit healthy immune architecture and immune homeostasis without widespread loss of $CD4^+$ T cells despite high plasma viral loads.

Many subsets of cells fit this model. Effector $CD4^+$ T cells and effector memory (T_{em}) $CD4^+$ T cells are a front line of defense against HIV and SIV infection. These cells are clearly essential to the immune response to SIV as some of the most successful vaccines against SIVmac 239 and SIVmac 251 in rhesus macaques specifically elicit potent CD4+ T_{em} cell responses (41, 42). These cells are also early targets of HIV-1 and SIV infection in both natural and non-natural hosts; gastrointestinal $CD4^+$ T_{em} are rapidly depleted in the acute phase of infection (7). Effector $CD4^+$ T cells and $CD4^+$ T_{em} cells are expendable in the sense that they are terminally differentiated and may be replenished by $CD4^+$ central memory T cells (T_{cm}) (39, 40, 71). $CD4^+$ effector T cells and $CD4^+$ T_{em} cells from humans, rhesus macaques, and sooty mangabeys are infected *in vivo* (72, 87). Importantly, sooty mangabey CD4+ T_{em} cells and rhesus macaque $CD4^+$ T_{em} cells are infected at similar levels, despite low CCR5 expression on sooty mangabey $CD4^+$ T_{em} cells (72). This likely indicates that high levels of SIV alternative coreceptor expression support high levels of SIV infection in sooty mangabey $CD4^+$ T_{em} cells.

Central memory $CD4^+$ T_{cm} cells are antigen-experienced cells found in the blood and lymphoid tissues that may replenish $CD4^+$ T_{em} and effector $CD4^+$ T cells lost to infection (39, 40, 71). These cells are critical to immune homeostasis because they maintain effector cell functions in the face of rampant viral replication and cell death (66). Preserved $CD4^+$ T_{cm} cells were associated with survival in vaccine challenge studies in macaques (57). Human $CD4^+$ T_{cm} cells harbor high levels of HIV-1 (8, 18). The rate of $CD4^+$ T_{cm} decline is 20-fold greater in SIV-infected rhesus macaques than in SIV-infected sooty mangabeys (67) and rhesus macaque $CD4^+$ T_{cm} harbor significantly more SIV viral DNA than sooty mangabey $CD4^+$ T_{cm} *in vivo* (72). Accordingly, rhesus macaque $CD4^+$ T_{cm} cells express high levels of CCR5 whereas sooty mangabey $CD4^+$ T_{cm} cells express exceedingly low levels of CCR5. Given the observed protection of sooty mangabey $CD4^+$ T_{cm} cells, we hypothesize that SIV alternative coreceptor expression is negligible in sooty mangabey $CD4^+$ T_{cm} cells.

Central memory stem $CD4^+$ T cells (T_{scm}) are a more recently defined subset of long-lived cells in blood and secondary lymphoid tissues that are uniquely able to self-renew as well as differentiate into $CD4^+$ T_{cm}, T_{em}, and other memory T cell subsets (31, 61, 62), making these cells critical to immune homeostasis. HIV-1 DNA is found in $CD4^+$ T_{scm} cells from infected patients both on and off therapy (13). HIV-1-infected patients that maintain normal $CD4^+$ T cell counts despite persistently high viremia (i.e. viremic non-progressors), exhibit low levels of $CD4^+$ T_{scm} cell infection relative to patients that eventually progress to $CD4^+$ T cell loss (52). SIV-infected rhesus macaques display high levels of direct infection of $CD4^+CCR5^+$ T_{scm} cells whereas the majority of SIV-infected sooty mangabeys have no detectable infection in the $CD4^+$ T_{scm} subset (14). We hypothesize that SIV alternative coreceptor expression is negligible in sooty mangabey $CD4^+$ T_{cm} cells.

Critical T-helper 17 (Th17) $CD4^+$ T cells maintain immune homeostasis, support gastrointestinal mucosa-associated lymphoid tissue (GALT), and stabilize the gastrointestinal epithelial barrier to microbial translocation. Th17 cells are rapidly depleted in human HIV-1 and rhesus macaque SIVmac 239 infections whereas Th17 cells are not lost in sooty mangabey SIVsmm infection (9, 15). Human peripheral blood Th17 cells express CCR5 and CXCR6 (60). We hypothesize that SIV alternative coreceptor expression on sooty mangabey Th17 cells is negligible.

CD4+ T follicular helper cells (Tfh) reside in the germinal center of lymph nodes. They provide T cell help for adaptive B cells responses and maintain lymph node architecture. Lymph node pathology is observed in non-natural hosts but not in natural hosts of SIV (10, 20, 84). Notably, germinal center Tfh cells are infected at higher frequencies in non-natural host macaques and humans than in sooty mangabeys (11, 64, 90). We hypothesize that SIV alternative coreceptor expression is low in sooty mangabey Tfh cells.

Currently, little is known about the expression of CXCR6 or GPR15 in primary natural host $CD4^+$ T cell subsets. The limited data we have comes from prior expression studies in human cells. There currently is no antibody to primate CXCR6 molecules, and, therefore, we did not assay CXCR6 expression in sooty mangabey immune subsets. Quantitative real-time PCR revealed that CXCR6 mRNA was expressed at similar levels in sooty mangabey $CD4^+$ PBMCs and rhesus macaque $CD4^+$ PBMCs. Expression levels generally increased in $CD4^+$ PBMCs from both species following stimulation with PHA and IL-2 (unpublished, data not shown) (78). We are in search of an antibody to define alternative coreceptor CXCR6 expression in sooty mangabey $CD4^+$ T cells relative to CCR5 expression in rhesus macaque $CD4^+$ T cells.

In humans, CXCR6 is found on $CD4^+$ effector T cells and on subsets of $CD4^+$ T helper cells. CXCR6 is generally associated with inflamed tissue sites, consistent with our hypothesis that sooty mangabey effector cells express high levels alternative coreceptor. CXCR6 is generally not co-expressed with T_{cm} cell markers such as CCR7 or L-selectin in human PBMCs (34, 51, 54, 60, 81, 86). GPR15 is expressed in human $CD4^+$ T cells from blood and lymphoid tissues (21, 25, 28, 46, 59) and has been found at variable levels on peripheral human $CD4^+$ T_{cm} and T_{em} cells (49, 50). Notably, CXCR6 and GPR15 are both expressed in human intestinal tissue (21, 59), an important tissue effector site of viral replication in both natural and non-natural hosts.

Future studies are needed to define the expression of CXCR6 and other alternative coreceptors on sooty mangabey $CD4^+$ cell subsets. These studies will ideally focus on $CD4^+$ T cell subsets from tissue sites as well as peripheral blood. Various cellular activation protocols *ex vivo* can result in unique coreceptor expression, as has been observed with CXCR6, and must be considered in future studies (54). It is also important to determine if natural host $CD4^+$ cells that express alternative coreceptors are infected *in vivo*. Notably, a recent study discovered that even abortive infections of $CD4^+$ T cells may lead to pyroptosis, a highly inflammatory form of cell death that could contribute to overall immune activation (23).

Alternative coreceptors and acute viral reservoir formation

Patients on long-term antiretroviral therapy (ART) with undetectable plasma viral loads typically experience viral recrudescence only weeks after therapy is terminated, indicating that long-lasting reservoirs of HIV-1 persist in the absence of easily detectable viral replication. Persistent reservoirs of HIV-1 infection are formed very early in the acute phase of infection. In 2013, there was hope that very early ART treatment would "cure" HIV-1-infected infants; an infant received ART within hours of HIV-1 exposure and remained free of plasma HIV-1 over two years after therapy was stopped (76). Unfortunately, this patient and other infants treated similarly have since experienced viral rebound following ART cessation (12, 82), indicating that persistent reservoirs of HIV-1 are formed very early following exposure. Viral rebound also occurs in non-natural host rhesus macaques receiving ART during the eclipse phase of SIV infection. SIVmac 251-infected rhesus macaques were treated with ART three days post-infection, before viral RNA or proviral DNA were detected in the blood. Viral rebound occurred in these animals shortly after ART cessation, demonstrating that a persistent SIV reservoir was seeded rapidly in these non-natural hosts (88).

The field lacks full understanding of mechanisms supporting acute viral reservoir formation. Efforts are needed to identify specific $CD4^+$ T cell subsets that mediate persistent reservoir formation. Resting memory $CD4^+$ T cells harbor latent virus in patients on therapy (30). Some studies propose that $CD4^+$ T_{cm} may represent one of the largest reservoirs of HIV-1 infected cells in patients treated with ART (17). Intriguingly, in the study of SIVmac 251-infected rhesus macaques receiving ART three days post-infection, proviral DNA was primarily found in $CD4^+$ T_{cm} and $CD4^+$ T transitional memory cells in the three days before ART was initiated. Therefore, infection of these specific $CD4^+$ T cell subsets may drive persistent reservoir formation and result in viral recrudescence when therapy is terminated.

Because $CD4^+$ T_{cm} from natural host sooty mangabeys are protected from direct SIV infection relative to $CD4^+$ T_{cm} from non-natural host rhesus macaques (67, 72), future studies are warranted to determine if persistent viral reservoir formation is slower in natural hosts than in non-natural host rhesus macaques and humans. (Of note, it would be difficult to perform such an experiment in sooty mangabeys because regulations forbid SIV challenge studies in these protected animals.) The exceedingly low CCR5 expression on sooty mangabey $CD4^+$ T_{cm} is associated with low SIV infection in this subset (72), and, accordingly, we hypothesize that low alternative coreceptor expression in $CD4^+$ T_{cm} of natural hosts is also generally associated with protection. If persistent reservoir formation occurs slowly in natural hosts, the expression patterns of SIV coreceptors in natural and non-natural host $CD4^+$ cells may help identify cell subsets that mediate rapid and persistent viral reservoir formation.

In summary, I determined that non-natural host rhesus macaque SIV infection is highly CCR5-dependent whereas natural host sooty mangabey SIV infection is partially CCR5-independent and can be mediated by alterative coreceptors, including sooty mangabey CXCR6. The discovery that SIVmac 239 and SIVmac 251 infections in rhesus macaque PBMCs are highly CCR5-dependent substantiates the rhesus macaque as a model of CCR5-tropic HIV-1 infection in humans. Because CCR5 expression is ubiquitously low on the surface of natural host $CD4^+$ T cells, alternative coreceptors likely support SIVsmm infection and replication *in vivo*. Defining the expression of CXCR6 and other alternative coreceptors on the surface of $CD4^+$ T cell subsets from sooty mangabeys may add to our current understanding of critical and expendable $CD4^+$ T cell subsets, and could possibly identify novel $CD4^+$ T cell subsets capable of maintaining viral replication without leading to widespread $CD4^+$ T cell loss in natural hosts of SIV. Further studies of acute natural host SIV infection and alternative coreceptor-mediated $CD4^+$ T cell subset tropism in natural hosts may also elucidate mechanisms of persistent viral reservoir formation.

FIGURES

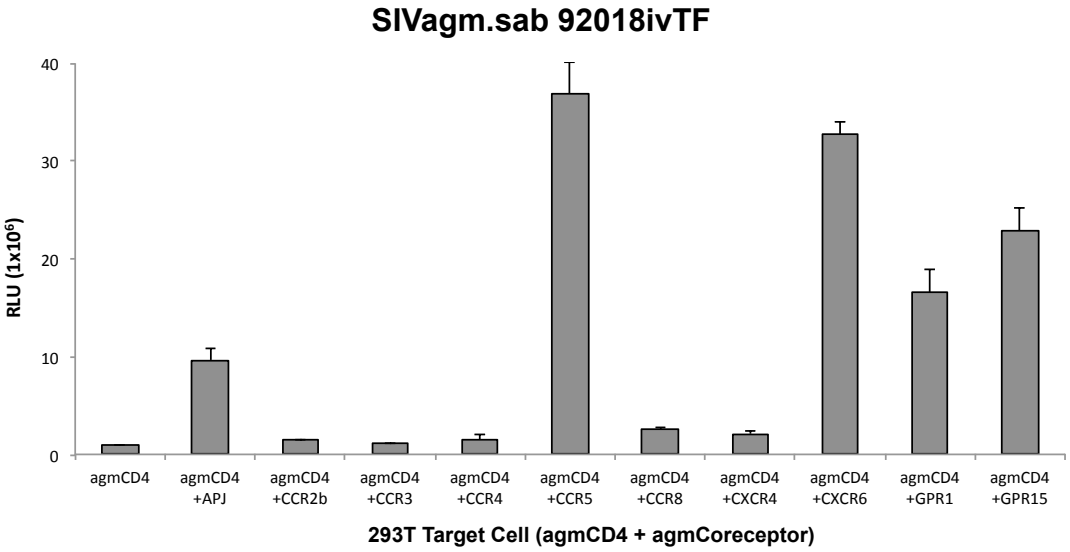

Figure 5.1. African green monkey alternative coreceptors support SIVagm infection *in vitro*. African green monkey sabaeus SIVagm.sab 92018ivTF Env was cloned from an infectious molecular clone of SIVagm.sab 92018ivTF derived by others following passage of SIVagm.sab in sabeaus animals (22, 35). Luciferase-expressing pNL-Luc-$E^{-}R^{+}$ Env pseudotypes were used to infect target 293T cells transfected with sabaeus agmCD4 and sabaeus putative coreceptors. Infection was measured three days post-infection by luciferase expression in relative light units (RLU). (N=3, error bars represent standard deviation.) *Data courtesy of Katie Sheehan-Wetzel, Collman Laboratory, University of Pennsylvania.*

Figure 5.2. Amino acid alignment of primate CXCR6 N-terminal domains. The amino acid sequences of CXCR6 N-termini from natural host African green monkey and sooty mangabey are aligned to those from non-natural host rhesus macaque, pig-tailed macaque, and cynomolgus macaque. The arrow highlights the S31/R31 in natural hosts and non-natural hosts. Human CXCR6 is included for comparison; however, note that HIV-1 does not use CXCR6 as a coreceptor. (*, conserved residue between all species.)

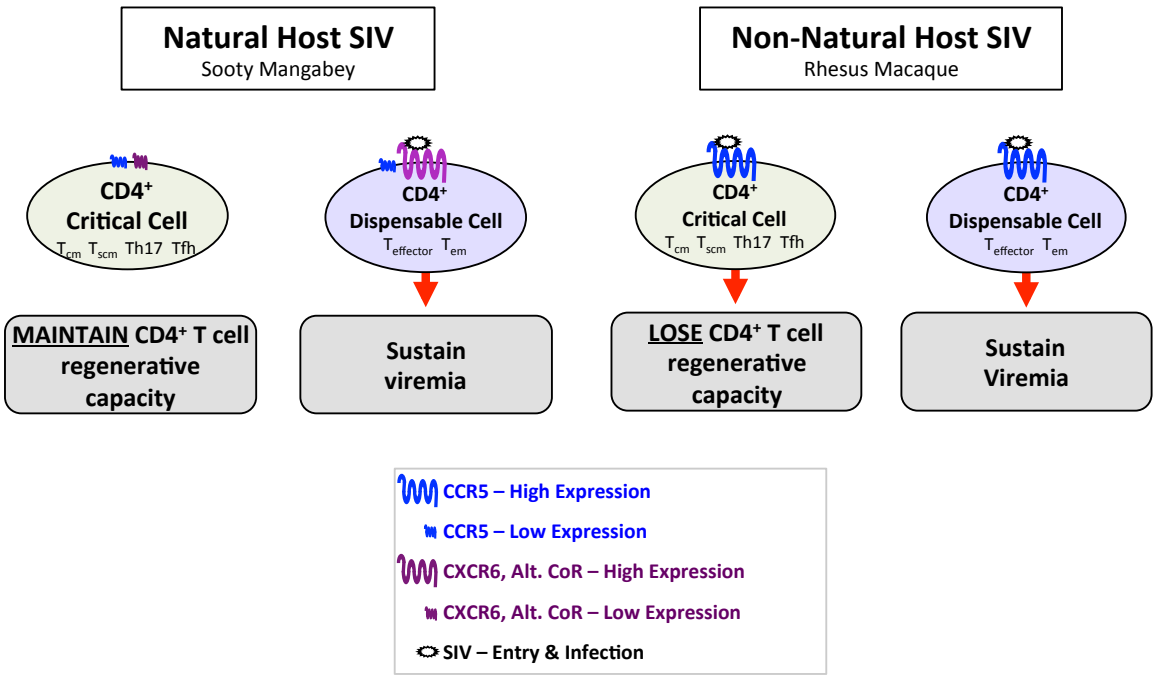

Figure 5.3. Model of SIV cellular tropism and outcomes in natural and non-natural hosts. A hypothetical model of SIV tropism in natural (left) and non-natural (right) primate hosts and subsequent impacts on disease progression. Natural host SIV infection, including SIVsmm infection in sooty mangabeys, occurs primarily in dispensable $CD4^+$ cell subsets leading to sustained viremia without loss of immune homeostasis. By contrast, non-natural host SIV infection, including SIV infection in rhesus macaques, occurs in both critical and dispensable cell subsets leading to sustained viremia with loss of immune homeostasis. T_{cm}, T central memory cells; T_{scm}, T stem central memory cells; Th17, T-helper 17 cells; Tfh, T follicular helper cells; T_{em}, T effector memory cells; Alt. CoR, other alternative coreceptors such as GPR15.

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