WIDESPREAD USE OF CXCR6 FOR ENTRY BY NATURAL HOST SIVS: IMPLICATIONS FOR CELL TARGETING AND INFECTION OUTCOME

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Dedication

To my parents, Bruce and Peggy Sheehan, and my husband, Chris Wetzel, who have always supported my pursuit of learning.

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ABSTRACT

WIDESPREAD USE OF CXCR6 FOR ENTRY BY NATURAL HOST SIVS: IMPLICATIONS FOR CELL TARGETING AND INFECTION OUTCOME

Katherine S. Wetzel

Ronald G. Collman, M.D.

Natural hosts of simian immunodeficiency virus (SIV) are African primates that have coevolved with species-species SIVs and do not progress to AIDS despite high viral loads. This is in stark contrast to the immunodeficiency observed in infection of "non-natural" hosts of SIV/HIV, Asian macaques and humans. Certain critical CD4+ T cell subsets and anatomic niches that are required for maintaining immune system homeostasis and function are infected less frequently in natural hosts than in non-natural hosts, suggesting that the determinants of virus target cells contribute to the outcome of infection. SIV and HIV target cells are largely defined by the expression of the receptor CD4 and a coreceptor. Our lab recently discovered that the entry coreceptor CCR5 is dispensable for SIV infection of the natural host sooty mangabey (SM), and then identified CXCR6 as an additional coreceptor for this SIV. In this thesis, I defined entry coreceptors of a second natural host virus, SIVagmSab that infects sabaeus African green monkeys and found that CXCR6 was a robust coreceptor for this virus as well. I also investigated coreceptor use by the HIV-1 forerunners: the natural host virus SIVmus that infects mustached monkeys and crossed into chimpanzees; and SIVcpz that infects chimpanzees and causes AIDS-like disease and crossed into humans to found HIV-1. SIVmus infected cells expressing CXCR6 and CCR5, while SIVcpz was restricted to use of CCR5, indicating that loss of CXCR6 use coincided with the emergence of pathogenesis in this lineage. Lastly, I defined expression of CXCR6 on SM lymphocytes, and found little or no CXCR6 expression on CD4+ T cell subsets that are critical in lymphocyte homeostasis, but enrichment on replenishable effector memory CD4+ T cells. CXCR6+ CD4+ T cells were largely distinct from CCR5+ CD4+ T cells, thus forming a previously unappreciated SIV target cell population in SM. These data support a model where use of CXCR6 is a common feature among natural host SIVs that targets the virus towards more expendable cell subsets, and away from critical subsets and anatomic niches that are required to maintain immune system function, thus permitting high viral replication without immunodeficiency.

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CHAPTER 1

Introduction

Introduction to HIV-1 and AIDS

Nearly forty years after its emergence, Acquired Immune Deficiency Syndrome (AIDS) remains a major public health threat, causing the death of over 1 million people in 2015 worldwide (1). AIDS is marked by the deterioration of the immune system, defined by the occurrence of opportunistic infections due to this immunodeficiency and CD4+ T cell loss. Human immunodeficiency virus type 1 (HIV-1) was identified in 1983 (2-4) as the etiologic agent that causes the majority of AIDS cases, while HIV type 2 (HIV-2), a related virus largely confined to West Africa, accounts for few cases (5). In 2015, the most recent year for which data are available, 36.7 million people worldwide were living with HIV-1, and 2.1 million people were newly infected (6). The United States population accounts for 1.2 million infected patients (as of 2013) and an estimated 37,600 new HIV-1 infections (as of 2014) (7).

HIV is transmitted to a new host when a bodily fluid containing the virus (blood, semen, vaginal secretions, or breast milk) comes into contact with a mucosal surface of the genital or gastrointestinal tract, or via direct contact to blood by intravenous drug use or needle stick (8). Most new infections are established by a single virion, termed transmitted-founder viruses (9-13). While early post-transmission events are hard to study in detail, it is known that the virus must infect a cell expressing the virus receptor and a coreceptor at or near the mucosal surface, which is followed by focal, then systemic, virus replication. The virus life cycle requires integration into the host genome; as such, an infected person remains infected for their lifetime. After up to one to two weeks of undetectable viral replication, acute HIV-1 infection presents with flu-like symptoms, and virus titers expand to 10^7 copies or more of viral RNA per milliliter of blood (Figure 1.1). After several more weeks, viral load declines roughly 100-fold with the emergence of the adaptive immune response to the virus and depletion of virus target cells, and reaches a set point. This acute infection is followed by a chronic phase that can last several years or decades, where viral loads are steady or slowly rising, and the immune system remains largely intact. Eventually,

patients who are not treated with antiviral drugs will suffer a loss of immune system function marked by reduced CD4+ T cell counts that results in the emergence of opportunistic infections, thus marking the development of AIDS (Reviewed in (14)).

HIV/SIV genome and replication

HIV is a member of the retrovirus family, as it is enveloped and encodes a single stranded RNA genome that replicates through a DNA intermediate. It is closely related to simian immunodeficiency virus (SIV), a family of species-specific viruses that infect nonhuman primates (NHP). Both HIV and SIV are more specifically termed lentiviruses due to the long incubation periods in their hosts. The main genome structure of HIV and SIV are typical of retroviruses in that they encode *gag*, *pol* and *env* genes, flanked by long-terminal repeats (LTR) (Figure 1.1). *gag* gene products include the structural components of the virion, such as the capsid and matrix proteins, while *pol* encodes the virus enzymes: integrase, protease and reverse transcriptase. The *env* gene encodes the virus glycoprotein that interacts with cell surface proteins to permit entry. In addition to the three main genes, HIV and SIV encode *tat*, which promotes transcription from the LTR, and *rev*, which is required for export of unspliced and partially spliced viral RNAs from the nucleus (15, 16). Finally, the HIV and SIV genomes contain a variety of accessory genes that encode for proteins that promote infectivity within host cells, largely by antagonizing host restriction factors including APOBEC3G, TRIM5a, SAMHD-1 and tetherin. The specific genes contained in each genome and roles thereof vary between different HIVs and SIVs, yielding the three virus groups shown in Figure 1.2. All HIV and SIVs encode *nef*, *vif* and *vpr*, although these proteins can have different functions between different lineages of SIV (Reviewed in (17)). In addition, HIV and its relatives also encode *vpu*, while HIV-2 and its relatives encode *vpx* (Figure 1.2). Some SIV genomes contain neither of these genes.

HIV and SIV enter cells by first binding to a receptor, CD4, using the virus surface glycoprotein Env. On the virion, Env forms a trimer of heterodimers made up of gp120, the surface unit that

consists of 5 conserved regions and 5 variable loops, and gp41, the fusion peptide. CD4 binding changes the conformation of Env and reveals the coreceptor binding site. Env then binds the coreceptor, which is a seven transmembrane G protein coupled receptor (7TMR) such as the chemokine receptor CCR5, to initiate further conformational changes that promote fusion of the viral and cellular membranes to deliver the viral genome (Figure 1.3) (18). Virus target cells that express both CD4 and a 7TMR coreceptor include CD4+ T cells and macrophages of the immune system.

Post-fusion, the reverse transcription of the RNA genome to DNA by the virus reverse transcriptase begins, and the capsid disassembles (likely simultaneously, although the precise order of events is debated in the field). The DNA enters the nucleus as part of the preintegration complex, and integrates preferentially into actively transcribed genes using the virus integrase. Virus replication is initiated from the LTR, and RNAs for host proteins as well as the genome for nascent virions are transcribed. HIV/SIV assembles at the plasma membrane, and budding occurs. Once the virus buds, the virus protease cleaves the immature structural proteins to give rise to the mature virion (15, 16).

Pathogenesis of HIV-1 infection and the animal model infection SIVmac

Productive HIV-1 infection results in death of the target CD4+ cell (19-21). However, viral load is an incomplete predictor of the severity of disease course, suggesting that immunodeficiency is not solely due to direct virus cytopathic effect (22). Instead, a stronger correlate of disease progression and CD4+ T cell loss is the frequency of activated CD8+ T cells, as measured by expression of the surface markers CD38 and HLA-DR (22-24). Other markers of immune activation associated with disease progression include elevated CD4+ and CD8+ T cell replication, increased macrophage activation (as measured by sCD14) and elevated inflammatory plasma markers such as IL-10, among others (25-27). Thus, it is now widely thought that chronic activation of the immune system that follows HIV-1 infection largely drives CD4+ T cell loss and

immunodeficiency. Hypothesized mechanisms of this immune activation include loss of CD4+ T cell homeostasis due to constant need to regenerate lost CD4+ T cells, and disruption of structure and immune function of the lymph node and gut barrier, the latter of which results in translocation of microbial products into circulation (27). However, many studies investigating these mechanisms are correlative, and the relative contribution of these mechanisms, in addition to others, remains unclear.

The complexity of HIV-1 pathogenicity is evidenced by observation of HIV-1 infected patients on combination antiretroviral therapy (cART). The introduction of cART in 1996 drastically decreased the morbidity and mortality for patients who had access and adhere to their daily regimens, with nearly 50% reduction in AIDS-related deaths in the first several years following cART introduction in the United States and other developed countries (28). However, those who maintain undetectable viral loads can still experience enduring immune-mediated complications due to residual immune activation, such as organ failure or HIV-associated neurocognitive disorders, despite undetectable viral replication (29-32). In order to address such complications therapeutically, it will be necessary to completely define the drivers of immune activation and HIV-1 pathogenesis.

A valuable tool for studying HIV pathogenesis and treatment strategies has been experimental infection of rhesus, cynomolgus and pigtail macaques with SIVmac. SIVmac infection recapitulates many features of HIV-1 infection described above, such as CD4+ T cell loss and chronic immune activation, although immunodeficiency arises at a faster rate than what is observed in HIV-1 infection of humans (33, 34). This model has allowed interrogation of early infection events, which is rarely possible in HIV-1 infection, as well as a platform for testing vaccines and other therapies.

SIV+ African monkeys do not progress to AIDS

However, the pathogenic phenotype of SIVmac infection of rhesus macaques (RM, *Macaca mulatta)*, which only occurs in experimental settings, is an exception in regard to SIV infections. Most simian immunodeficiency viruses (SIVs) belong to a diverse, ancient family of viruses that naturally infect African, but not Asian or New World, nonhuman primates (NHP). Of the 70 known African NHP species, 45 species (representing ~90% of those tested thus far) have been identified as infected with a species-specific SIV (35). These species are termed "natural hosts" of SIV, as they do not progress to AIDS, in contrast to "non-natural hosts" such as HIV-infected humans and SIVmac-infected macaques. Of the natural hosts, the sooty mangabey (SM, *Cercocebus atys*) infected with SIVsmm and the African green monkey (AGM, *Chlorocebus spp*.), infected with SIVagm are the best studied as they are housed in primate research centers in the United States and abroad. Additionally, AGM of the subtype sabaeus have been surveyed in the wild, with results supporting asymptomatic infection (36, 37). This lack of pathogenesis is thought to be the product of at least hundreds of thousands of years of coevolution between virus and host (36, 38). A fundamental goal of the greater HIV field is to understand how such monkeys are able to maintain immune system function despite viral replication, and, in addition, how those features differ in non-natural host infections, which would inform mechanisms of HIV-1 pathogenesis.

Natural host SIV origin of HIV-1 and SIVmac

Although natural host viruses are nonpathogenic in their respective hosts, cross-species transmission of these viruses into new hosts originated HIV and SIVmac infection (Figure 1.4A). HIV-1 Group M, the group responsible for the AIDS pandemic, is the result of a cross-species transmission event where SIVcpz crossed the species barrier into humans from chimpanzees (39). SIVcpz, which is pathogenic in its host (40), was shown by phylogenetic analyses to be the product of a recombination event between two viruses that crossed the species barrier into chimpanzees: SIVrcm and an SIV of the lineage SIVgsn/mus/mon (41). As shown in Figure 1.4B,

the 5' half of the virus that encodes *gag* and *pol* originates from SIVrcm that infects red-capped mangabeys (RCM, *Cercocebus torquatus*), while the 3' half that encodes *env* originates from a virus of the lineage SIVgsn/mus/mon, which infects greater spot-nosed monkeys (GSN, *Cercopithecus nictitans*), mustached monkeys (MUS, *Cercopithecus cephus*) and mona monkeys (MON, *Cercopithecus mona*), respectively. The less prevalent HIV-1 groups, groups N, O and P, also originate from SIVcpz, with O and P first crossing from chimpanzees into gorillas before infecting humans (42-45). HIV-2, which is less pathogenic than HIV-1, is the product of the cross-species transmission of SIVsmm, which infects the sooty mangabey, into humans (46). At least 8 different cross-species transmissions from sooty mangabeys into humans have occurred, with two of these transmissions founding the majority of HIV-2 cases (47-49).

Akin to HIV-2, SIVmac is the product of SIVsmm crossing the species barrier into a new host, in this case, Asian-origin macaques (46). However, unlike humans, macaques do not naturally encounter sooty mangabeys, as the former inhabits Asia and the latter Africa. Instead, this cross-species transmission occurred inadvertently in the California National Primate Research Center where rhesus macaques and SIV-infected sooty mangabeys were cohoused, and possibly during the generation of an animal model for kuru that included inoculating rhesus macaques with tissue homogenate from sooty mangabeys (50). SIVmac was later isolated from animals at the New England National Primate Research Center who had origins in the California Primate Center (51). Despite its unusual origin, SIVmac infection of macaques recapitulates many of the features of HIV-1 infection of humans as mentioned, making it the best animal model to date (52).

Main features that vary between natural and non-natural host infection

SIV-infected natural hosts are distinct from SIVmac-infected macaques and HIV-1-infected humans due to their lack of disease. However, natural hosts do not avoid disease by controlling the virus; they maintain viral loads as high, if not higher, than non-natural hosts (similar to viral loads shown in Figure 1.1), and infected cells turnover at a rapid rate, akin to non-natural hosts

(53-55). Furthermore, natural host SIVs are not inherently nonpathogenic, as cross-species transmission can cause immunodeficiency in a new host, as described for the transmission of SIVsmm into macaques. Therefore, factors beyond viral replication itself contribute to the lack of pathogenesis observed in natural hosts.

Studies of captive SM and AGM have revealed that these natural hosts do not experience either the profound CD4+ T cell depletion and the chronic immune activation that are thought to be the main drivers of AIDS. Comparative studies of these natural hosts to RM and humans have identified distinct features of benign infection outcomes; these include distinct patterns of infection among anatomic compartments, namely the lymph node and the gut mucosa, as well as particular CD4+ T cell subsets. In non-natural hosts, lymph nodes support substantial virus replication and are marked by fibrosis and a loss of architecture that impedes normal interactions between immune system cells (56, 57). In contrast, natural host infections do not damage lymph node architecture, and virus burden is generally lower, particularly in the follicles (53, 58, 59). The gut harbors the majority of virus replication in both natural and non-natural host infections (60-63). A hallmark of non-natural host infection is loss of gut barrier integrity, which permits the translocation of microbial products such as lipopolysaccharide (LPS) from the intestinal lumen to the circulation, inciting chronic activation of the immune system (64-66). In natural host infection, the gut barrier remains intact such that microbial translocation does not occur, and markers of immune activation return to baseline after acute infection (67-69).

Both within and outside of the aforementioned compartments, certain critical CD4+ T cell subsets are infected or lost at a lower frequency in natural hosts than in non-natural hosts. HIV and SIV primarily infect activated and memory CD4+ T cells (70, 71). Less differentiated memory CD4+ T cells subsets that reside in the lymph node, including central memory CD4+ T cells (Tcm), and stem-cell memory CD4+ T cells (Tscm), are infected at a lower frequency and turnover less often in natural hosts than in non-natural hosts (72-75). In fact, the rate of decline of Tcm is a strong

correlate of the rate of disease progression (76), and it is thought that continued proliferation to replenish lost Tcm contributes to immune activation as well as the disruption of CD4+ T cell homeostasis. An additional subset, TH17 cells, which produce IL-17 and IL-22 and are crucial for maintaining gut immunity and barrier integrity, are lost in non-natural host, but not natural host infection (77-79). Lastly, T follicular helper (Tfh) cells that mediate lymph node germinal reactions are infected less frequently in sooty mangabey than rhesus macaque lymph nodes, which likely contributes to the lack of inflammation and fibrosis observed in this tissue in the former (56). These anatomic and cellular differences, summarized in Figure 1.5, clearly suggest that the cells that serve as targets for natural and non-natural host infection differ and therefore contribute to the divergent infection outcomes observed.

Coreceptor use of natural and non-natural HIV and SIV

As mentioned, HIV and SIV engage a receptor, CD4, which causes the envelope protein (Env) to change conformation and subsequently bind to the coreceptor, permitting membrane fusion and entry into cells. These coreceptors are seven-transmembrane G protein-coupled receptors (7TMRs) whose ligands are usually chemokines. Studies of HIV-1 suggest that Env engages these coreceptors by binding the N-terminal domain and the second-extracellular loop, in a manner akin to that of their ligands (80). While 7TMRs are quite diverse at the amino acid level, they share general characteristics such as their 7 transmembrane topology and a signaling motif in the second intracellular loop (81).

The discovery of CD4 as the receptor for HIV-1 revealed that it was necessary, but not sufficient, for viral entry (82). Nearly ten years later, studies finally identified the HIV-1 coreceptors: CCR5 and CXCR4. CXCR4, then termed fusin (83), was identified first, and the discovery of CCR5 quickly followed as a second, and ultimately more frequently used, HIV-1 coreceptor (84-88). New HIV-1 infections are established by CCR5 (R5) using viruses, and later in infection, viruses that use CXCR4 exclusively (X4), as well as dual CCR5/CXCR4 using (R5X4) viruses,

sometimes emerge. The importance of CCR5 (and coreceptors in general) to infection was underscored by the discovery that humans homozygous for a CCR5-null mutation (CCR5Δ32) are highly resistant to HIV-1 infection (89-91). Coreceptor use is also tightly linked to pathogenesis, as the emergence of CXCR4 using strains is associated with a more rapid disease progression, likely due to a broader range of HIV-1 target cells that express this coreceptor, particularly naïve T cells, which are critical for immune homeostasis (92).

Natural Host SIVs can use non-CCR5 coreceptors to enter cells

Early studies of both natural and non-natural SIVs quickly identified CCR5 as a common coreceptor of SIV, but SIVs rarely used CXCR4 (93-95). Other coreceptors, typically of human origin, were observed to permit SIV entry in vitro, but their relevance was not appreciated given the rare use of these alternative coreceptors by HIV-1. The most robust of these coreceptors included CXCR6 (previously called STRL33, Bonzo, or TYMSTR), GPR15 (previously called Bob) and GPR1, while use of APJ, and CCR3 and others were described less frequently (96-98).

One noted exception occurred in SIVrcm, the natural host virus that infects red-capped mangabeys (RCM). RCM are frequently genetically CCR5-null due to a common 24-base pair deletion allele, and SIVrcm evolved to use the coreceptors CCR2b and CXCR6, and not CCR5 (99, 100). However, aside from this exception, CCR5 was thought to be the main SIV coreceptor in both natural and non-natural host infections.

A link between CCR5 use by natural host viruses and infection outcome was determined when a survey of various primate species revealed that CCR5 expression on CD4+ T cells was very limited in natural hosts when compared to non-natural hosts (101). More detailed analyses of SM CD4+ T cell subsets found that the frequency of CCR5 expression was especially restricted on Tscm and Tcm cells, providing a mechanism for their observed protection from SIV infection (72, 73). A low frequency of CCR5+ CD4+ cells in infant and juvenile SM and AGM has also been

correlated with infrequent mother to infant transmission in sooty mangabeys and African green monkeys (37, 102, 103). While these observations highlight the importance of coreceptor expression in defining SIV target cells, they also raise the question of how natural hosts are able to maintain high viral loads with seemingly limited target cell availability and high turnover of infected cells.

Several years ago, our lab discovered a 2-base pair deletion CCR5 allele that was present at high allele frequency (25%) in the sooty mangabey colony at Yerkes National Primate Research Center (YNPRC), and also present in wild animals native to West Africa (104). When considered in combination with a previously identified but less common 24-base pair deletion CCR5 allele, it was discovered that animals homozygous for CCR5-null alleles (n=14) were infected at a similar frequency to wild type (WT) CCR5 animals (n=97) at YNPRC (Figure 1.6A). Furthermore, these CCR5 null animals were able to maintain high viral loads of 4.37log10, in comparison to 4.83log of CCR5-WT animals and 4.65log10 of CCR5 heterozygous animals (n=81) (Figure 1.6B). These data clearly demonstrated that coreceptors aside from CCR5 must be used in SIVsmm infection (104).

To determine which 7TMRs might act as additional SIVsmm coreceptors, the lab cloned a panel of nine candidate coreceptors, in addition to CCR5, from SM nucleic acids (105). To accurately assess SIV coreceptor usage, it is essential to test viral entry through 7TMRs of the same species, as single amino acid changes can alter coreceptor functionality (106). The lab tested the ability of each candidate coreceptor, with SM CD4, to facilitate entry of SIVsmm pseudotyped luciferase reporter viruses and found that in addition to CCR5, SM CXCR6 is a robust coreceptor of SIVsmm (Figure 1.6A). GPR15 facilitated modest entry, while minimal entry was observed through APJ and GPR1. Entry through CCR2b, CCR3, CCR4, CCR8, and CXCR4 was not observed. This pattern held true for Envs isolated from both CCR5 wild type (FFv and FPm) and

CCR5-null animals (FNp) (Figure 1.7A), demonstrating that use of non-CCR5 coreceptors is not specific to virus infecting CCR5-null animals (105).

Use of CXCR6 by SIVsmm for infection of SM primary lymphocytes was confirmed by infecting peripheral blood mononuclear cells (PBMC) in the presence or absence of the CCR5-blocker maraviroc (MVC) and the CXCR6-blocker CXCL16, which is the ligand for CXCR6 (Figure 1.7B) (107). SIVsmm replication was limited when blocking each coreceptor individually, and limited further when both blockers were used together (mean of 57% for CXCR6 blocking, 53% for CCR5 blocking, and 78% for both together). While the degree of blocking conferred was animal dependent, these data demonstrate that both CXCR6 and CCR5 are used for SIVsmm entry into SM PBMC. Use of these two blockers was not sufficient to completely abrogate SIVsmm entry; this could be due to use of additional alternative coreceptors, such as GPR15, or incomplete blocking by maraviroc and CXCL16, as these treatments are specific but incomplete against SM molecules. In contrast, for the non-natural host virus SIVmac, MVC treatment is sufficient to block replication in rhesus macaque PBMC (Figure 1.7C) (107). Concordant results have been found in studies where SIV-infected macaques were treated with CCR5 blockers (108, 109). Thus, SIVmac is restricted to use of CCR5 as a coreceptor, and like HIV-1, does not use CXCR6 or other alternative coreceptors to infect host cells.

While these studies indicate that use of CXCR6 for entry is a feature of the natural host virus SIVsmm, but not SIVmac, the breadth of this phenotype among natural host viruses is not known. It is also not known whether CXCR6 use was a feature of natural host ancestors in the HIV-1/SIVcpz lineage, and whether or how it changed during cross-species transmission and emergence of the pathogenic SIVcpz/chimpanzee and HIV-1/human infections. Furthermore, it is not known what CD4+ SIV target cells express the coreceptor CXCR6, and thus potentially support high-level replication in natural hosts without disrupting immune homeostasis.

Goals of this thesis

In this thesis, I address several key questions and hypotheses raised by the discovery that the natural host virus SIVsmm uses CXCR6 in addition to CCR5 for entry into sooty mangabey lymphocytes. First, I investigated the breadth of CXCR6 use by natural host SIVs and hypothesized that CXCR6 is used by a second natural host virus, SIVagmSab that infects African green monkeys of the subtype sabaeus (Chapter 2). I found that SIVagmSab also used CXCR6 to enter sabaeus lymphocytes. Secondly, the forerunners of the HIV-1 *env* are the pathogenic SIVcpz, and the natural host virus lineage SIVgsn/mus/mon. While HIV-1 is known to use CCR5 and/or CXCR4 for entry, the coreceptor use of its SIV predecessors was undetermined. I hypothesized that use of CXCR6 by this *env* lineage was lost through cross-species transmission. I found that SIVmus could use species-matched CXCR6 like other natural host viruses, but pathogenic SIVcpz was restricted to use of CCR5 like its direct descendent HIV-1 (Chapter 3). Finally, while use of CXCR6 by SIVsmm has been demonstrated, the expression of this coreceptor on CD4+ T cells had not been determined. I hypothesized that CXCR6 would be expressed on more expendable CD4+ T cell subsets and not on more critical subsets, such as Tcm, which could permit high viral replication without causing immunodeficiency. As there were no existing antibodies that detected CXCR6 of any natural host species, I generated such an antibody. I stained SM memory CD4+ T cells and found that CXCR6 expression was restricted on Tcm, but enriched on effector memory CD4+ T cells (Tem). Additionally, CXCR6+ CCR5- CD4+ T cells formed a distinct subset (Chapter 4). Together, the data presented here demonstrate that species-matched CXCR6 use is a common feature of natural host viruses, but absent among non-natural host viruses. Therefore, entry by natural host SIVs is fundamentally different from entry by non-natural host SIVs. This likely defines distinct target cells between these two types of infections and contributes to the lack of disease progression observed in natural hosts.

Figures

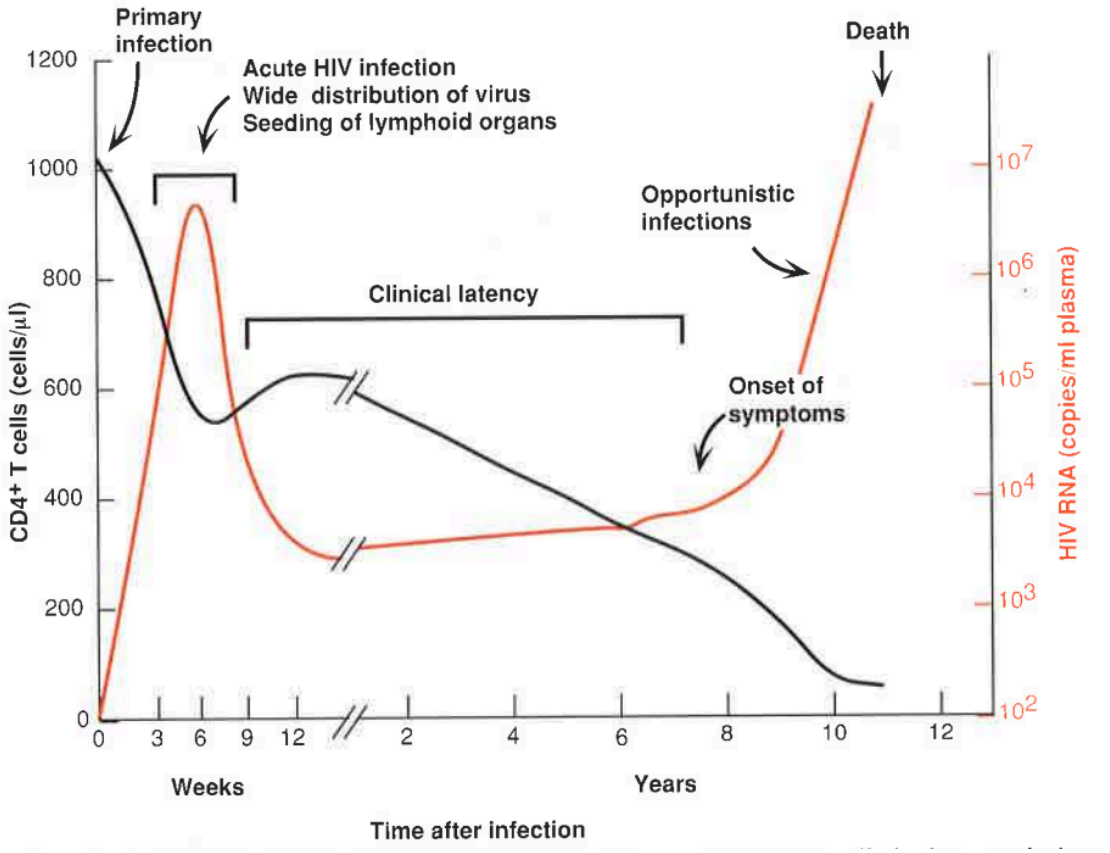


Figure 1.1: Typical progression of HIV-1 infection. Typical changes in viral load (red) and CD4+ T cell counts (black) are shown for HIV-1 infected individuals over time. Patient-specific variability between CD4+ T cell counts and viral RNA copy numbers is observed. Figure from *Fauci AS, Desrosiers RC. 1997. Pathogenesis of HIV and SIV. In Retroviruses (ed. Coffin JM, Hughes SH, Varmus HE*), p. 600. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. (Modified from Pantaleo et al. 1993a.) Reprinted with permission from Cold Spring Harbor Laboratory Press.

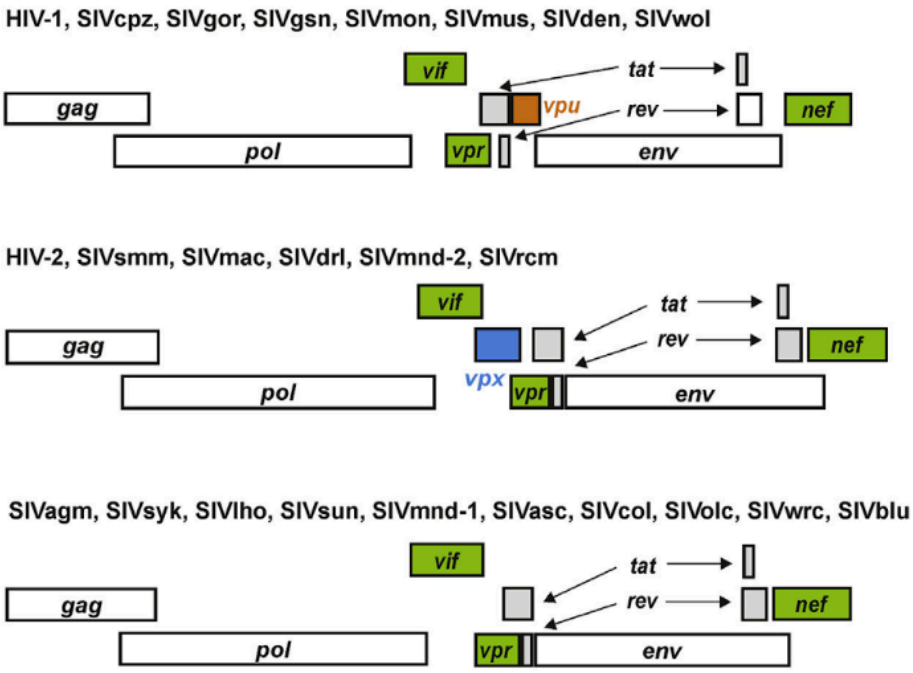


Figure 1.2: Primate lentivirus genomes. The three genome structures of primate HIV/SIV are shown. All viruses encode genes for the structural proteins Gag, Pol, Env (in white), the regulatory proteins Rev and Tat (grey), and the accessory proteins Vif, Vpr and Nef (green). Primate lentiviruses vary in the presence of genes for the accessory proteins Vpu (orange), and Vpx (blue). Figure from *Sauter D, Kirchhoff F. Properties of Human and Simian Immunodeficiency Viruses*. In: Ansari A, Silvestri G, editors. Natural Hosts of SIV: Implication in AIDS. 1 ed: Elsevier Inc.; 2014. p. 69-84. Reprinted with permission from Elsevier.

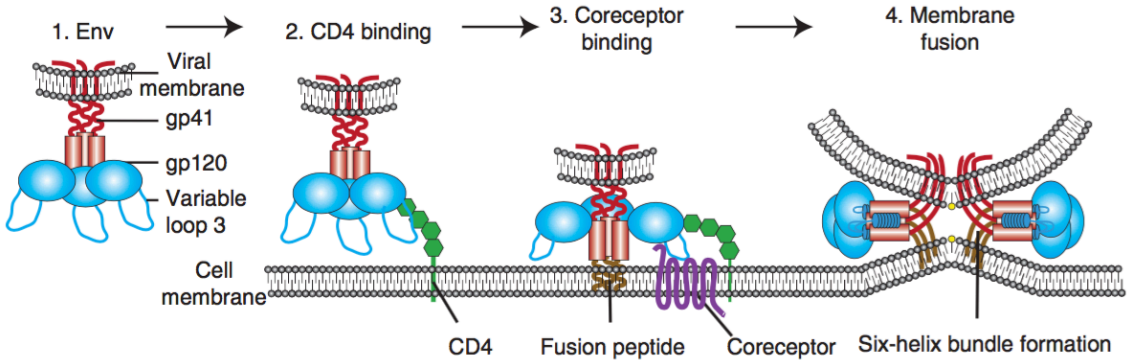
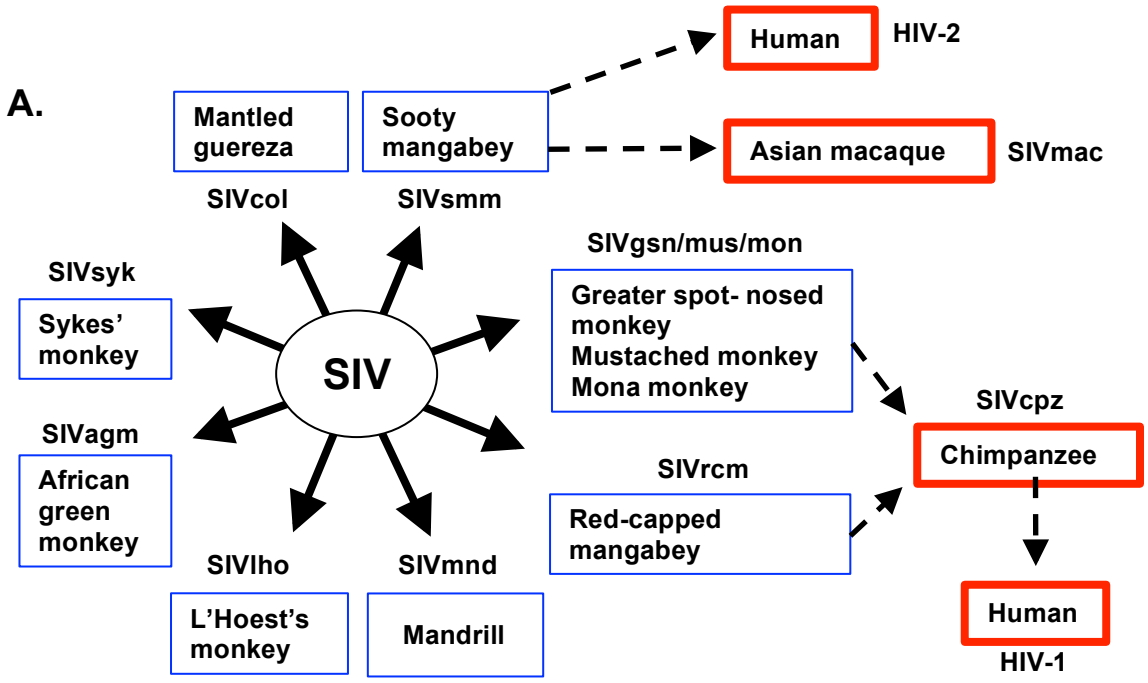


Figure 1.3: The HIV/SIV Env glycoprotein interacts with cell surface CD4 and coreceptor to mediate entry. The HIV/SIV Env consists of gp120 and gp41. To enter cells, gp120 first binds CD4. This induces conformational changes that permit binding of a coreceptor, which is largely mediated by Env variable loop 3. Further conformational changes permit insertion of the fusion peptide of Env gp41 into the host cell membrane and six-helix bundle formation to permit membrane fusion and delivery of the virus genome to the cell. Figure from *Cold Spring Harb Perspect Med 2012;2:a006866*. Reprinted with permission from Cold Spring Harbor Laboratory Press.



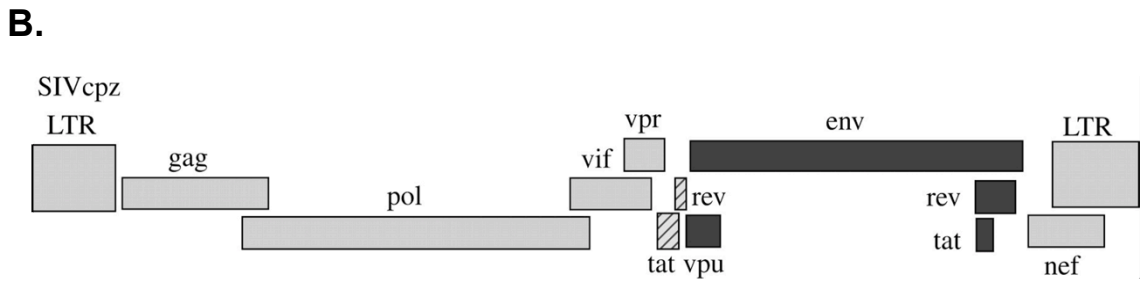


Figure 1.4: Relationships between natural host and non-natural host SIVs. A. A selection of diverse African natural host primate species and their SIVs are shown in blue boxes to the left, and dashed arrows indicate known SIV transmissions into other species. Hosts of pathogenic HIV/SIV infections are shown in red boxes. Figure modeled after *Sharp and Hahn, CSH Perspectives in Med., 2012* (5). B. The genome of SIVcpz is the product of a recombination event between SIVrcm (grey) and SIVgsn/mus/mon (black). The origin of the first exons of *rev* and *tat* is uncertain, thus shown as striped. Figure 1.3B from reprinted from Sharp and Hahn, Philos Trans R Soc Lond B Biol Sci., 2010 Aug 27;365(1552):2487-94. Reprinted with permission from the Royal Society.

Phenotype of infection	Natural hosts	Non-natural hosts
Viral load	High	High
Turnover of infected cells	High	High
Immunodeficiency	NO	YES
Chronic Immune activation	NO	YES
Peripheral CD4+ T cell loss	NO	YES
Robust lymph node infection, lymphoid inflammation & fibrosis	NO	YES
Gut barrier breakdown & microbial translocation	NO	YES
Tscm, Tcm, Tfh infection	Low	High
Th17 loss	NO	YES
CCR5+ expression	Low	High

Figure 1.5: Key features of natural host compared to non-natural host SIV infections

A.

CCR5 Genotype	Total SM	# SIV positive SM	% SIV positive SM
WT/WT	97	63	65
WT/Δ	81	50	62
Δ/Δ	14	7	50

B.

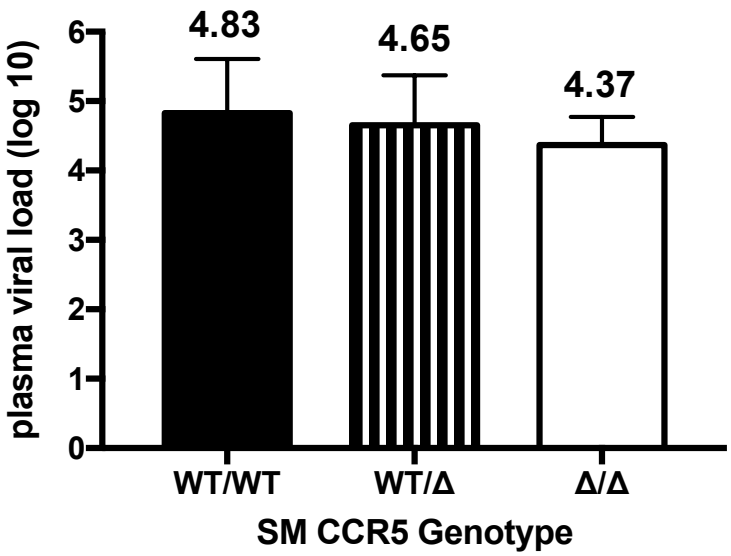


Figure 1.6: Non-CCR5 entry pathways are used in SIVsmm infection *in vivo*. A. Frequency of naturally SIVsmm-infected sooty mangabeys (SM) at Yerkes National Primate Research Center (YNPRC) does not differ based on CCR5 genotype. B. SM at YNPRC have high plasma viral loads whether they have two wild type CCR5 alleles (WT/WT, n=60), one wild type CCR5 allele (WT/ Δ, n=49) or are CCR5-null (Δ/ Δ, n=7). Error bars represent one standard deviation and means are displayed above each bar.

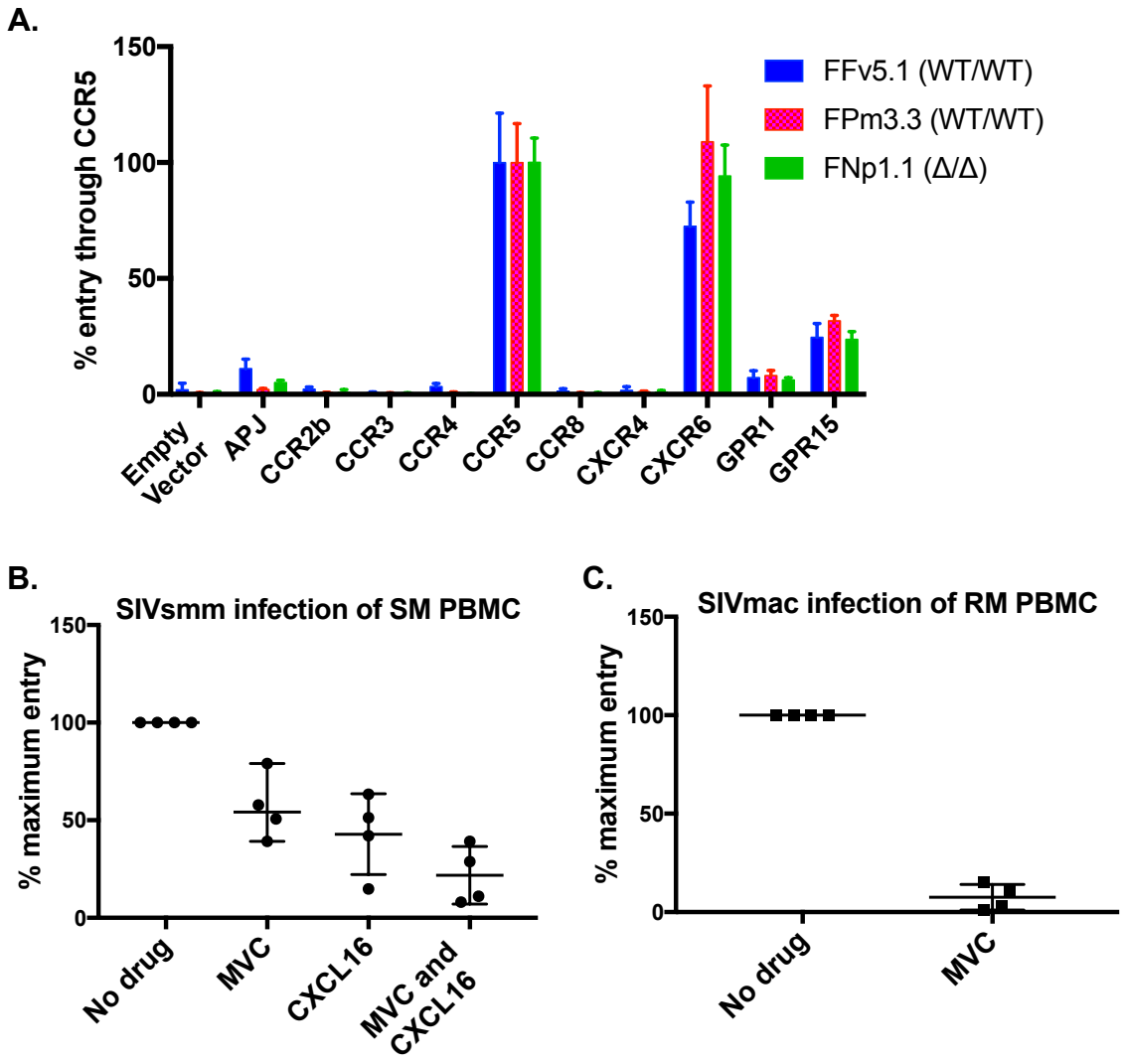


Figure 1.7: SIVsmm uses coreceptors in addition to CCR5. A. 293T cells expressing 7TMRs of sooty mangabey (SM) origin and SM CD4 were infected with luciferase reporter viruses carrying SIVsmm Envs. Legend indicates the name of the SIVsmm Env and the CCR5 genotype of the SM from which the Env was isolated in parentheses. B. SM PBMC were infected with SIVsmm D215 in the presence of vehicle alone (no drug), the CCR5 blocker maraviroc (MVC), the CXCR6 blocker CXCL16, or both blockers together. Replication was measured by p27 Gag production in the supernatant and data is shown as percent of replication in the presence of vehicle alone (no drug) at day 7 post infection. C. RM PBMC were infected with SIVmac239 in the presence of vehicle alone (no drug) or MVC, and replication was measured and data shown as in (B).

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CHAPTER 2

CXCR6-Mediated Simian Immunodeficiency Virus SIVagmSab Entry into Sabaeus African Green Monkey Lymphocytes Implicates Widespread Use of Non-CCR5 Pathways in Natural Host Infections

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Abstract

African green monkeys (AGM) and sooty mangabeys (SM) are two well-studied natural hosts of SIV that do not progress to AIDS when infected with their species-specific viruses despite maintaining high viral loads. Natural hosts are characterized by a low frequency of CD4+ T cells expressing the entry coreceptor CCR5. Recent studies from our lab and others have shown that CCR5 is not required for SIV infection of SM, and that the chemokine receptor CXCR6 permits entry of the SIV that infect SM *in vitro* and *ex vivo*. Furthermore, entry pathways in addition to CCR5 are used by SIV to infect AGM of the subspecies vervet, and CXCR6 is a robust coreceptor for this SIV *in vitro*. Here, I analyzed use of species-matched CXCR6 and other alternative coreceptors by a third natural host virus, SIVagmSab which infects AGM of the subspecies sabaeus. I cloned sabaeus AGM CXCR6, CCR5 and eight other candidate coreceptors and found that CXCR6, CCR5 and GPR15 permitted entry of pseudotypes expressing a previously characterized SIVagmSab Env, 92018ivTF. Importantly, I also tested coreceptor use of two novel *envs* cloned from plasma of wild-infected sabaeus AGM and found robust CXCR6 use as well. Finally, blocking entry through CXCR6 with the ligand CXCL16 significantly blocked replication of SIVagmSab92018ivTF in sabaeus AGM lymphocytes, indicating use of CXCR6 for entry. Furthermore, blocking CXCR6 had a quantitatively greater impact on infection than did blocking CCR5. Together these data support a paradigm for natural host infection in which use of CXCR6 (and possibly other coreceptors) is a common feature of natural host SIVs. Limited expression of CCR5 is seen in natural host CD4+ T cells, and thus use of CXCR6 by natural host SIVs could target infection towards certain CD4+ T cell subsets that support viral replication while preserving immune system homeostasis and function.

Introduction

Over 40 species of African nonhuman primates (NHPs) have been identified as "natural hosts" of SIV, as evidence suggests they do not progress to AIDS, which is likely due to prolonged coevolution between virus and host (35). Natural hosts maintain high viral loads and a rapid turnover of infected cells, like "non-natural hosts" such as Asian macaques infected with SIVmac (54, 55, 111). Despite this, infected natural hosts maintain normal peripheral CD4+ T cell counts, maintain gut barrier integrity and immunity and preserve lymph node structure and functions (112, 113). In contrast, non-natural host infection is marked by progressive CD4+ T cell loss, gut barrier disruption and translocation of microbial products, and lymph node fibrosis and inflammation (114). Understanding how natural hosts of SIV are able to avoid immunodeficiency is crucial to define mechanisms of pathogenesis in non-natural hosts, including HIV-1 infected humans.

A key mechanism implicated in the benign outcome of infection in natural hosts is the protection of certain CD4+ T cell target populations and anatomic niches from infection (and conversely, viral replication in cells other than those that support infection in hosts that develop AIDS). In the natural host sooty mangabey (SM, *Cercocebus atys*), central memory (Tcm) and stem-cell memory (Tscm) CD4+ T cells, which are required for maintenance of memory T cell homeostasis, are infected at a lower frequency than in rhesus macaques (RM, *Macaca mulatta*) (72, 73). Also, natural host lymph nodes carry a low viral burden, particularly in the follicles and T follicular helper cells (Tfh), and also maintain their structure and function (53, 56, 58, 59). In infected RM and humans, lymph nodes carry a high viral burden and are marked by inflammation, fibrosis and collagen deposition (56, 115). Lastly, infected natural hosts maintain gut barrier and immunity, likely due to maintenance of Th17 cells (67, 77). In contrast, non-natural host infection is marked by a loss of gut Th17 cells and disruption of the barrier integrity, causing microbial products to translocate into circulation and drive immune activation (64-66, 78, 79). Therefore, to understand pathogenesis, it is essential to define determinants of cell targeting by SIV in natural and non-natural hosts.

HIV/SIV target cells are largely defined by the expression of the virus receptor, CD4, and a seven-transmembrane coreceptor. Historically, CCR5 was thought to be the only coreceptor used by SIV, although *in vitro* experiments had demonstrated use of other chemokine receptors (usually of human origin) for entry (96-98). Natural hosts maintain a low frequency of CCR5+ CD4+ T cells, suggesting that reduced expression of this coreceptor protected CD4+ T cells from infection (72, 101). However, this finding also raised the question of how natural hosts are able to maintain such high viral loads with limited target cell availability and high turnover of infected cells (54, 55, 111). Recently, our lab identified a common CCR5 deletion allele in SM that prevents CCR5 surface expression on the cells of homozygous animals (104). Despite this, CCR5-null SM are frequently infected and maintain viral loads near that of CCR5-wildtype SM, indicating that non-CCR5 entry pathways are used by SIVsmm. We then identified species-matched CXCR6 as a robust coreceptor for SIVsmm infection *in vitro*, and confirmed use of CXCR6 and CCR5 by SIVsmm to infect SM peripheral blood mononuclear cells (PBMC) using blocking agents (105, 107). A subsequent study of AGM of the subspecies vervet (*Chlorocebus pygerythrus*, which inhabit East and Southern Africa) has shown that use of CCR5 is not required for infection of vervet PBMC, and that species-matched CXCR6 and GPR15 are efficient coreceptors *in vitro* (116). Although use of CXCR6 in vervet AGM PBMC infection was not directly examined in that study, these data suggest that use of coreceptors in addition to CCR5 is a common feature of natural host SIVs.

Here, I investigated the entry pathways of a third natural host virus, SIVagmSab that infects sabaeus African green monkeys (*Chlorocebus sabaeus*, which inhabit West Africa). Previous studies of SIVagmSab entry have used human coreceptors and CD4 (98). However, knowing that single amino-acid changes can alter coreceptor use by SIVs (106), I first cloned candidate coreceptor and CD4 genes from sabaeus AGM cells. Also, many SIVagm isolates have been passaged on human cells, which could have altered the ability of such viruses to use coreceptors

of sabaeus origin (117, 118). Therefore, I cloned novel field isolate *envs* from plasma from sabaeus AGM that were infected upon their capture in West Africa, and tested these in addition to a recently described unpassaged SIVagmSab transmitted-founder clone 92018ivTF (119). Since coreceptor function can depend on its degree of expression, I measured entry across a range of coreceptor levels (120, 121). Also, I tested coreceptor use at two levels of CD4 expression, in part because AGM lymphocytes downregulate CD4 (122). Finally, I examined the contribution of CCR5 and CXCR6 use to SIVagmSab infection of sabaeus PBMC by using blocking agents against these two coreceptors. I found that sabaeus CXCR6 is an efficient coreceptor of SIVagmSab, and substantially contributes to SIVagmSab infection of sabaeus PBMC. These data identify another natural host virus that can use CXCR6 for entry and support an emerging paradigm of CXCR6 use as a common feature of natural host SIVs.

Results

Cloning and sequencing of Sabaeus African Green Monkey CD4 and candidate coreceptors.

Recent work from our lab and others has described use of species-matched non-CCR5 coreceptors by natural host viruses SIVsmm and SIVagmVer (105, 107, 116). I asked whether the SIV of a third natural host, SIVagmSab of sabaeus AGM, also utilized species-matched non-CCR5 coreceptors for entry with species-matched CD4. I first amplified CD4 from cDNA from two sabaeus AGM, and isolated two alleles (CD4-30 and CD4-31) that differed from each other by 5 amino acids (Table 1). These alleles contained several previously described AGM CD4 polymorphisms (123, 124), and CD4-30 also contained a unique C-terminal Ser. Two polymorphisms fell within domain 1 of CD4, but not within residues implicated in human CD4/ HIV-1 interactions (125). Like other reported AGM CD4 alleles, these alleles share 90% amino acid identity to human CD4 and are most closely related to the CD4 molecules of other African green monkey subspecies, including vervet and grivet.

I next amplified APJ, CCR2b, CCR3, CCR4, CCR5, CCR8, CXCR4, CXCR6, GPR1 and GPR15 from genomic DNA or cDNA from one AGM sabaeus using previously described primers that lie outside the open reading frames of each gene (105). The non-CCR5 coreceptors cloned are identical in sequence to the genes described in the recently sequenced sabaeus genome (126) (BioProject: PRJNA215854), except for CCR2b, which contained a conservative V340A difference. Sabaeus CCR5 is polymorphic, and the allele analyzed here is unique to published sequences but contains the previously described AGM polymorphisms N57S and R163G (98).

I then aligned the amino acid sequences of these ten sabaeus candidate coreceptors to those of human, rhesus macaque and sooty mangabey. As shown in Table 2, these coreceptors all vary in amino acid sequence from those of human, including in the N-terminus and second extracellular loop (ECL2), the two domains expected to interact with Env, based on studies of

CCR5 and HIV-1 (80). These differences underscore the need to use species-matched coreceptors to accurately assess SIV entry pathways.

The sabaeus candidate coreceptor sequences were more similar to those of other nonhuman primates than to those of human origin. Some sequences were identical at the amino acid level, such as GPR1 and CXCR4 (100% amino acid identity between sabaeus AGM and RM) and CCR4 (100% amino acid identity among sabaeus AGM, RM, and SM). Of note, sabaeus CXCR6 codes a Ser at position 31, as do SM and many other primate species. This residue is associated with robust use of CXCR6, while an Arg at that position is responsible for the poor coreceptor activity for SIVmac of CXCR6 for several macaque species, including rhesus, pigtail, and cynomolgus (106). The sabaeus CXCR6, GPR15 and CXCR4 amino acid sequences described here are identical to described alleles for vervet AGM (116), while vervet APJ, CCR2b, CCR3, CCR4, CCR8 and GPR1 have not been reported.

SIVagmSab Envs use non-CCR5 coreceptors in vitro

I then tested the ability of each candidate coreceptor to facilitate SIVagmSab entry *in vitro* in conjunction with each cloned CD4 allele. I transfected 293T cells with CD4 and each candidate coreceptor in turn, and infected cells with a pseudotyped reporter virus carrying the SIVagmSab92018ivTF Env on a pNL43E-R+Luciferase backbone. The SIVagmSab92018ivTF *env* was cloned from the previously described infectious molecular clone that is the transmitted/founder virus of an intravenous infection with an unpassaged SIVagmSab92018 stock (119). Pseudotype virus entry was measured by luciferase production in 293T target cells. SIVagmSab92018ivTF readily entered cells expressing sabaeus CCR5 and CXCR6, and robust entry through GPR15 was also observed (Figure 1). Low-level entry was measured through sabaeus GPR1 and APJ. Notably, although use of human CXCR4 has been reported, no substantial entry through sabaeus CXCR4 was observed here (119, 127). This pattern of

coreceptor use was consistent between CD4-30 and CD4-31, and subsequent experiments were performed using CD4-31 only.

I then sought to expand this analysis to additional SIVagmSab envelopes. However, aside from SIVagmSab92018ivTF, all available SIVagmSab isolates have been passaged on human cell lines (117, 118), and it is possible that adaptation occurred that has altered native coreceptor use patterns. Therefore, I cloned *envs* from plasma collected from two sabaeus AGM (89042 and 89044) that were already infected upon their capture in West Africa. The sequences of *rev*, *tat*, *nef* and *vpr* from SIV infecting AGM 89042 have been described previously (128), while SIV from 89044 has not been previously characterized. I cloned *env* from vRNA isolated from the two plasma samples. As shown in Figure 2.2A, the two *envs* share 78% nucleotide sequence homology and represent distinct sequences among characterized SIVagmSab *envs*.

I pseudotyped these SIVagmSab Envs onto a luciferase reporter backbone and tested their ability to enter cells via the ten sabaeus coreceptors and CD4-31 that I cloned (Figure 2B). Like 92018ivTF, both 89042 and 89044 robustly used CCR5, CXCR6 and GPR15 for entry, with low-level entry observed through GPR1. While 89042 showed some use of APJ (like 92018ivTF), 89044 did not enter via this coreceptor. None of the Envs tested were able to use sabaeus CXCR6, CCR5 or GPR15 independent of CD4. Thus, 92018ivTF reflects the alternative coreceptor use pattern of diverse SIVagmSab isolates from naturally infected monkeys, although use of APJ is variable.

Efficient Use of CXCR6 by SIVagmSab92018ivTF occurs at varying CD4 levels

The *in vitro* experimental conditions used here result in high levels of expression of CD4 and coreceptor on transfected 293T cells; while these conditions allow easy identification of possible coreceptors, they provide little information on efficiency of use. Additionally, since coreceptor function can depend on their level of expression, and AGM lymphocytes downregulate CD4

expression as they enter the memory pool (120-122), I tested entry through CXCR6, CCR5, GPR15, GPR1 and APJ at various expression levels of both coreceptor and CD4.

I titrated the amount of transfected plasmid in tenfold serial dilutions from 250ng to 0.25ug for the functional coreceptors (CCR5, CXCR6, GPR15, GPR1 and APJ) and then infected cells with the SIVagmSab92018ivTF luciferase reporter virus. Changes in surface expression as a result of titration could not be directly measured due to a lack of cross-reactive antibodies for all coreceptors shown here except for sabaeus CCR5. However, I did a parallel analysis using human molecules to ensure that the plasmid titration protocol would result in differing levels of surface expression. Cells were transfected with plasmids encoding human CCR5, CXCR6 and GPR15 at the same concentrations and stained for analysis via flow cytometry using antibodies that detect the human molecules. This revealed similar levels of staining for each coreceptor, and decreasing the amount of plasmid led to decreasing levels coreceptor expression across the titrations that was similar for the three molecules (data not shown).

As shown in Figure 2.3A, titrating sabaeus CXCR6 and CCR5 down tenfold from 250ng to 25ng of plasmid did not reduce entry; however, entry through GPR15, APJ and GPR1 all decreased. At a 1:100 dilution of coreceptor plasmid, entry through CXCR6, CCR5 decreased, and entry through GPR15 decreased even further, but all remained well above baseline, while entry through APJ and GPR1 could no longer be detected. Thus, sabaeus CXCR6 and CCR5 permitted entry quite similarly in all conditions, while entry through GPR15 appeared more sensitive to reduced coreceptor levels, and entry through APJ and GPR1 even more so.

AGM are known to express low levels of CD4, such that CD4 availability may be a limiting factor for SIVagmSab entry *in vivo* (101). Therefore, to test whether these results would reflect a similar pattern in the context of low CD4 expression, and that high expression of CD4 wasn't masking subtle differences in coreceptor use, I performed the same experiment (using CCR5, CXCR6 and

GPR15) in conjunction with both “high” (250ng, as in Figure 3.3A) and “low” (2.5ng) CD4 conditions. Transfected cells were stained for CD4 expression and a comparable decrease thereof was observed across the coreceptor conditions (data not shown).

This reduction in CD4 resulted in about 50% reduction in maximum entry for the three coreceptors (Figure 2.3B-D). However, of the three coreceptors, entry through CXCR6 appeared least sensitive to reduced coreceptor levels in the presence of low CD4, as entry remained consistent despite a 100-fold decrease in transfected coreceptor plasmid. This suggests that reduced CD4 may not be an obstacle for CXCR6 mediated entry. Collectively, these data show that SIVagmSab entry through sabaeus CXCR6 is as efficient as entry through sabaeus CCR5, including in conditions of reduced coreceptor and CD4, and sabaeus GPR15 is also used moderately well.

Maraviroc and CXCL16 specifically but incompletely block entry through Sab CCR5 and CXCR6

Recently published work from our lab demonstrated that blocking entry through CCR5 using maraviroc (MVC) and through CXCR6 using recombinant human CXCL16, the ligand for CXCR6, reduced replication of SIVsmm in SM PBMC, confirming that CXCR6 (in addition to CCR5) is an entry coreceptor for SIVsmm in primary cells (107). Others have shown that blocking entry through CCR5 does not hinder replication of SIVagmVer in vervet PBMC, indicating use of non-CCR5 entry pathways by this virus as well (116). Therefore, having shown that AGM sabaeus CXCR6 functions as a coreceptor *in vitro*, I asked whether CXCR6 was used by SIVagmSab92018ivTF for entry into sabaeus PBMC.

First, I asked whether maraviroc and CXCL16 could specifically block entry through AGM sabaeus CCR5 and CXCR6 respectively *in vitro*, as these coreceptors vary in amino acid sequence from human and sooty mangabey homologs (Table 1). I transfected 293T cells with

plasmids expressing sabaeus CD4 and CXCR6, CCR5, GPR15, GPR1 and APJ, and pretreated cells with 15um MVC, 500ng/mL CXCL16 or vehicle alone for one hour prior to infection with the SIVagmSab9201ivTF reporter pseudotype (Figure 4A). Both maraviroc and CXCL16 were specifically able to block entry through their respective sabaeus coreceptors. However, blocking was incomplete; 18% entry remained through CCR5 despite MVC treatment, and 11% through CXCR6 despite CXCL16 treatment. Therefore, use of these inhibitors is specific, but likely underestimates the use of each coreceptor by SIVagmSab. Unfortunately, no small molecule inhibitor or other blocker is available against the orphan receptor GPR15, preventing similar analysis of this coreceptor.

SIVagmSab92018ivTF uses CXCR6 to enter cells

I then investigated the use of CXCR6 and CCR5 in SIVagmSab infection of PBMC from five animals. Sequencing of the CXCR6 and CCR5 genes from the monkeys revealed identical CXCR6 sequences, but divergent CCR5 sequences. The CCR5 amino acid sequences contained previously described polymorphisms at residues 57, 93, and 163 (129), with three heterozygous for R93, which has been reported to support SIVagm entry less efficiently (24). No animals possessed CCR5 sequences predicted to be nonfunctional.

Sabaeus PBMC were stimulated with phytohemagglutinin (PHA) and interleukin-2 (IL-2) and then pretreated with 15uM MVC, 500ng/mL CXCL16, both blockers together, or vehicle alone, and infected with replication competent SIVagmSab9201ivTF derived from an infectious molecular clone. Virus replication was quantified by measuring p27 in the culture supernatant by ELISA. At day 7 post-infection, blockade of CCR5 resulted in a slight but statistically significant 8% decrease in supernatant p27 (range: 2-14%; p=0.026 compared with vehicle alone). Blockade of CXCR6 decreased replication by 36% (range: 22-52%; p=0.004 compared with vehicle alone). The treatments showed an additive effect, such that replication was decreased 52% when both agents were used (range: 40-72%; p=0.005 and p=0.001 compared with CXCL16 alone and

CCR5 alone, respectively). CXCL16 had no effect on infection of RM PBMC by SIVmac239 (which uses RM CXCR6 poorly and enters RM PBMC entirely through CCR5 (107)), nor on infection of human PBMC by HIV-1 BaL, suggesting that SIVagmSab/sabaeus PBMC blocking by CXCL16 was not an effect of the chemokine unrelated to entry (data not shown). While this study only included PBMC from 5 animals, we noticed no obvious defect in replication or blocking due to CCR5 allele.

These data demonstrate that CXCR6 is used for SIVagmSab entry into sabaeus PBMC. Furthermore, the blocking studies suggest that while CCR5 is used, the contribution of CXCR6 to entry is quantitatively greater. It is unclear whether residual infection is due to incomplete blocking of entry through CCR5 and CXCR6, or use of another coreceptor such as GPR15.

Tables

CD4 clone:	Human	Rhesus Macaque	Sooty Mangabey	AGM Grivet (*C. Aethiops*)	AGM Vervet (*C. Pygerythrus*)
AGM Sab 30	90.6	95.4	95.0	99.1	98.9
AGM Sab 31	90.8	95.6	95.2	99.3	99.1

Table 1: Sabaeus African Green Monkey CD4 molecules. CD4 was cloned from cDNA from PBMC of two individual animals, yielding two AGM CD4 clones that are 98.9% identical at the amino acid level (differing by 5 amino acids). (Table originally printed in Wetzel et al, JVI 2017.)

	% AA identity to 7TMR from:			# AA differing from human:	
AGM Sab 7TMR:	**Human**	**Rhesus Macaque**	**Sooty Mangabey**	**NTD**	**ECL2**
APJ	98.7	99.7	99.5	0	1
CCR2b	96.7	99.2	99.4	2	2
CCR3	93.5	98	97.2	6	4
CCR4	98.6	100	100	0	1
CCR5	97.4	99.1	98.9	2	1
CCR8	94.6	99.2	98.9	8	1
CXCR4	98.6	100	99.7	3	1
CXCR6	95.3	98.8	98.3	6	1
GPR1	98	100	99.7	1	1
GPR15	97.2	99.4	99.4	1	2

Table 2: Sabaeus African Green Monkey candidate coreceptors. Seven transmembrane receptors (7TMR) were cloned from PBMC genomic DNA or cDNA, and the amino acid identity is shown compared with the human, rhesus macaque and sooty mangabey molecules. Also shown is number of amino acids differing from the human molecule in the N-terminal domain (NTD) and extracellular loop 2 (ECL2), which are implicated in coreceptor function based on studies with CCR5 and HIV. (Table originally printed in Wetzel et al, JVI 2017.)

Figures

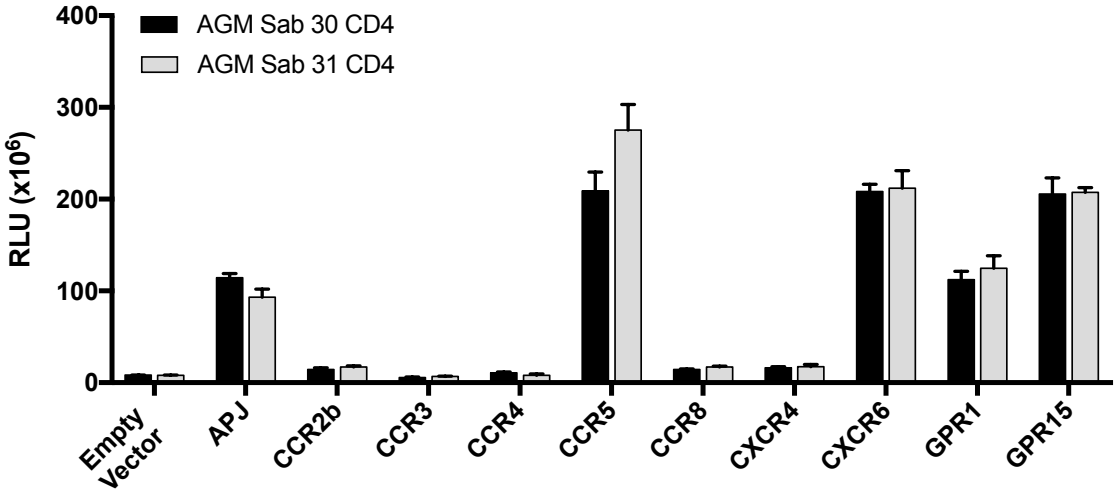

Figure 2.1: SIVagmSab92018ivTF Env uses non-CCR5 coreceptors *in vitro*, independently of CD4 allele. A) 293T cells were transfected with one of two AGM Sab CD4 allele clones and each sabaeus candidate coreceptor. Cells were infected 48 hours later with the SIVagmSab 92018ivTF Env luciferase reporter pseudotype. Cells were lysed 72 hours post-infection and luciferase content was measured by relative light units (RLU). Infections were carried out in triplicate and data (means ± standard deviation) are representative of 4 replicate experiments. (Figure originally printed in Wetzel et al, JVI 2017.)

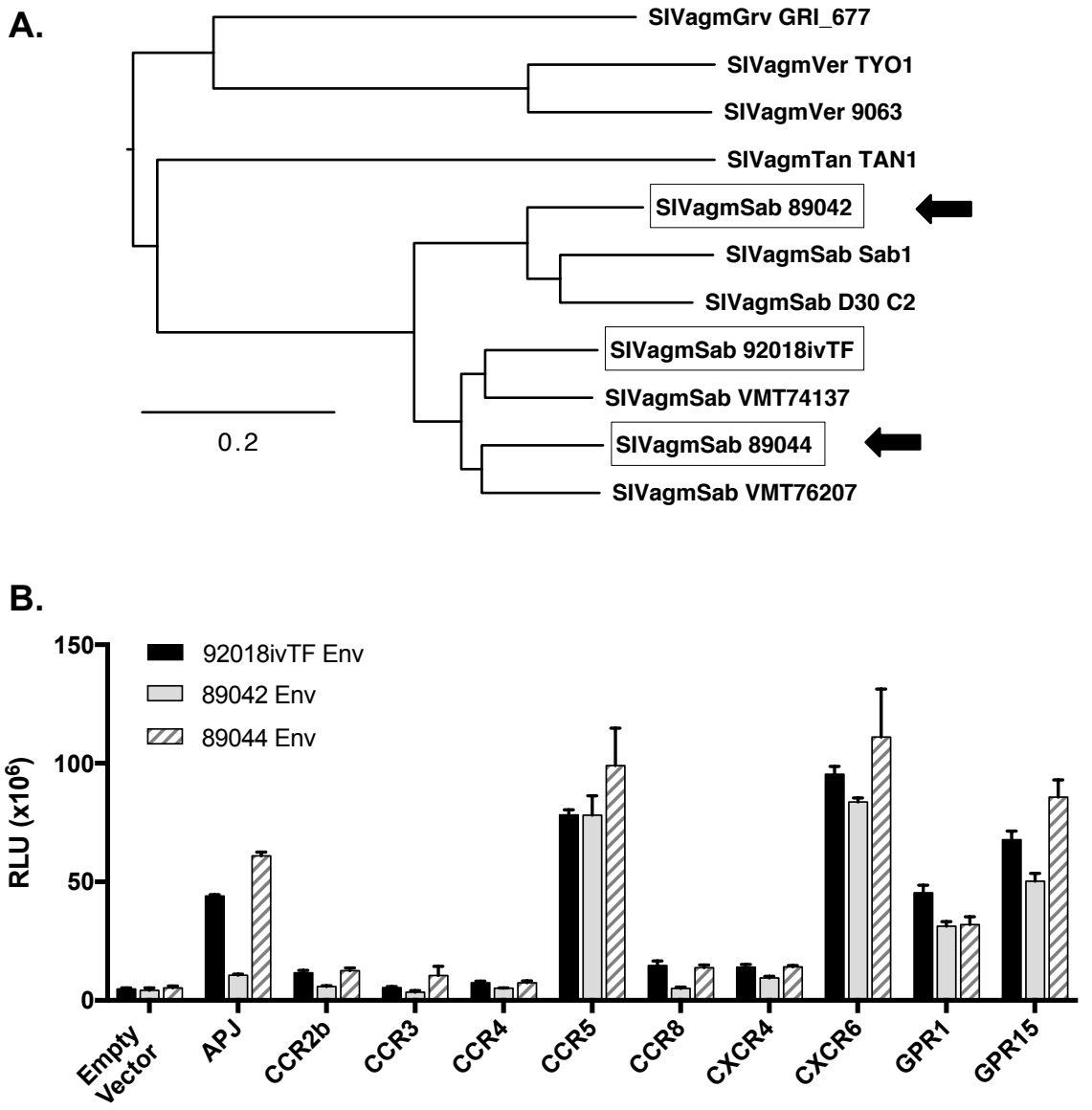

Figure 2.2: Diverse field isolate SIVagmSab Envs use non-CCR5 coreceptors *in vitro*. SIVagmSab Envs were amplified from plasma isolated from two wild-infected sabaeus AGM (89042 and 89044). A) Maximum likelihood phylogenetic tree showing novel envs cloned in this study (89042 and 89044), the previously characterized env 92018ivTF, and other reported SIVagm Env sequences. Envs used in this study are boxed, and the two envs cloned here from wild naturally infected animals are indicated by arrows. Bootstrap values >70% are indicated by an asterisk. The scale bar indicates 0.2 nucleotide substitutions per site. B) 293T cells were transfected with AGM Sab CD4-31 and each sabaeus candidate coreceptor. Cells were infected 48 hours later in triplicate with luciferase reporter pseudotypes containing the field isolate SIVagmSab Envs or the 92018ivTF Env. Cells were lysed 72 hours post-infection and luciferase content was again measured by relative light units (RLU). Infections were carried out in triplicate and data (means ± standard deviation) are representative of 3 replicate experiments. (Figure originally printed in Wetzel et al, JVI 2017.)

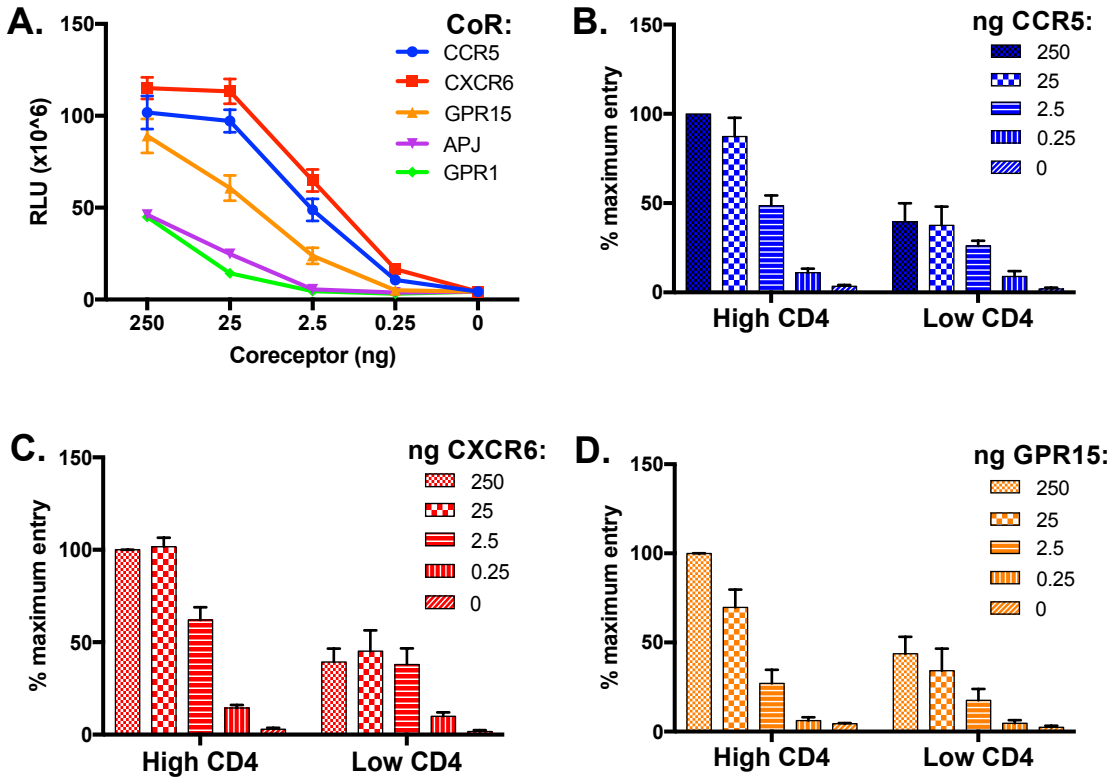

Figure 2.3: SIVagmSab92018ivTF efficiently uses sabaeus CXCR6 at high and low CD4 levels. A) 293T cells were transfected with sabaeus CD4-31 (250ng of plasmid) and each AGM Sab CCR5, CXCR6, GPR15, GPR1 and GPR15 at tenfold serial dilutions, from 250ng to 0.25ng plasmid. Empty pcDNA3.1 was used as filler such that cells in each condition were transfected with an equal quantity of DNA (500ng). Cells were infected 48 hours later with the SIVagmSab92018ivTF Env luciferase reporter pseudotype. Cells were lysed 72 hours post-infection and luciferase content was measured by relative light units (RLU). B) The experiment was performed as in panel A, but with two amounts of CD4 (250ng, high and 2.5ng, low) and ten-fold dilutions of CCR5 (B), CXCR6 (C) and GPR15 (D). Data is normalized to 100% entry based on RLU at maximum CD4 and coreceptor amounts for each coreceptor. Data represent means ± standard deviations of three replicate experiments carried out in triplicate. (Figure originally printed in Wetzel et al, JVI 2017.)

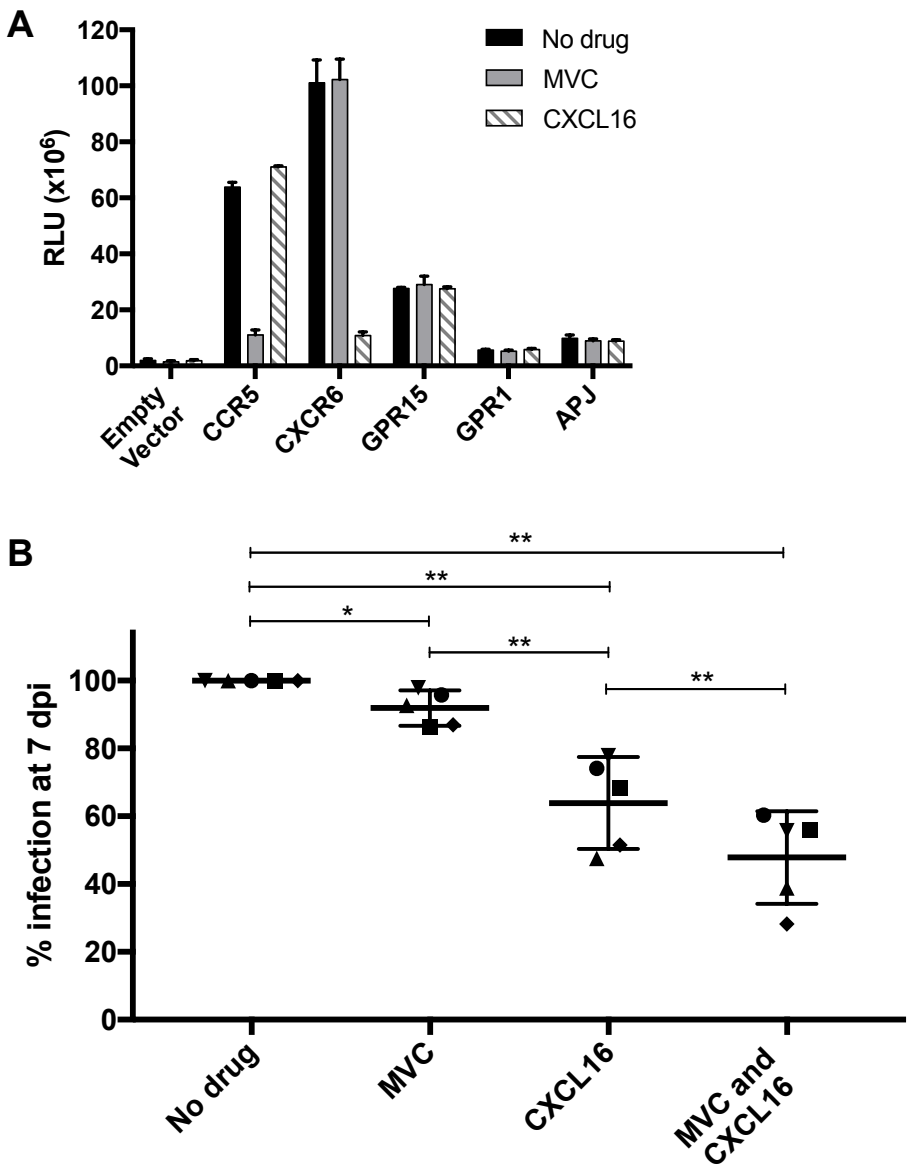

Figure 2.4: Blocking entry through CXCR6 limits SIVagmSab92018ivTF replication in sabaeus lymphocytes. A) 293T cells were transfected with plasmids encoding sabaeus CD4-31 and one of the following: sabaeus CCR5, CXCR6, GPR15, GPR1, APJ or empty vector. Cells were treated with 15uM maraviroc, 500ng/mL human CXCL16 or vehicle alone for one hour and then infected with the luciferase reporter pseudotype containing the SIVagmSab 92018ivTF Env. Cells were lysed 72 hours post-infection and luciferase content was read by measuring relative light units (RLU). Infections were carried out in triplicate and data (means ± standard deviation) are representative of 3 replicate experiments. B) Sabaeus PBMC were stimulated for 3 days with 5ug/mL PHA and 100U/mL IL-2, then treated with vehicle alone, 15uM maraviroc, 500ng/mL human CXCL16 or both blocking agents for one hour. Cells were then infected in duplicate with SIVagmSab92018ivTF generated from an infectious molecular clone. Infection was measured by p27 production in supernatant at day 7, and shown for each treatment as a percent of vehicle alone (no drug). Each symbol represents data from a different animal's PBMC, and shown are median and standard deviation values for each condition. *, $P<0.05$; **, $P< 0.01$ (two-tailed paired t-test). (Figure originally printed in Wetzel et al, JVI 2017.)

Discussion

In contrast to HIV-1 and SIVmac-infected nonprogressors that avoid pathogenesis mainly by controlling viremia, natural hosts do not experience disease while maintaining high viral loads, which is likely the result of prolonged coevolution with their SIVs. Recent evidence suggests that one mechanism for this benign coexistence is linked to patterns cell targeting within such hosts (130). Since cell targeting is determined largely by entry, in this study I aimed to identify entry pathways used by the natural host virus SIVagmSab. I found that CXCR6 was used as a coreceptor, in addition to CCR5, by diverse SIVagmSab Envs, including those isolated from wild, naturally infected animals. GPR15 was also used, albeit less efficiently. Robust CXCR6 use occurred at both high and low CD4 levels. Blocking CXCR6 inhibited SIVagmSab replication in PBMC, confirming that it not only functions *in vitro*, but also mediates infection of primary target cells relevant to infection *in vivo*.

In vitro use of alternative coreceptors by various SIVs was first observed years ago, and many studies used human, not species-matched molecules (96-98). However, CCR5 was long thought to be the only coreceptor relevant *in vivo*. One initial exception was SIVrcm that infects the natural host red-capped mangabeys (RCM). Many RCM are CCR5-null due to a common deletion allele, yet SIVrcm was found to use CXCR6 and CCR2b, and not CCR5, to enter cells (99, 100). More recently, our lab identified a common CCR5 deletion allele in sooty mangabeys (SM) (104); we found that homozygous, CCR5-null animals were infected at a similar frequency to wild-type CCR5 SM and maintained nearly equivalent viral loads. These data demonstrate a lack of CCR5-dependence of SIVsmm, and later studies identified CXCR6 as an additional coreceptor for SIVsmm isolated from both wild type CCR5 and CCR5-null SM (105, 107). A lack of CCR5-dependence was also observed for the natural host virus SIVagmVer, as blocking entry through CCR5 did not hinder replication in vervet PBMC (116). Alongside these data demonstrating non-CCR5 pathway use by SIVsmm and SIVagmVer, this study bolsters a paradigm of alternative coreceptor usage by natural host viruses. This provides a stark contrast to

pathogenic SIVmac infection of macaques and HIV-1 infections of humans, which is largely restricted to use of CCR5 as a coreceptor (89-91, 107, 116, 131, 132).

Natural hosts species, including African green monkeys, are characterized by a low frequency of CCR5+ CD4+ T cells, and reduced expression of CCR5 on critical CD4+ T cell subsets has been implicated as a mechanism by which such subsets are protected from infection (67, 72, 73). However, this observation also raised the question as to how natural hosts are able to sustain high viral loads with a reduced pool of target cells. The identification of CXCR6 as an additional coreceptor for SIVagmSab (and SIVsmm) may answer this question, as CD4+ T cells that lack CCR5 but express CXCR6 (or other alternative coreceptors) would be target cells that were previously unappreciated.

The use of alternative coreceptors in CCR5-low natural hosts likely also contributes to distinct outcomes of infection as compared to the CCR5-dependent infection of non-natural hosts. Unique features of natural host infection that contribute to the lack of pathogenesis include resolution of immune activation after acute infection, lack of microbial translocation, maintenance of gut immunity and integrity, reduced lymph node infection and inflammation and a maintenance of peripheral CD4+ T cells (112, 113). One identified mechanism that contributes to these distinct features is divergent cell targeting by natural host SIVs. While both natural and non-natural SIVs replicate in cells that turnover quickly, central memory (Tcm) and stem cell memory (Tscm) CD4+ T cells are relatively spared from infection in sooty mangabeys, but not rhesus macaques (72, 73). This preservation likely supports the maintenance of CD4+ T cell homeostasis in natural hosts. In lymph nodes, T follicular helper cells (Tfh) that participate in germinal center reactions are infected at a lower frequency in natural hosts than non-natural hosts, likely contributing the absence of inflammation and fibrosis in this compartment (56). In the gut, the SIV infection status of Th17 cells in the gut of natural hosts has not been characterized, but Th17 cells are maintained in SIV infection of SM and AGM (77). In SIVmac infection of RM and HIV-1 infection in humans,

Th17 cells are lost, which is associated with the disruption of barrier integrity, microbial translocation and chronic immune activation (77-79). Thus, expression of these alternative coreceptors such as CXCR6 is likely restricted on and within these critical, relatively protected CD4+ subsets and tissues, and instead found on more expendable CD4+ cells.

To accurately define targets cells of SIV, it will be critical to determine CXCR6 expression patterns of CD4+ cells in natural hosts. CXCR6 is thought to be an extralymphoid homing receptor, contributing to adhesion in the periphery via interactions with its ligand CXCL16 (133-135). In humans, enrichment of CXCR6+ cells is common at sites of inflammation, and CXCR6 expression has been described mainly on memory CD4+ T cells, although expression on naïve cells has also been reported (136-139). Studies of CXCR6 surface expression on nonhuman primate cells have not been performed due to a lack of cross-reactive reagents. Recently, Riddick *et al* measured CXCR6 mRNA in vervet AGM CD4+ T cells and found expression in both naïve and memory CD4+ T cell subsets (116). However, to accurately identify natural host SIV target cells, it will be necessary to define CXCR6 surface expression on CD4+ cells in these animals. Such CXCR6+CD4+ T cells likely support viremia, while CD4+ T cells that lack CXCR6 (and CCR5) are protected from infection and able to maintain immune system function.

Blocking entry through CCR5 and CXCR6 using MVC and CXCL16, respectively, was not sufficient to completely inhibit replication of SIVagmSab92018ivTF in sabaeus lymphocytes. This may be due to incompletely blocking of these pathways by maraviroc and CXCL16 (Figure 2.4A), but SIVagmSab could also use additional coreceptors. Of the coreceptors tested, GPR15 is the most likely candidate as it is used nearly as efficiently as CCR5 and CXCR6 (Figure 2.3) and is expressed on human CD4+ T cells (140). Interestingly, SIVmac, the descendent of SIVsmm, is able to use RM GPR15 in an *in vitro* transfection system (107). However, use of GPR15 *ex vivo* has not been observed, as blocking CCR5 is sufficient to inhibit replication in RM PBMC (107, 116), and concordant results have been described *in vivo (141)*. Therefore, use of GPR15 *in vitro*

may not reflect use *in vivo* and must be tested directly. In this study, I was unable to block entry through GPR15 as its ligand has not been identified and no small molecule against this receptor is available. Future studies using GPR15 knockdown or deletion approaches in sabaeus PBMC would address its use. It is unlikely that GPR1 and APJ permit entry of SIVagmSab in sabaeus PBMC, as their use *in vitro* was minimal and studies of humans and rodents do not describe expression on relevant CD4+ target cells (142-146). Lastly, while the panel of coreceptors tested here encompassed the major non-CCR5 coreceptors for HIV and SIV described to date, it is possible that entry is permitted by a currently unknown 7TMR.

The sabaeus AGM whose cells were included in this study were polymorphic for CCR5, concordant with previous observations, and several animals were heterozygous for a polymorphism that has been shown to limit SIVagm replication *in vitro* (93R) (98, 129). However, no obvious replication defect was observed in PBMC from these animals. Also, AGM maintain a low frequency of CCR5+CD4+ T cells (98, 101). Together, these observations suggest a long history of coevolution between SIVagm and its host, driven by deleterious consequences of CCR5-mediated infection. In contrast, all CXCR6 alleles sequenced here were identical, suggesting distinct evolutionary pressures have been exerted on these two coreceptors. I speculate that the lack of AGM CXCR6 diversity despite prolonged AGM/SIVagm coexistence reflects a benign outcome when CXCR6 is used to enter target cells.

Use of non-CCR5 pathways has now been observed for three distinct natural host viruses, SIVagmVer, SIVsmm, and SIVagmSab, and CXCR6 use in lymphocyte infection has been confirmed for the latter two. Importantly, these viruses are quite diverse; SIVagm and SIVsmm represent two distinct SIV lineages and molecular clock analyses combined with biogeographic calibration place their most recent common ancestor to over 100,000 years ago (36, 38). Furthermore, SIVagmSab and SIVagmVer infect nonsympatric subspecies of African green monkeys in West and East/Southern Africa, respectively, and also diverged over 100,000 years

ago (36). Despite this distance, use of non-CCR5 entry pathways is a common feature among these viruses. This suggests that use of such pathways is the norm among natural host SIVs that have coevolved with their hosts. In contrast, restriction to CCR5 use only occurs in more recent or zoonotic infections, such as SIVmac or HIV-1. In these newer hosts, CCR5+ CD4+ cells are abundant, perhaps due to lack of evolution with CCR5-using viruses, and immunodeficiency ultimately develops. It is not clear why CXCR6 use might be lost, and virus thus restricted to CCR5, but may be linked to factors required to overcome cross-species transmission barriers. For SIVmac, which is the product of cross species transmission of SIVsmm into macaques, the source of the restriction is known; RM CXCR6 encodes a polymorphism in the N terminus of CXCR6, S31R, which renders it a poor coreceptor (106). For HIV-1, features of Env prevent CXCR6 use, although rare CXCR6-using isolates have been reported (97, 147). To determine changes that may have occurred in coreceptor usage in the emergence of HIV-1, it will be necessary to define the coreceptor usage patterns of SIVgsn/mus/mon, the natural host forerunner of *env* of SIVcpz, which gave rise to HIV-1 (39, 41, 45, 148).

In summary, this study supports a model of SIV/HIV tropism where in non-natural hosts, an abundance of CCR5+ cells results in the infection and disruption of many CD4+ T cell subsets and tissues and leads to immunodeficiency. In contrast, in natural host infection, where CCR5+ cells are infrequent, use of CXCR6 (and possible other coreceptors) directs SIV towards cells that are able to support viremia without immunodeficiency. Defining CXCR6+ cells, both in blood and tissues will be essential to understanding natural host cell targeting.

Materials and Methods

Sabaeus African green monkey PBMC

PBMC from sabaeus AGM (from a Caribbean- born colony) were isolated from whole blood as previously described (101), cryopreserved and thawed before use. Animals were housed at the University of Pittsburgh in accordance with the Guide for the Care and Use of Laboratory Animals (149), the Association for Assessment and Accreditation of Laboratory Animal Care, and the Animal Welfare Act. Animal procedures were approved by the IACUC of the University of Pittsburgh.

Cloning of sabaeus CD4 and coreceptor molecules

Genomic DNA was isolated from resting sabaeus AGM PBMC (n=1, AGM 30) using the QIAamp DNA Blood Mini Kit (Qiagen) and RNA was isolated from both resting and concanavalin A (ConA)/IL-2 stimulated PBMC (n=2, AGM 30 and AGM 31) using the RNeasy Plus Kit, (Qiagen). cDNA was synthesized using the Superscript III First Strand Kit (Invitrogen) using random hexamers (for CD4), oligodT (for CXCR4) or gene-specific primers (for CCR2b and CCR4). Primers for 7TMR cDNA synthesis and PCR amplification were previously described (28) as were the CD4 primers (AGM CD4 for2 and AGM CD4 rev) (29). Sabaeus CD4 and full-length 7TMRs were amplified from genomic DNA (APJ, CCR3, CCR5, CCR8, CXCR6, GPR1, GPR15) or cDNA (CD4, CCR2b, CCR4, and CXCR4) using Phusion High Fidelity DNA polymerase (New England BioLabs). Coreceptors were amplified from AGM 30. Reactions included 50ng gDNA or 2uL cDNA synthesis reaction, and cycling conditions followed kit protocol as previously reported (105), but annealing temperatures were modified as follows: 64°C (APJ, CCR3, CCR4, CCR8, CXCR6, GPR1, GPR15, CXCR4) 60°C (CCR2b) 61°C (CCR5) or 72°C (CD4). Amplified genes were ligated into pcDNA3.1+ using dual restriction enzyme digest (CCR2b, CCR3, CCR4, CCR8 or CXCR6), or into the pcDNA3.1D/V5-His-TOPO vector by TOPO Directional cloning (CD4, APJ, GPR1, CCR5, CXCR4 or GPR15) using the pcDNA3.1 Directional TOPO Expression Kit (Invitrogen). Clones were screened by restriction digest analysis and confirmed by Sanger

nucleotide sequencing. Nucleotide and amino acid alignments were performed using the ClustalW algorithm in MacVector 13.5.2. 7TMR membrane topology predictions were made using TMpred (http://www.ch.embnet.org /software/TMPRED_form.html). Expression of coreceptor molecules was ensured by measuring entry into target cells by previously described promiscuous SIVsmm pseudotypes (27) or by flow cytometry (CCR2b, CCR4).

Cloning of SIVagmSab envelopes

Plasma samples were collected in Senegal from sabaeus AGM (89042 and 89044) that were SIV+ upon capture, and stored at the Institut Pasteur. Viral RNA was isolated from plasma samples using Viral RNA Mini Kit (Qiagen) and cDNA was synthesized using Superscript III reagents and a gene-specific primer (5'-CTCCWCCCTGGAAAGTCCCKCT-3') modified from a previously reported primer (119). SIVagmSab Envs were amplified in 50uL PCR reactions using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and forward (5' **CACC**CCSCTCCAGGCCTGTRNCAATA-3') and reverse (5'-CCARCCATCSACWATDCCCC-3') primers designed from published SIVagmSab sequences using Primer3 (150), with the bases required for TOPO Directional cloning in bold. . 3kb amplicons were cloned into pcDNA3.1D/V5-His-TOPO using the pcDNA3.1 Directional TOPO Expression Kit (Invitrogen). Clones were screened by colony PCR, and successfully ligated clones were tested for function on transfected 293T cells expressing sabaeus CD4 and CCR5 or CXCR6.

SIVagmSab Env sequencing and phylogenetic analyses

SIVagmSab envelopes 89042 and 89044 were sequenced using a MiSeq sequencer (Illumina) and reads were aligned using Geneious 7.1.7 software (Biomatters Ltd). A maximum likelihood phylogenetic tree was generated using only the overlapping portion (1882 bp) of previously published SIVagm sequences and the two novel *envs*. Sequences were first aligned using MAFFT (35) and jModelTest (36) was used to determine the best-fit substitution model. The phylogenetic tree was generated with PhyML v3.0 (37, 38) using the General Time Reversible

(GTR) substitution model and concordantly estimating the proportion of variable sites and the gamma distribution parameter from the sequence data and the phylogeny. 1000 bootstrap replicates were performed. Figtree v1.4.2 was used to visualize the tree, which is rooted at the midpoint (http://tree.bio.ed.ac.uk/software/figtree/). The scale bar represents 0.2 nucleotide substitutions per site.

Coreceptor functional analyses using pseudotyped virus

Pseudotype reporter viruses were generated by transfecting 293T cells with an SIVagmSab Env plasmid and a backbone pNL43-Luc-E-R+ plasmid using Fugene 6 transfection reagent (Promega) as previously described (105). The following day, cells were washed with PBS and fresh supplemented DMEM was added. 48 hours later, supernatant was collected and spun with 5% sucrose to pellet cell debris. p24 ELISA was performed on all stocks to allow normalization for infections.

HEK 293T cells were maintained in Dulbecco's modified Eagle medium supplemented with 10%FBS, 1% L-glutamine and 1% penicillin/streptomycin. 2.5e5 293T cells/ well were plated in 12-well plates and transfected with 250ng CD4 and 250ug coreceptor/empty pcDNA3.1+ (except where otherwise noted) using Fugene 6 reagent. The following day, cells were lifted, washed and replated at 2e4 cells/well in 100uL/well in 96-well plates. The following day, SIVagmSab Env pseudotypes were incubated with 50 units/mL DNase (Roche) for 15 minutes, and pseudotypes normalized by p24 amount were added/well. Amount of virus/well varied slightly between experiments, as follows: CD4 comparison, (Fig 1) 34ng; Env comparison and titration experiments (Figs. 2B, 3) 10ng; Coreceptor blocking (Fig. 4A) 4ng. Plates were spinoculated for 2 hours at 1200g and incubated for 72 hours at 37°C 5% CO2. To measure entry, cells were lysed with 50uL 0.5% Triton X in PBS, and luciferase content read by adding 50uL luciferase reagent (Luciferase Assay System, Promega) and reading RLU on a Luminoskan Ascent (Thermo Labsystems). For all experiments, target cells were infected in parallel with pseudotypes

expressing the vesicular stomatitis virus glycoprotein (VSVg) to ensure differences in entry were coreceptor specific.

To confirm that equivalent amounts of the coreceptor plasmid yielded similar levels of expression, and that titration experiments resulted in similar decreases in expression levels for different coreceptors, 293T cells were transfected with human CCR5, CXCR6, and GPR15 and stained by FACS analysis using anti-human coreceptor antibodies 3A9 (CCR5; BD Pharmingen), K041E5 (CXCR6; BioLegend), and 367902 (GPR15; R&D Systems). Validation of AGM CD4 plasmid titration experiments was done similarly, using antibody L200 (BD Pharmingen).

To test the function and specificity of coreceptor blocking agents, transfected target cells were pretreated for one hour at 37°C with the following treatments (values represent final concentration): No drug (PBS and DMSO), 15uM maraviroc (in DMSO, with PBS added) (NIH AIDS Reagent Program) 500ng/mL human recombinant CXCL16 (in PBS, with DMSO added) (R&D Systems) and both maraviroc and CXCL16.

Sabaeus PBMC infection

Cryopreserved sabaeus PBMCs were thawed and stimulated with 5ug/mL PHA-P (Sigma) in RPMI Medium 1640 supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin. 100U/mL IL-2 was added the following day. After 72 hours of stimulation, 2e5 cells/well were plated in a 96-well round bottom plate in duplicate and incubated for one hour at 37°C with the following treatments (values represent final concentration): No drug (PBS and DMSO), 15uM maraviroc (in DMSO, with PBS added), 500ng/mL human recombinant CXCL16 (in PBS, with DMSO added) and both maraviroc and CXCL16. Treatments occurred in complete RPMI supplemented with 100U/mL IL-2. Following incubation, 5ng p27 SIVagmSab 92018ivTF was added and cells were spinoculated at 1200g for 90 minutes, then incubated at 37°C/5%CO2 overnight. The following morning, cells were lifted and washed two times in complete RPMI, and

the final wash was collected for Day 0 measurement. Cells were resuspended in 200uL complete RPMI plus 100U/mL IL-2 and appropriate treatment, and 100uL (50%) supernatant was collected and replaced with IL-2/treatment media on days 3, 7, 10, and 14. Virus replication was measured by p27 Gag ELISA (Perkin-Elmer). For infections lacking blocking agents, p27 values at day 7 ranged from 5000-16000pg/mL.

Accession numbers. Sabaeus AGM CD4 and coreceptor sequences cloned here were deposited in GenBank under accession numbers KY225904 to KY225915. SIVagmSab *env* sequences were deposited in GenBank under accession numbers KY225916 and KY225917.

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CHAPTER 3

CXCR6 Use by SIVmus, but not SIVcpz, Suggests Coreceptor Bottleneck Occurred in HIV-1 Emergence

Abstract

HIV-1 originated from the cross-species transmission of SIVcpz, which infects chimpanzees, into humans. SIVcpz is the product of a recombination event between two viruses that crossed the species barrier, with a virus of the SIVgsn/mus/mon lineage contributing the envelope gene (*env)*. SIVgsn/mus/mon infects greater spot-nosed monkeys, mustached monkeys and mona monkeys, respectively, which are considered "natural hosts" of these species-specific SIVs. While these species have not been studied in detail, SIV-infected natural hosts in general maintain high viral loads but do not progress to AIDS. In contrast, SIVcpz is pathogenic in its host, like HIV-1 in humans. Recent studies from our lab and others have indicated that use of the entry coreceptor CXCR6 in addition to CCR5 is a common feature of natural host SIVs, including SIVsmm that infects sooty mangabeys and SIVagm that infects African green monkeys. However, the use of species-matched coreceptors by SIVcpz and SIVgsn/mus/mon are not known. In this study, I defined the coreceptor usage patterns of the two HIV-1 *env* forerunners. I found that SIVcpz was restricted to use of species-matched CCR5 for entry, like HIV-1 in humans, whereas SIVmus was able to use both CCR5 and CXCR6, like other natural host viruses. Coreceptor use was determined by Env, not coreceptor sequence, with a V3 loop Pro residue contributing to CCR5 restriction. These data bolster the finding that CXCR6 use is common among natural host viruses, extending it to the natural host forerunners of the HIV-1/SIVcpz lineage, and show that use thereof can be lost upon cross-species transmission. This coreceptor bottleneck in chimpanzees may have altered cellular targeting, thereby disrupting the equilibrium between virus and host observed in natural host infections and instead promoting pathogenicity.

Introduction

HIV-1, the virus that causes AIDS, originated from the cross-species transmission of the simian immunodeficiency virus (SIV) that infects chimpanzees (*Pan troglodytes*), SIVcpz, into humans (1, 2). SIVcpz is the product of the recombination of two SIVs that crossed the species barrier from their natural hosts into chimpanzees: SIVrcm that infects red-capped mangabeys (RCM, *Cercocebus torquatus*) and an SIV of the lineage SIVgsn/mus/mon which infects greater spot-nosed monkeys (GSN, *Cercopithecus nictitans*), mustached monkeys (MUS, *Cercopithecus cephus*) and mona monkeys (MON, *Cercopithecus mona*), respectively (3-5). The 5' half of SIVcpz, which encodes *gag* and *pol,* is derived from SIVrcm, while the 3' half of SIVcpz, which encodes *env,* is derived from SIVgsn/mus/mon. RCM, GSN, MUS and MON are termed natural hosts of SIV, as they are infected with species-specific strains of virus, but are not thought to progress to immunodeficiency, akin to the well-studied natural hosts sooty mangabeys (SM, *Cercocebus atys*) and African green monkeys (AGM, *Chlorocebus spp*.) (6, 7). This lack of pathogenicity is thought to be the product of many years of virus-host coevolution, and contrasts with "non-natural" HIV-1 infection in humans that leads to AIDS without treatment, as well as SIVcpz infection of chimpanzees, where less severe but clear pathology has been observed in the wild (7, 8).

Like non-natural hosts, infected natural hosts maintain high viral loads and frequent turnover of infected cells, demonstrating that lack of disease progression is not due to control of the virus (9-11). Despite high viral loads, natural hosts do not endure many of the features of pathogenic infection observed in infected humans and SIVmac-infected rhesus macaques (RM, *Macaca mulatta*), the animal model for HIV-1 infection, such as widespread loss of virus target CD4+ T cells, lymph node inflammation and fibrosis, gut barrier breakdown and microbial translocation, and chronic immune activation (12). Instead, CD4+ T cell loss is limited to the gut and recovers, lymph node structure and function is maintained, gut integrity is preserved, and immune activation resolves after acute infection (6, 13). One mechanism implicated in the lack of disease

progression is limited infection and/or loss of certain critical CD4+ T cells, which preserves immune function. Such critical CD4+ T cell subsets include central memory (Tcm) and stem-cell memory (Tscm) CD4+ T cells, which maintain memory CD4+ T cell homeostasis, T follicular helper cells (Tfh) that reside in lymphoid tissues and provide B cell help, and Th17 cells, which maintain the gut barrier and immune functions (14-17). This subset protection from infection and loss likely allows natural hosts to avoid immunopathology despite infection of immune system cells, and clearly suggests that to understand pathogenesis, we must first understand the determinants of SIV cellular targeting in natural hosts, and how they may have changed as HIV-1 emerged from several cross-species transmission events.

HIV and SIV target cells are largely defined by expression of the receptor CD4, and a 7 transmembrane G protein coupled receptor (7TMR) coreceptor, both of which are engaged by the Env glycoprotein to permit entry. HIV-1 enters cells using the chemokine receptor CCR5 as a coreceptor, which is required for human infection (18-20), although HIV-1 sometimes evolves later to use CXCR4 in addition to or instead of CCR5 (21, 22). SIVs use CCR5 for entry, but very rarely use CXCR4 (23-25). While it was long known that SIV can enter cells using additional 7TMRs *in vitro*, including CXCR6 and GPR15, these studies often used coreceptors of human origin, and accepted dogma was that CCR5 was the sole coreceptor used by SIVs (26-28). Studies of several natural host species have revealed that natural hosts CD4+ T cells infrequently express CCR5, possibly as a mechanism to protect CD4+ T cells from SIV infection (29). However, this raised the question as to how natural hosts are able to maintain such high viral loads with limited target cells and rapid turnover of infected cells.

One exception to the established dogma was SIVrcm that infects the natural host red-capped mangabey (RCM). RCM are frequently CCR5-null due to a common deletion allele, and SIVrcm cannot use CCR5 for entry, but instead enters cells via CXCR6 and CCR2b. More recently, our lab identified a CCR5 deletion allele that is common among sooty mangabeys at Yerkes National

Primate Research Center, which also rendered homozygous animals CCR5-null. Despite lacking CCR5 on the cell surface, these animals were infected with SIVsmm at a similar frequency to wild type animals and were able to maintain high viral loads, indicating that CCR5 is not necessary for infection of sooty mangabeys. Further investigation determined that CXCR6 permits SIVsmm entry in addition to CCR5, as studies showed robust use of CXCR6 of SM origin by SIVsmm *in vitro,* and blocking use of CXCR6 with its ligand CXCL16 limited SIVsmm replication in SM lymphocytes (30, 31). Our study of a second natural host, AGM of the subtype sabaeus, revealed that sabaeus CXCR6 is a robust coreceptor for SIVagmSab *in vitro*, and like in SM, blocking use of CXCR6 via CXCL16 treatment limited replication in sabaeus lymphocytes (Chapter 2) (32). *In vitro* use of species-matched CXCR6 was found in a third natural host, AGM of the subtype vervet (33). Therefore, CXCR6 use (in addition to CCR5 use) appears to be a common feature of viruses that infect CCR5-low natural hosts.

As mentioned above, the SIV that gave rise to *env* of pathogenic SIVcpz and HIV-1 is the natural host virus SIVgsn/mus/mon. However, it is not known if SIVgsn/mus/mon can use species-matched CXCR6 for entry like other natural host viruses. Furthermore, the ability of SIVcpz to use species-matched coreceptors in addition to CCR5 is also unknown. Therefore, in this study, I tested the ability of SIVmus and SIVcpz to use species-matched CCR5, CXCR6, GPR15, and CXCR4 *in vitro*. I found that like HIV-1, SIVcpz was restricted to use of CCR5 for entry. In contrast, SIVmus was able to enter cells using species-matched CXCR6 in addition to CCR5, and experiments where species coreceptors were swapped indicated that CXCR6 use was determined by Env. Surprisingly, SIVmus was unable to enter cells expressing chimpanzee CD4. I also compared these closely related viruses to probe Env determinants of coreceptor usage and found that residue 326P, located in the V3 loop of SIVcpz and HIV-1 Env, is incompatible with CXCR6 use. Together, these data demonstrate that CXCR6 use is a feature of the natural host virus SIVmus, and that loss of use of this coreceptor coincided with the development of pathogenesis as the virus transmitted between hosts to give rise to SIVcpz, and then HIV-1.

Results

Cloning and analysis of candidate coreceptors and CD4 from chimpanzee, mustached monkey and greater spot-nosed monkey

I first aimed to define coreceptor usage patterns of HIV-1 ancestors SIVcpz and SIVgsn/mus/mon. Given that single amino acid changes can alter coreceptor functionality (34), it is necessary to test SIVcpz and SIVgsn/mus/mon on species-matched candidate 7TMR and CD4 to accurately identify virus coreceptors. I focused the analysis on CXCR6, a robust coreceptor for several natural host SIVs, GPR15, a more moderate natural host SIV receptor, and CXCR4, which is used by certain HIV-1 isolates but not SIVs, in addition to the canonical coreceptor CCR5 (30-32).

I amplified cpzCCR5, cpzCXCR6, cpzGPR15 and cpzCXCR4 from genomic DNA or cDNA from one *Pan troglodytes verus* chimpanzee using primers that lie outside the open reading frame (ORF) of each gene (30), and cloned each into an expression vector. I cloned the candidate coreceptors from the *P.t. verus* subspecies of chimpanzee because they are housed at Yerkes National Primate Research Center and the stored samples were readily available; however, *P.t. verus* are not infected with SIVcpz in the wild like the subspecies *P.t. troglodytes* or *P.t. schweinfurthii* (2, 35). Therefore, I asked whether the cloned *P.t. verus* coreceptors were indeed representative of the latter two subspecies. The cloned *P.t. verus* coreceptor genes were sequenced and aligned to published deep sequencing data from four *P.t. troglodytes* and six *P.t. schweinfurthii* chimpanzees (data not shown) (36). The cpzCCR5 and cpzCXCR4 clones were also compared to published *P.t. troglodytes* alleles (37). This analysis revealed that the *P.t. verus*. candidate coreceptors represented alleles of the *P.t. troglodytes* and *P.t. schweinfurthii* at the amino acid level; I identified at least 1 synonymous SNPs between the chimpanzee subspecies per coreceptor, but only one nonsynonymous SNP, which occurred in GPR15. Two *P.t. troglodytes* chimpanzees were heterozygous for GPR15, with one allele matching the *P.t. verus* clone by encoding S275, while the other encoded P275; this residue lies in the third extracellular

loop (ECL3). Also, the CXCR6 and GPR15 amino acid sequences cloned here match previously published chimpanzee alleles (subspecies unknown) (38). In summary, the *P.t. verus* candidate coreceptor genes cloned here also reflect *P.t. troglodytes* and *P.t. schweinfurthii* coreceptors, and are thus appropriate for SIVcpz analysis. For cpzCCR5 and cpzCXCR6, the sequences of the N-terminus and second extracellular loop (ECL2), the domains likely involved in coreceptor-Env interactions based on studies of HIV-1 and human coreceptors (39), are shown in Figure 3.1.

The *env* gene of SIVcpz is derived from a member of the lineage SIVgsn/mus/mon. Available biological samples of these species are limited to stored gDNA extracted from rare bushmeat samples (5, 40, 41). Therefore, I focused on MUS and GSN for analysis because we were able to obtain bushmeat specimens from these species.

Coreceptors of interest that are encoded by a single exon (CCR5, CXCR6 and GPR15) were cloned from MUS 1085 and GSN 1365 using previously described primers (30). To clone CXCR4, which has two exons, both exons were amplified individually and spliced together via PCR as described in Methods. Clones were sequenced and when possible, compared to previously described MUS and GSN protein sequences. musCCR5 was different from the one previously sequenced allele by encoding a Pro at position 35 instead of a Leu (42). gsnCCR5 is polymorphic, and the molecule cloned here matches several previously described alleles at the amino acid level (42). No sequences for mus or gsnCXCR6, GPR15 or CXCR4 have been previously reported. musCXCR6 and gsnCXCR6 were nearly identical, but musCXCR6 encoded Arg at position 31, which is the same amino acid that renders rmCXCR6 a poor coreceptor for SIVmac (34). In contrast, gsnCXCR6 encoded the more common Ser residue at position 31 that is associated with robust coreceptor function. The alignment of the N-terminus and ECL2 of mus and gsnCCR5 and CXCR6 with those of human and chimpanzee are shown in Figure 3.1. The N-termini of mus and gsnCXCR6 are quite distinct from the N-termini of the human and chimpanzee molecules, differing in identity by at least five residues.

To generate musCD4 and gsnCD4, I amplified and sequenced the nine CD4 exons from gDNA from two MUS (1085 and 1246) and two GSN (42 and 1289), and musCD4 and gsnCD4 were then synthesized and inserted into expression vectors. Both musCD4 and gsnCD4 differed from human CD4 at 16 of 109 amino acid residues in domain 1, which interacts with the SIV/HIV Env (43). Chimpanzee CD4 has been cloned previously and was provided by B. Hahn and F. Bibollet-Ruche (44). cpzCD4 differs from human CD4 at three residues in this domain. Overall, the differences in CD4 sequence, as well as coreceptor sequence, underscore the importance of testing coreceptor usage of viruses on their species-matched molecules.

SIVcpz is restricted to use of cpzCCR5 for entry, while SIVmus can enter using musCXCR6 as well as musCCR5

To test coreceptor usage of SIVcpz, expression vectors containing cpzCD4 and each CPZ candidate coreceptor were transfected into CF2th-Luc cells that contain a Tat-driven luciferase reporter. Cells were then infected with SIVcpz derived from four diverse infectious molecular clones: SIVcpz*Pts* BF1167, isolated from *P.t. schweinfurthii,* or SIVcpz*Ptt* EK505, MB897, or MT145, all isolated from *P.t. troglodytes.* Virus entry was quantified by luciferase production measured in relative light units. For all SIVcpz isolates tested, only cells expressing cpzCD4 and cpzCCR5 permitted greater entry than cells transfected with cpzCD4 alone (Figure 3.2A, left panel). To confirm expression of the candidate coreceptors from the expression plasmids, I transfected 293T cells with these constructs, and infected them with a luciferase reporter virus carrying an unusual promiscuous SIVsmm Env that can use multiple coreceptors for entry (Figure 2A, right panel)(30). I observed robust entry through all of these CPZ coreceptors, demonstrating functional expression. Therefore, SIVcpz is similar to HIV-1 but unlike natural host SIVs, and is restricted to use of species-matched CCR5 as a coreceptor and does not use CXCR6 or GPR15.

Since no SIVmus or SIVgsn envelopes or infectious molecular clones have been isolated for use in functional experiments to date, testing coreceptor usage first required amplification of functional *envs*. I was able to amplify *envs* from gDNA from two SIVmus-infected MUS (1085 and 1246) (5, 40), and cloned these *envs* into expression vectors. Five *envs* (SIVmus1085) cloned from MUS 1085 functioned robustly, while those from MUS 1246 (SIVmus1246) functioned only very weakly (data not shown). I therefore proceeded to test coreceptor use of 3 *envs* amplified from MUS 1085 (1-54, 4-1 and 4-12), which ranged from 96 to 98% amino acid identity to each other and to the previously described SIV *env* sequence from this animal (5).

To test SIVmus1085 coreceptor use, 293T cells were transfected with expression plasmids containing musCD4 and MUS coreceptor, or gsnCD4 and GSN coreceptor, and infected with luciferase reporter pseudotypes carrying the SIVmus1085 Envs. In contrast to SIVcpz, SIVmus was able to enter cells expressing species-matched CXCR6 in addition to CCR5; this was observed for the species-matched MUS coreceptors as well as the closely related GSN coreceptors (Figure 3.2B-C, left panels). The R31 residue that restricts entry of SIVmac through rmCXCR6 did not restrict entry of SIVmus1085 through musCXCR6. Neither mus/gsnGPR15 nor mus/gsnCXCR4 were used for entry. Robust entry of the previously mentioned promiscuous SIVsmm (30) into target cells expressing all tested coreceptors demonstrates expression of these molecules on the cell surface (Figure 3.2B-C, right panels). Therefore, SIVmus1085 enters cells expressing species-matched CXCR6 and CCR5 (as well as gsnCXCR6 and gsnCCR5), like other natural host viruses. In contrast SIVcpz only enters cells expressing species-matched CCR5, like HIV-1. Since I observed consistent coreceptor use patterns among the all SIVcpz and SIVmus strains tested, I performed further analysis with pseudotyped SIVcpzMT145 and SIVmus1085 1-54.

Coreceptor usage pattern is determined by Env, not coreceptor species origin

The inability of SIVcpz to use cpzCXCR6 could be attributable to either the SIVcpz Env, or the species origin (and thus amino acid sequence) of the CXCR6 molecule. Therefore, I first probed coreceptor origin by testing the ability of SIVcpz to enter cells expressing musCD4 and musCXCR6, which permitted SIVmus entry (Fig 1B). I transfected 293T cells with musCD4 along with empty vector, musCCR5 or musCXCR6, or cpzCD4 along with empty vector, cpzCCR5 or cpzCXCR6. Cells were then infected with a luciferase reporter virus expressing the SIVcpz MT145 Env. The SIVcpz MT145 pseudotype entered cells expressing CD4 and CCR5 from both MUS and CPZ, but not those expressing CD4 and CXCR6 (Figure 3.3A). This result suggests that the inability of SIVcpz to enter through CXCR6 is Env determined. To ask if cpzCXCR6 could function as a coreceptor for viruses in this lineage, I then infected 293T cells expressing musCD4 and empty vector, musCCR5 or CXCR6, or cpzCCR5 or CXCR6, with reporter pseudotypes expressing SIVmus1085. SIVmus1085 entered cells expressing musCD4 and each of the coreceptors, although entry through cpzCXCR6 was the least robust (Figure 3B). Together, these data suggest that the inability of SIVcpz to use CXCR6 is a property of Env, and is not because of intrinsic poor coreceptor function of cpzCXCR6.

Finally, I tested the ability of SIVmus to enter cells expressing cpzCD4 and cpzCXCR6, in case this combination of CD4 and coreceptor is not permissive to viruses of this lineage. Surprisingly, while SIVmus1085 could enter cells expressing musCD4 and CPZ coreceptors, it could not enter cells expressing cpzCD4 and CPZ coreceptors, as no entry above background RLU was observed (Figure 3.4A). I then tested additional cpzCD4 alleles that vary at positions 52, 55 and 68, but found that none were able to permit entry (data not shown). This SIVmus entry defect could be slightly rescued when human CD4, which varies from cpzCD4 by 4 total amino acid residues, was used instead (Figure 3.4A). In sharp contrast, SIVcpz exhibited no apparent species CD4 preference, readily entering cells expressing musCD4, cpzCD4, or huCD4, in

conjunction with cpzCCR5. Therefore, although SIVmus can enter cells via multiple coreceptors, it strongly prefers species-matched CD4 to CPZ or human CD4.

SIVcpz V3 loop 326P is not compatible with CXCR6 use

Identifying distinct capabilities of CXCR6 use between the closely related SIVcpz and SIVmus Envs provided a unique opportunity to probe Env determinants of CXCR6 usage. Given that the V3 loop of Env is a key determinant of coreceptor usage in general (45), I aligned this region of the Envs used in this study (Figure 3.5A, bold text) as well as additional published Envs in the SIVgsn/mus/mon lineage (Figure 3.5A, plain text). I observed that residue 326 (SIVmac239 numbering), which falls in the crown of the V3 loop, encodes Pro for the SIVcpz strains, but Ala for the members of the SIVgsn/mus/mon lineage. I then compared this residue across SIV and HIV sequences for which the ability to use CXCR6 is known, and found that like SIVcpz, Pro was typically encoded by HIV-1 isolates, among which CXCR6-using strains are very rare (46). In contrast, no SIVs (or HIV-2) that can use CXCR6 encode Pro. Instead, Ala (SIVmus, SIVagm), Thr (SIVrcm) or Ser (SIVsmm, SIVmac, HIV-2) is encoded at that position.

Based on these observations, I hypothesized that 326P restricts use of CXCR6. To test this, I used site-directed mutagenesis to generate SIVmusA326P, which encodes Pro instead of the native Ala at position 326, and the converse, SIVcpzP326A, which encodes Ala instead of Pro. SIVmusA326P was unable to enter cells expressing musCD4 and musCXCR6, despite entering cells expressing musCD4 and musCCR5, demonstrating that P326 is incompatible with CXCR6 usage (Figure 3.6A). In contrast, SIVcpz P326A was not able to enter cells expressing cpzCD4 and cpzCXCR6, or mus CD4 and mus CXCR6, indicating that 326A is not sufficient to confer CXCR6 usage to SIVcpz.

I then asked if the entire SIVmus V3 loop was sufficient to confer CXCR6 use to SIVcpz, and swapped the SIVmus 1085 1-54 V3 loop (Figure 5A) into the SIVcpz MT145 Env. The resulting

virus was poorly infectious (entering cells at 4-5% of the RLU observed for the same amount of wild type SIVcpz as measured by p24 capsid protein). Nevertheless, I did not observe rescue of either musCXCR6 or cpzCXCR6 usage, suggesting that Env domains beyond the SIVmus V3 loop likely contribute to CXCR6 usage.

Figures

CCR5

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N term  1                                                           31
HU      M D Y Q V S S P I Y D I N Y Y T S E P C Q K I N V K Q I A A R
CPZ     • • • • • • • • • • • • D • • • • • • • • • • • • • • • • • •
MUS     • • • • • • • • T • • • D • N • • • • • • • • • • • • • • • •
GSN     • • • • • • • • T • • • D • • • • • • • • • • • • • • • • • •

ECL 2   169                                                     197
HU      S Q K E G L H Y T C S S H F P Y S Q Y Q F W K N F Q T L K
CPZ     • • • • • • • • • • • • • • • • • • • • • • • • • • • • •
MUS     • • R • • • • • • • • • • • • • • • • • • • • • • • • • •
GSN     • • R • • • • • • • • • • • • • • • • • • • • • • • • • •
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CXCR6

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N term  1                                                               33
HU      M A E H D Y H E D Y G - F S S F N D S S Q E E H Q D F L Q F S K V
CPZ     • • • • • • • • • • • - • N • • • • • • • • • • • • • • • • • • •
MUS     • • • Y • H Y • • D • F • N • • • • • • • • • • • • • • • • R • •
GSN     • • • Y • H Y • • D • F • N • • • • • • • • • • • • • • • • • • •

ECL 2   170                                   190
HU      Y G N V F N L D K L I C G Y H D E A I S T
CPZ     • • • • • • • • • • • • • • • • • • • • •
MUS     • • • • • • • • • • • • • • • • • E • • •
GSN     • • • • • • • • • • • • • • • • • E • • •
```

CD4

```
        1                                                      55
HU      KKVVLGKKGDTVELTCTASQKKSIQFHWKNSNQIKILGNQGSFLTKGPSKLNDRA
CPZ     •••••••••••••••••••••••••••••••••T••••••••••••••••••••I
MUS     •••••••••G••••••N•P•••NT•••••H••••••••I••••••••••••S•••
GSN     •••••••••G••••••N•P•••NT•••••H•••T•••••••••••••••••S•••

        56                                                  109
HU      DSRRSLWDQGNFPLIIKNLKIEDSDTYICEVEDQKEEVQLLVFGLTANSDTHLL
CPZ     •••••••••••••••••••••••••••••••G••••••••••••••••••••••
MUS     •••K••••••CFSM••••••••••E••••••••K••••E•••••••••••••••
GSN     •••K••••••CFSM••••••••••E••••••••K••••E•••••••••••••••
```

Figure 3.1: Coreceptor and CD4 sequences vary in sequence between human, CPZ, GSN and MUS. CCR5 and CXCR6 were cloned from CPZ, GSN and MUS genomic DNA. Sequences were aligned to the human sequence, and shown are the N-terminus (N term) and the second extracellular loop (ECL2), the two domains that interact with the virus glycoprotein (based on studies of human CCR5 and HIV-1 gp120). GSN and MUS CD4 exons were sequenced from genomic DNA and aligned to known sequences of human CD4 and chimpanzee CD4; shown is domain 1 that interacts with HIV/SIV gp120. Residues identical to the human sequence are represented as dots.

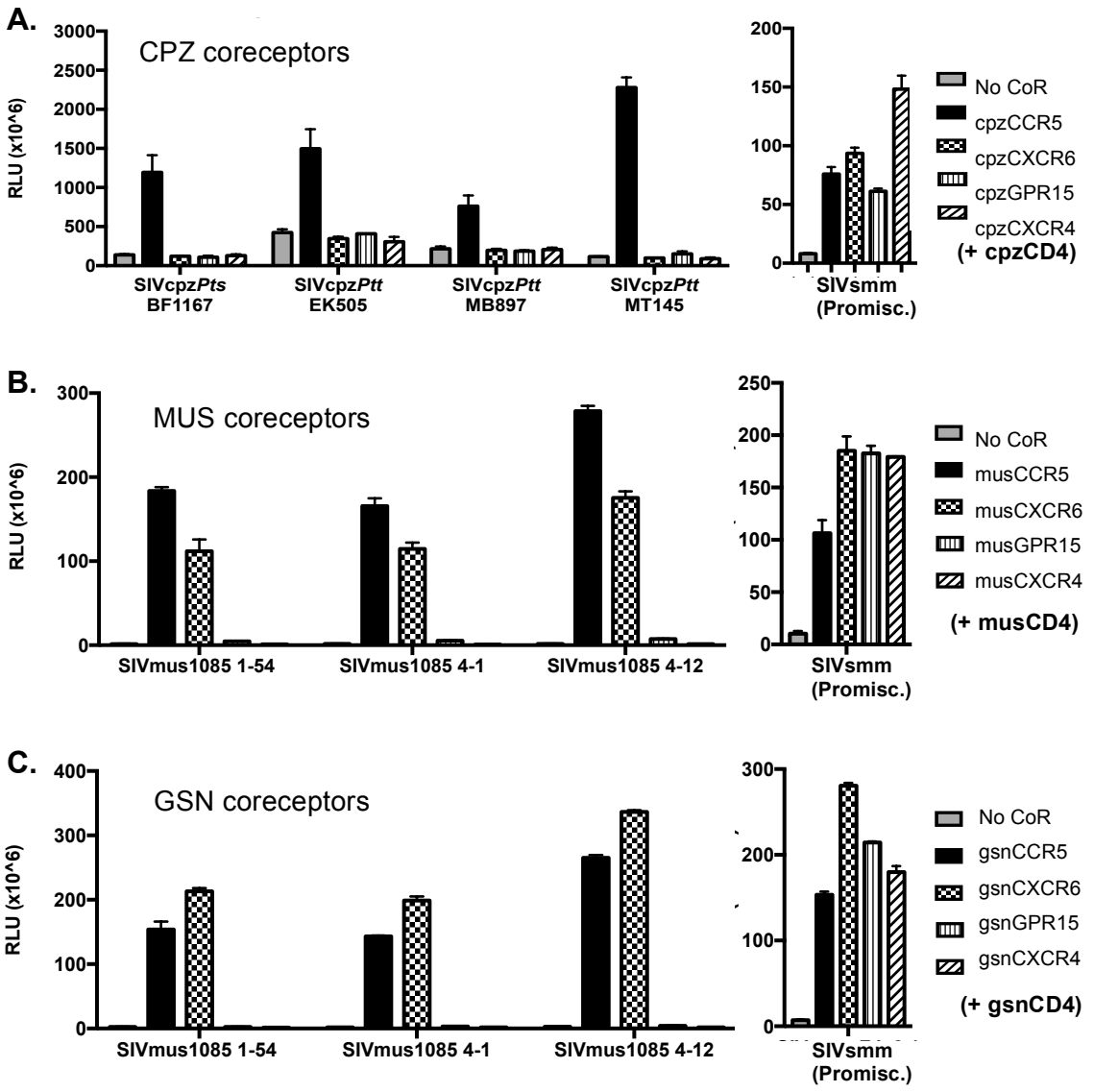


Figure 3.2: SIVcpz is restricted to CPZ CCR5, while SIVmus1085 can use MUS and GSN CCR5 and CXCR6 for entry. A) Left panel: CF2thLuc cells that contain a Tat-driven luciferase reporter were transfected with expression plasmids containing chimpanzee (CPZ) CD4 and coreceptor or empty vector (No CoR). 48 hours later cells were infected with one of four diverse SIVcpz isolates derived from infectious molecular clones. Entry was quantified 48 hours later by lysing cells and measuring luciferase content by relative light units (RLU). Right panel: 293T cells were transfected with CPZ CD4 and coreceptor and infected with luciferase reporter pseudotypes carrying a promiscuous SIVsmm Env that can use a wide repertoire of coreceptors for entry. B-C) 293T cells were transfected with expression plasmids containing CD4 and coreceptor of MUS (B) or GSN (C) origin, or empty vector (No CoR). 48 hours later cells were infected with luciferase reporter viruses carrying SIVmus1085 Envs (left panel) or a promiscuous SIVsmm (right panel). Entry was quantified 72 hours later by lysing cells and measuring luciferase content by relative light units (RLU). A-D) Infections were carried out in triplicate and error bars represent one standard deviation. One representative experiment is shown.

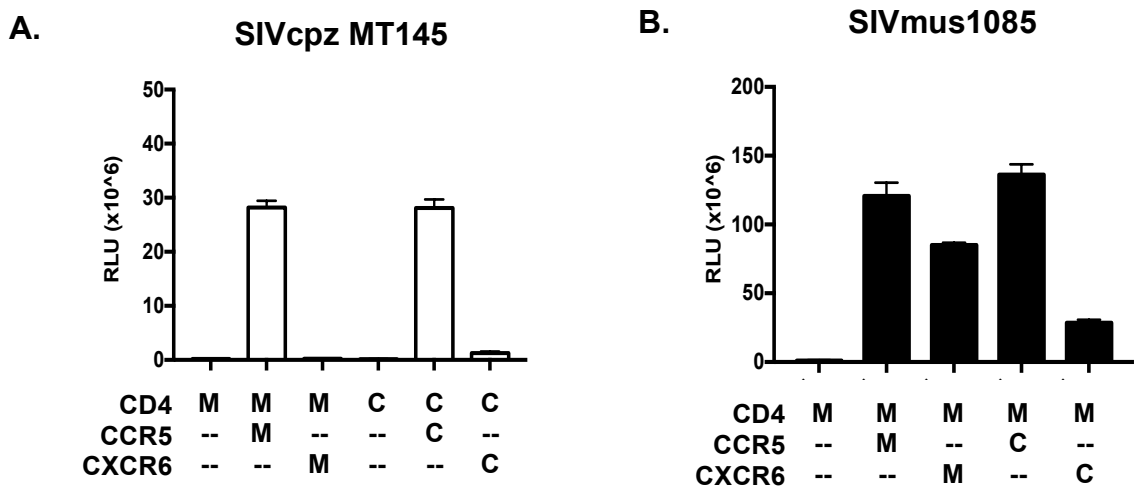


Figure 3.3: The inability of SIVcpz to use CXCR6 for entry is determined by Env, not CXCR6 species origin/sequence. 293T cells were transfected with expression plasmids containing CD4 and coreceptor. The species origin of the CD4 and coreceptor are indicated below the graph and abbreviated as M (mustached monkey) C (chimpanzee) or – for no plasmid. 48 hours post transfection, cells were infected with luciferase reporter pseudotypes carrying the SIVcpz MT145 Env (A) or the SIVmus1085 1-54 Env (B). Entry was quantified 72 hours later by lysing cells and measuring luciferase content by relative light units (RLU). Infections were carried out in triplicate and error bars represent one standard deviation.

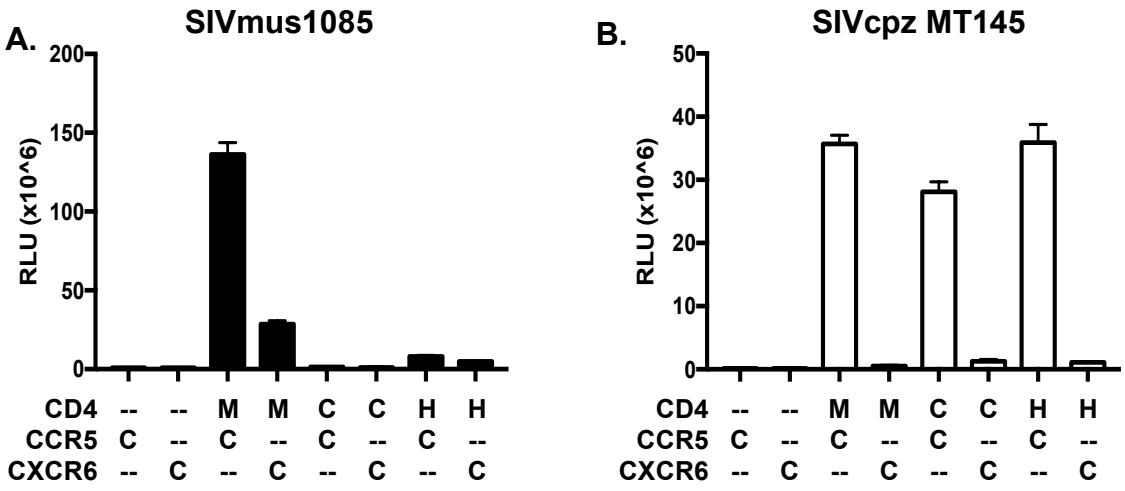
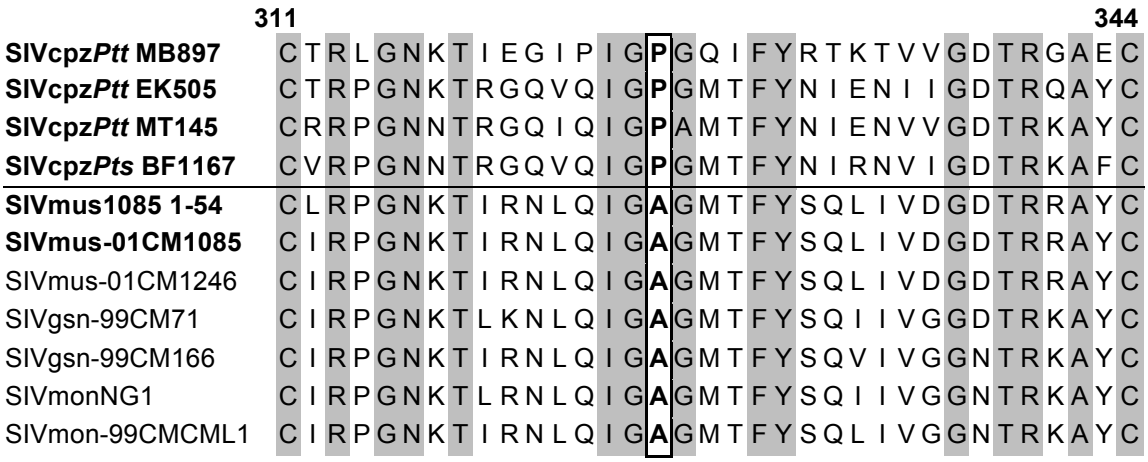


Figure 3.4: SIVmus1085 cannot use chimpanzee CD4 for entry. 293T cells were transfected with expression plasmids containing CD4 and coreceptor. The species origin of the CD4 and coreceptor are indicated below the graph and abbreviated as M (mustached monkey) C (chimpanzee) H (human) or – for no plasmid (coreceptor rows) or empty vector (CD4 rows). 48 hours post transfection, cells were infected with luciferase reporter pseudotypes carrying the SIVmus1085 1-54 Env (A) or the SIVcpz MT145 Env (B). Entry was quantified 72 hours later by lysing cells and measuring luciferase content by relative light units (RLU). Infections were carried out in triplicate and error bars represent one standard deviation.

A.



B.

CXCR6 Use Phenotype	Virus	V3 residue (326/313)
Cannot use CXCR6	HIV-1	P
	SIVcpz	P
Can use CXCR6	SIVmus	A
	SIVagm	A
	SIVsmm	S
	SIVrcm	T
	SIVmac*	S
	HIV-2	S

Figure 3.5: Amino acid sequences of SIV and HIV V3 loops. A) The V3 loop amino acid sequences of SIVcpz and SIVmus sequences used in this study (bold text) were aligned with other members of the SIVgsn/mus/mon lineage (plain text) using the ClustalW algorithm. The V3 loop of the previously published SIVmus-01CM1085 is identical to SIVmus1085 4-1 and 4-12. Residue 326 (SIVmac239 numbering) of the V3 loop is boxed. Conserved residues are shaded in grey. B) The predominant amino acid residues that occur at V3 crown residue 326 (SIVmac 239 numbering) or 313 (HIV HXB2 numbering) for SIVs and HIVs where use of CXCR6 has been tested. *SIVmac can use other species' CXCR6 for entry, but does not efficiently enter through rmCXCR6 due to a S31R polymorphism in the N terminus of rmCXCR6.

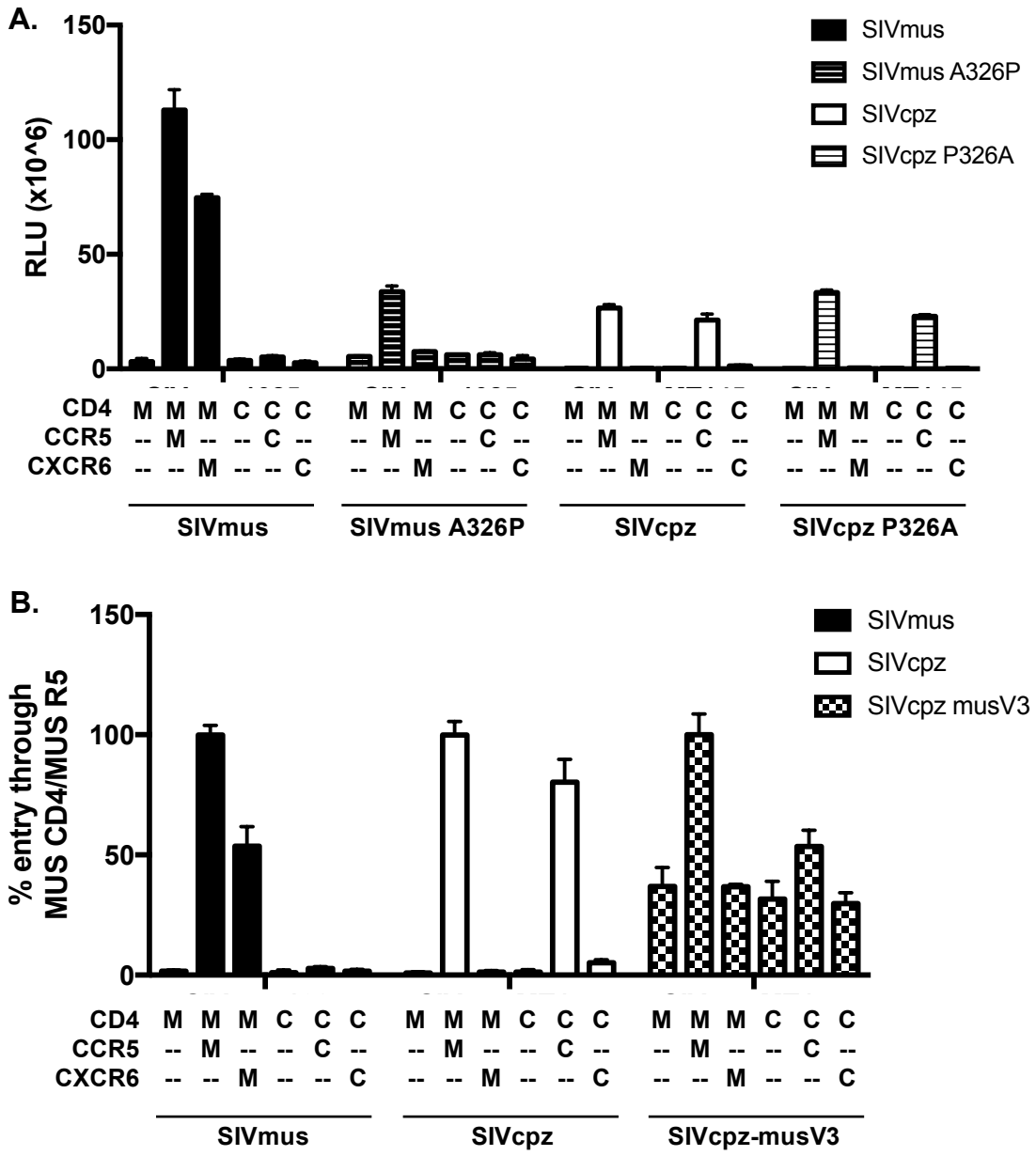


Figure 3.6: SIVmus1085 Env determinants of CXCR6 usage: V3 loop residue 326P is not compatible with CXCR6 usage, but neither 326A nor the SIVmus V3 loop is sufficient to confer CXCR6 usage. A) SIVmus1085 1-54 and SIVcpz MT145 *env* constructs were mutated via site-directed mutagenesis to generate V3 crown mutants SIVmusA326P and SIVcpzP326A. Luciferase reporter pseudotyped viruses carrying these Envs, or SIVmus 1085 1-54 or SIVcpz MT145, were used to infect 293T cells expressing musCD4 and coreceptor (M) or cpzCD4 and coreceptor (C). Entry was quantified 72 hours post-infection by relative light units (RLU). B) The V3 loop of SIVcpz MT145 was replaced with the V3 loop of SIVmus1085 1-54 to generate SIVcpz-musV3. Pseudotypes carrying the SIVcpz-musV3 Env, or SIVmus1085 1-54 or SIVcpz MT145, were used to infect 293T cells expressing musCD4 and coreceptor (M) or cpzCD4 and coreceptor (C). Due to low infectivity of SIVcpz-musV3, entry was normalized to % entry through musCD4 and musCCR5 to enable comparison. Infections were carried out in triplicate and error bars represent one standard deviation.

Discussion

Humans in Africa continue to be exposed to SIV-infected African primates, largely due to bushmeat hunting (41). Therefore, it is critical to understand the transmission events that lead to the emergence of HIV-1 and its ancestor, SIVcpz. One of the earliest requirements for cross-species transmission is entry into the cells of a novel host; therefore, I investigated coreceptor usage of SIVcpz and its *env* ancestor SIVmus. Furthermore, studies from our lab and others have linked use of the entry coreceptor CXCR6 (in addition to CCR5) to a benign disease course for several natural host SIVs, while virus dependence on CCR5 (but not CXCR6) is associated with progression to immunodeficiency. I hypothesized that as HIV-1 evolved from its natural host ancestors and pathogenesis emerged, use of CXCR6 was lost.

In this study, I found that SIVcpz, which is pathogenic in its chimpanzee host, is restricted to use of CCR5 like HIV-1 and the HIV-1 model SIVmac. In stark contrast, its natural host ancestor SIVmus enters cells expressing CXCR6 as well as CCR5. These coreceptor-use phenotypes were determined by Env, not coreceptor origin, and I identified a Pro residue on the V3 crown of SIV as a contributor to this restriction to CCR5 use. Lastly, SIVmus was unable to enter cells expressing cpzCD4.

While SIVmus was able to use species-matched CXCR6 for entry, SIVcpz, the descendent of the SIVgsn/mus/mon lineage, was restricted to use of CCR5, demonstrating that a coreceptor bottleneck occurred with the emergence of SIVcpz. This pattern held for four diverse SIVcpz isolates, including SIVcpz*Ptt* (MT145, EK505 and MB897), the SIVcpz subspecies that crossed into humans, and SIVcpz*Pts* (BF1167), which has not crossed into humans (2, 35, 47).

SIVcpz was long thought to be nonpathogenic in chimpanzees, but this was largely due to observations of very few SIVcpz-infected chimpanzees, as well as experimentally HIV-1-infected chimpanzees (1, 48-51). However, a thorough non-invasive survey of habituated chimpanzees in

West Africa revealed an increased death hazard and CD4+ T cell depletion among SIV+ chimpanzees when compared to their uninfected counterparts, indicating that SIVcpz is indeed pathogenic for the two subspecies of chimpanzees in which it is found (8). While similar studies have not been undertaken for MUS, GSN or MON monkeys, which are not held in captivity, studies of SM, AGM and mandrills that have been long-infected with their species-specific SIVs have led to the conclusion that SIV infections of African monkeys, like SIVmus of MUS, are likely nonpathogenic (6, 7). Thus, the bottleneck from CCR5 and CXCR6 use by SIVmus to CCR5 use by SIVcpz coincides with the emergence of pathogenesis within this virus lineage.

A parallel observation is made when comparing SIVsmm/SM and SIVmac/RM. The cross-species transmission of SIVsmm to RM occurred in the lab and not the wild, but the same bottleneck is observed: natural host SIVsmm/SM infection uses CCR5 and CXCR6 (and possibly other coreceptors) for entry, while pathogenic SIVmac/RM infection is restricted to CCR5 (31, 33, 52). Unlike SIVcpz, in the case of SIVmac, the cause for the bottleneck is coreceptor mediated, as RM CXCR6 encodes an Arg residue in the N-terminus that renders it a poor coreceptor for SIVmac (34). For the SIVmus-SIVcpz transition, where loss of CXCR6 use in SIVcpz is Env determined, it is unclear why the cross-species transmission would result in loss of CXCR6 use in SIVcpz emergence. Possible drivers include SIV Env adaptation to use chimpanzee CCR5 or CD4, or chimpanzee immune system pressures, and these and others are discussed at length in Chapter 5. While the mechanisms of restriction to CCR5 use are distinct for SIVmac and HIV-1, macaques and humans both maintain a high frequency of CCR5+ CD4+ T cells (29), such that use of this coreceptor for virus entry likely permits replication in cell types and anatomic compartments that promote immunodeficiency.

A caveat of this bottleneck observation is that MUS and related monkeys are not well studied, and as mentioned the lack of pathogenicity of these infections is presumed based on similarities to the well-studied natural hosts like SM, AGM subspecies, and mandrills. Extensive bushmeat

surveys have shown that SIV prevalence among MUS, GSN, and MON ranges from 0-7%, which is much lower than the 50% prevalence observed among adult SM and AGM (7, 41, 53). This low prevalence is closer to what is observed in more pathogenic infections, and some have hypothesized that this may suggest a sub-optimal host-virus relationship (41). Furthermore, like HIV-1 and SIVcpz, SIVgsn/mus/mon encodes *vpu*, which may be linked to the increased pathogenicity of HIV compared to other immunodeficiency viruses (54). Therefore, surveys of these monkeys in the wild, like those performed of sabaeus AGM (55, 56) and chimpanzees (8) would be required to definitively confirm whether MUS, GSN and MON actually experience nonpathogenic infection.

By identifying the distinct coreceptor usage patterns of the closely related SIVmus and SIVcpz, I was able to probe determinants of CXCR6 use. I found that CXCR6 use in this lineage was determined by the virus, not coreceptor sequence, and identified that a Pro residue at position 326/313 (SIVmac239/HIV HXB2 numbering), which is commonly found in the V3 loop of SIVcpz and HIV-1, as incompatible with CXCR6 use in SIVmus. As shown in Figure 3.5B, no known CXCR6-using virus encodes Pro at this position. Also, while experimental data on CXCR6 by SIVs beyond those shown in Figure 3.5B are unknown, it is worth noting that no natural host SIVs that have been sequenced to date encode a Pro at this position, such as SIVdrl that infects drills (*Mandrillus leucophaeus*) and SIVsyk that infects Sykes' monkeys (*Cercopithecus albogularis*), suggesting a possible capacity for CXCR6 use (46). Interestingly, several rare HIV-1 isolates that use CXCR6 *in vitro* code a Trp instead of a Pro at this position, although others retain the Pro (27, 57). Given that neither the SIVcpz P326A nor the SIVcpz-musV3 mutant viruses were able to use CXCR6 for entry, the data suggest that the requirements for CXCR6 use likely extend beyond the V3 loop of Env. This finding is in agreement with a previous study that found that the V1 and V2 domains, in addition to V3, were necessary to confer CXCR6 use from an SIV to HIV-1 (58). However, given the low infectivity of the SIVcpz-musV3 mutant, I did not generate additional mutants.

Like other SIVs, SIVmus1085 did not enter cells expressing species-matched CXCR4. More surprisingly, SIVmus1085 did not enter cells expressing species-matched GPR15. *In vitro*, GPR15 is frequently used by SIVs, including SIVmac, SIVsmm and SIVagm, albeit with varying degrees of efficiency (30, 32, 59). While I did not probe determinants of use of this coreceptor in this study, others have identified a Pro at position 321 in the V3 loop as necessary for use of GPR15 for SIVmac (60). This residue precedes the Pro that regulates CXCR6 use identified in this study (326P) by five amino acids. While 321P is found in SIVsmm and SIVagm as well, SIVmus1085 encodes Asn at this position, which may explain to the lack of GPR15 use observed by SIVmus.

Differences in cell and tissue targeting between natural and non-natural HIV/SIVs are strongly implicated in the distinct outcomes of infection. In non-natural host infections, critical memory CD4+ T cell subsets such as Tcm, Tscm, and Th17 are infected or lost at a greater frequency than in natural host infections (14-16). Similarly, non-natural host infection is marked by inflammation, structural disruption and fibrosis of lymph nodes, along with high viral burden in the follicles during chronic infection, which is not observed in natural host infections (17, 61). Limited infection of these critical subsets and tissue compartments in natural hosts is thought to preserve immune system homeostasis and function. Therefore, to understand SIV cell targeting and its contribution to pathogenesis, it is crucial to define expression patterns of these proteins. Initial studies identified low levels of CCR5 expression as a common feature of natural hosts such as SM and AGM compared to non-natural hosts such as RM and human, independent of infection. Interestingly, chimpanzees were found to have moderate levels of CCR5 expression (29). Furthermore, limited CCR5 expression has since been implicated in protection of certain CD4+ T cell subsets from infection in SM, such as Tcm and Tscm. While informative, these studies of CCR5 expression also raised the question of how natural hosts are able to support viremia equal or above that of non-natural hosts with apparently reduced target cells.

Our findings that a common feature of natural host viruses is use of species-matched CXCR6 provide an answer to that question. While the field has long known that SIVs can use a variety of human 7TMR coreceptors to enter cells *in vitro*, CCR5 was long thought to be the sole coreceptor used by SIVs *in vivo*. An early-recognized exception was SIVrcm, whose RCM host is frequently CCR5-null due to a common deletion allele. SIVrcm cannot use CCR5, and can instead enter cells using CXCR6 and CCR2b (62, 63). This deletion allele was also found in SM, but at a lower frequency such that homozygous SM were not identified. However, several years ago, our lab identified an additional, more common CCR5 deletion allele in SM, and found that animals that were homozygous for CCR5-null alleles and lacked CCR5 on the cell surface were infected with SIV at similar rates to CCR5 wild-type animals and maintained high viral loads, indicating that non-CCR5 entry pathways are used by SIVsmm (59). We then identified CXCR6 as a coreceptor used by SIVsmm to enter SM lymphocytes, and also found that CXCR6 is used by SIVagmSab to enter sabaeus AGM lymphocytes (30-32) (Chapter 2). *In vitro* studies of SIVagmVer, which infects the vervet subspecies of AGM, found that SIVagmVer can efficiently enter cells expressing vervet CXCR6 (33). In this study, I add to this mounting evidence of common natural host SIV CXCR6 use by demonstrating robust use of musCXCR6 by SIVmus to enter transfected cells, and furthermore extend this emerging paradigm to the natural host ancestor of the HIV-1/SIVcpz lineage. This study marks the first functional analysis of SIVmus Env. Unfortunately, unavailability of MUS lymphocytes prevented us from confirming use of CXCR6 by infecting cells in the presence of a CXCR6 blocking agent, akin to our SM and sabaeus AGM studies. Nevertheless, SIVmus represents a distinct lineage of SIV that is the ancestor of HIV-1 *env*, and therefore these findings expand the range of known CXCR6-using SIVs and bolster our hypothesis that CXCR6 use is common to natural host viruses. By identifying yet another natural host SIV that can use CXCR6 for entry, the need to identify CD4+ cells that express this coreceptor becomes more imperative to fully understand the implications of this additional entry pathway and define SIV target cells. This is a question that I address in Chapter 4 of this thesis.

Phylogenetic analyses have indicated that a member or ancestor of the SIVgsn/mus/mon lineage gave rise to *env* of SIVcpz with cross-species transmission (3-5). Therefore, the finding that SIVmus was unable to use chimpanzee CD4 for entry was quite unexpected, but might be explained several ways. First, it is possible that the virus that crossed the species barrier into chimpanzees was SIVgsn, SIVmon or a close relative thereof, rather than SIVmus; unfortunately, lack of availability of samples prevented us from testing envelopes of these two viruses. It's also possible that none of the present-day viruses of this lineage are able to use CD4, but the ancestor that actually made the jump could in fact use chimpanzee CD4. Third, it could be that the entire SIVgsn/mus/mon lineage uses chimpanzee CD4 poorly, but the virus that did cross could use it sufficiently well to replicate and adapt to use of this receptor in chimpanzee cells. Cross-species transmission of SIVs generally requires the virus to adapt to its new host; for example, the emergence of HIV-1 from SIVcpz required a mutation in the matrix protein to efficiently replicate in human cells (47). It's tempting to speculate that as SIVgsn/mus/mon Env adapted to replication using chimpanzee CD4, a casualty of adaptation was loss of CXCR6 use. Finally, it could be that chimpanzee CD4 has evolved away from an ancient allele that permitted SIVgsn/mus/mon entry. Given the nonlinearity of the CD4 binding site and the diversity thereof across SIV strains, it's difficult to pinpoint the Env residues responsible for the inability to use cpzCD4 by SIVmus merely by comparing sequences, but future mutagenesis studies could identify responsible residues. Additionally, restriction of cross-species transmission is a common theme among SIVs and HIVs. For example, restriction factors often prevent such transmission, such as TRIM5a restricting HIV-1 replication in Old World monkeys (64). However, to our knowledge, this is the first time that inability to use CD4 has been identified as a possible point of restriction in natural cross-species transmission. (Albeit, one that was seemingly overcome.) Overall, this finding underscores the need for functional analysis in addition to sequence analysis to fully understand virus/host relationships.

In summary, I found that CXCR6 could function as an entry coreceptor for yet another natural host virus, SIVmus, which is in the family of viruses that was the upstream ancestor of the HIV-1/SIV lineage. The ability to use CXCR6 was lost with cross-species transfer of this virus into chimpanzees, as SIVcpz was unable to enter cells expressing species-matched CXCR6, like HIV-1. Thus, the loss of CXCR6 use correlates with the emergence of pathogenicity in this lineage of viruses. Why this coreceptor bottleneck occurred between GSN/MUS/MON and chimpanzees is unclear, but the need to adapt to replicate efficiently in its new chimpanzee host may have contributed. Future experiments to define which natural host cells express CXCR6 will help determine the relationship between CXCR6 use and benign SIV infection.

Materials and Methods

Cloning MUS and GSN Coreceptors

For GSN and MUS, stored gDNA had been previously isolated from bushmeat samples in Cameroon. Samples used in this study were MUS 1085 (5) and 1246 (40) (both SIV+), GSN 1289 and 1365 (both SIV-)(41) and GSN42 (SIV+) (41). GSN CCR5, CXCR6 and GPR15 were cloned from GSN 1365 and CXCR4 was cloned from GSN 1289, and MUS coreceptors were cloned from MUS 1085.

Coreceptors were amplified as previously described (30, 32). Briefly, previously described primers that lie outside the open reading frames of the coreceptors that contained restriction digest sites were used to amplify desired genes from gDNA using Phusion polymerase (New England BioLabs). For CCR5, CXCR6 and GPR15, PCR products were digested and ligated into expression vector pcDNA3.1+.

For MUS and GSN CXCR4, the only multi-exon coreceptors amplified from gDNA, each exon was amplified using primers flanking exon 1 and exon 2. (Sequences that permit TOPO Directional and/or restriction enzyme digest cloning are underlined.):

CXCR4 Exon 1 Fwd: 5'-CACCGGATCCGCCTGAGTGCTCCAGTAGCCACCGCATCTGG-3'

CXCR4 Exon 1 Rev: 5'-CACATGCAGCCACTGGAACGCTCT-3'

CXCR4 Exon 2 Fwd: 5'-TCACTATGGGAAAAGATGGGGAGGA-3'

CXCR4 Exon 2 Rev: 5'-GTCCCTCGAGACATCTGTGTTAGCTGGAGTGAAAACTTGAA-3'

The amplicons were sequenced and a forward primer was designed that would amplify the second exon while splicing the two exons together; given that the GSN and MUS sequences were identical in this region, the same primer was used for both species. This forward primer was also designed with to permit TOPO Directional cloning into pcDNA3.1/V5-His-TOPO vector (Invitrogen).

5’-**CACC**ATGGAGGGGATCAGTATATACACTTCAGATAAC-3’. (Motif to permit TOPO Directional cloning is in bold. Exon 1 is underlined).

For all coreceptors, DH5a cells were transformed with the ligation products. Constructs were isolated via MiniPrep (Qiagen) and clones were sequenced using Sanger sequencing and aligned using the ClustalW algorithm with MacVector 15.5.0.

MUS and GSN CD4

To generate musCD4 and gsnCD4, primers were designed using published human and rhesus macaque sequence as a template in order to amplify the nine exons of CD4 from gDNA in four amplicons:

Exons 1-2 Fwd: 5’-CACCCAGCAAGGCCACAATGAAC-3’

Exons 1-2 Rev: 5’-TCAGACACCAAAGGCTTTCA-3’

Exons 3-4 Fwd: 5’-CCCAGCCAGGTAAATGGATA-3’

Exon 3-4 Rev: 5’-TCTCCACTCCTGACCTCCCA-3’

Exons 5-6 Fwd: 5’-GGAGAGGTAGGAAGGAACTGAAG-3’

Exons 5-6 Rev: 5’-GTCTCTGCCAACCACAGGAA-3’

Exons 7-9 Fwd: 5’-AAACCGATTCCCCAGCACT-3’

Exon 7-9 Rev: 5’-GGATCTGCTACATTCATCTGGT-3’

For samples with sufficient DNA, all nine exons were amplified (MUS 1085, GSN 42 and 1289). For less concentrated samples, only the exons 1-4, which include domain 1, were amplified (MUS 1246, GSN 1365). PCR was performed using Phusion polymerase and the product was run on an agarose gel. Amplicons of the proper size were purified using a Gel Extraction Kit (Qiagen) and sequenced using a MiSeq sequencer (Illumina) and sequences were analyzed using Geneious 7 software. For musCD4, all nonsynonymous SNPs occurred within one read-length (150bp), such that alleles could be determined, and one allele was chosen for synthesis. For gsnCD4, the nonsynonymous SNPs spanned the entire gene, and therefore a consensus sequence was

synthesized. The CD4 sequences were synthesized and cloned into the expression vector pcDNA3.1+ (GenScript).

Chimpanzee coreceptors and CD4

Stored nonviably frozen PBMCs (chimpanzee David) were thawed, and DNA and RNA were isolated using the QiaAmp Blood Mini Kit (Qiagen) and the RNeasy kit (Qiagen), respectively. cDNA synthesis from RNA was performed using the Superscript III kit (Invitrogen) using a gene-specific primer for CXCR4 (30). Chimpanzee CD4 was previously cloned (44).

SIVmus *env* cloning

Primers to amplify SIVmus Envs were designed using published sequence data for SIV from MUS 1085 (accession number AY340700) and MUS 1246 (accession number EF070329). PCR was performed in two rounds using Expand HF PCR System (Roche), following the manufacturer's protocol and using an annealing temperature of 55°C. Round one (35 cycles) contained outer primers and at least 100ng of gDNA. Round 2 (45 cycles) contained 2uL of the Round 1 reaction as input and was performed in duplicate with two different sets of inner primer pairs. Primer sequences are below: set 1 is the outer set, while sets 2 and 3 are inner sets. (The bases required for TOPO Directional Cloning are bolded.)

MusEnv1085Fwd1: 5'-GTGGAATTTGGAATGAGGTAACGG-3

MusEnv1085Rev1: 5'-TGGTCTAGGAGGTATTGGTCATCT-3

MusEnv1085Fwd2: 5'-**CACC**TGCTTTTCATTGCGTACTCTGTTT-3

MusEnv1085Rev2: 5'-TATCACAGGTCTTTTACTGGCTCC-3

MusEnv1085Fwd3: 5'-**CACC**ACCTCCTTTGAGTCCTTCTAGGTA-3

MusEnv1085Rev3: 5'-GAAATGCGACATATCCACCATCAG-3

MusEnv1246Fwd1: 5'-ATCCACAATGGTCTGTAGATCAGG-3

MusEnv1246Rev1: 5'-CAAAAGGAGAAGACCAGGAACTCT-3

MusEnv1246Fwd2: 5'-**CACC**ATGTTGCGCGTTTCACTGTATATT-3

MusEnv1246Rev2: 5'-AGTTTTTGCAGTCTGTCTATGCAC-3

MusEnv1246Fwd3: 5'-**CACC**TCTAATTCCATGCCAAATGCTGAC-3

MusEnv1246Rev3: 5'-GCACACAAACATTCCTTCTAGTCC-3

Amplification was confirmed by agarose gel and PCR products were column purified (Qiagen PCR Purification Kit) and ligated into pcDNA3.1/V5-His-TOPO vector using the TOPO Directional Cloning kit (Invitrogen). Ligation products were transformed into Stbl2 cells and cultured at 30°C. Colonies were screened for inserts of ~3kb by colony PCR using Taq polymerase and primers against T7 and BGH. Colonies containing plasmid with properly sized insert were cultured and plasmids were tested for functionality with backbone pNL4-3Luc E-R+ on 293T cells transfected to express CD4 and coreceptor. This yielded five distinct functional Envs from MUS 1085, three of which (1-54, 4-1 and 4-12) were chosen for this study. One PCR reaction yielded 1-54, and another yielded 4-1 and 4-12. For SIVmus1246, all Envs came from one Round 1 PCR reaction, but 2 different Round 2 reactions. This yielded five Envs, which were either nonfunctional or weakly functional.

SIVcpz isolates and *envs*

SIVcpz infectious molecular clones (IMCs) have been previously reported: SIVcpz*Ptt* strains MB897, EK505 and MT145 (47) and SIVcpz*Pts* strain BF1167 (35). SIVcpz IMCs were generated by transfecting IMC plasmids into 293T cells using Fugene 6 reagent. Cells were washed and media changed 24 hours post transfection, and supernatant was harvested 48 hours post wash.

SIVmus and SIVcpz *env* mutagenesis

Point mutations to SIVmus1085 1-54 and SIVcpzPtt MT145 *envs* were made using the Quickchange II XL Side-Directed Mutagenesis Kit (Agilent). Primers to introduce the mutations were designed using the manufacturer's protocol. Plasmids were grown in XL-10 Gold cells and

mutations were confirmed by sequencing the V3 loop via Sanger Sequencing. The constructs were also sequenced using a MiSeq sequencer (Illumina) and reads were aligned to the parent plasmid using Geneious 7 software to confirm that only the intended mutation was introduced.

To generate SIVcpz-musV3, the SIVcpzPtt MT145 Env plasmid was linearized by performing PCR using outward primers that flanked the V3 loop, such that the entire plasmid was amplified but the V3 loop was deleted. The V3 loop of SIVmus1085 1-54 was amplified using primers that contained ~20 bases of homology to SIVcpz MT145 at the regions directly flanking the V3 loop. The two amplicons were gel purified (Qiagen Gel Extraction Kit) and assembled using the NEBuilder HiFi DNA Assembly Cloning Kit (New England BioLabs). The assembly product was transformed into Stbl2s. Colonies were MiniPrepped (Qiagen Kit) and assembly was confirmed by digesting plasmid with BsteI and running the digest product on a gel.

Analysis of coreceptor function using SIVcpz derived from infectious molecular clones (IMCs)

CF2th-Luc cells that contain a Tat-driven luciferase reporter cells (8, 65, 66) were cultured in DMEM containing 3.5% FBS, 1% L-glutamine and 1% pen/strep and plated at 5e5/ well in 6-well plates for transfection. Cells were transfected with cpzCD4 and coreceptor plasmid (1ug each) using Fugene 6 reagent. 24 hours later, cells were washed, lifted and replated into 96-well plates. The following day, cells were infected with 50uL of IMC-derived virus stock. 48 hours post-infection, cells were lysed and luciferase content measured by adding luciferase substrate (Luciferase Assay system, Promega) and reading relative light units (RLU) on a Luminoskan Ascent instrument.

Analysis of coreceptor function using luciferase reporter pseudotyped virus

Pseudotyped virus was generated as previously described (30, 32). 293T cells were transfected with a plasmid containing SIVcpz, SIVmus or SIVgsn env and the luciferase reporter backbone

pNL4-3LucE-R+ using Fugene 6 reagent. Cells were washed and media changed 24 hours post transfection, and supernatant was harvested 48 hours post wash. Virus was quantified using a p24 enzyme-linked immunosorbent assay (ELISA).

Pseudotype infection of 293T cells expressing CD4 and coreceptor were performed as previously described (30, 32). Briefly, 293T cells were plated and the following day, transfected with expression plasmids containing species-specific CD4 and a coreceptor. 24 hours later, cells were lifted using 2mM EDTA and plated into 96-well plates. The following day, cells were infected with DNAse-treated pseudotyped viruses via spinoculation at 1200g for 2 hours, then incubated for 72 hours at 37°C. Cells were lysed using Triton X and luciferase content was measured by adding luciferase substrate (Luciferase Assay system, Promega) and reading relative light units (RLU) on a Luminoskan Ascent instrument. Pseudotyped viruses carrying the vesicular stomatitis virus glycoprotein (VSVg) served as a positive control to ensure any observed entry differences were coreceptor-specific. For infections using SIVcpzEK505 and MT145pseudotypes, SIVcpz MT145 P326A and SIVcpz-musV3, 40uL virus stock was used. For SIVmus1085 1-54, 4-1, 4-12, 5ng p24 was used.

To confirm expression of CD4 and coreceptor, 293T cells were transfected with CD4 and coreceptor and infected with pseudotypes expressing previously described SIVsmm Envs (FTv3.1 and FJV2.1) that use a broad repertoire of coreceptors (30).

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CHAPTER 4

Expression of CXCR6 on Natural and Non-natural Host Lymphocytes

Abstract

Nonpathogenic SIVsmm infection of the natural host sooty mangabey (SM) is characterized by limited infection of certain CD4+ T cell subsets, such as central memory CD4+ T cells (Tcm). In contrast, pathogenic SIVmac infection of the "non-natural host" rhesus macaque (RM) results in higher levels of Tcm infection. One mechanism implicated in this relative protection of Tcm in SM is reduced expression of the entry coreceptor CCR5 on such subsets compared with RM. However, high-level viremia in infected natural hosts despite low CCR5 levels suggests that other pathways may support infection, and may target virus to more "dispensable" cell types. Our lab has identified CXCR6 as a robust SIVsmm coreceptor, and demonstrated that CCR5 is unnecessary for SIVsmm infection of SM *in vivo* and *ex vivo*. In contrast, SIVmac does not use RM CXCR6. I hypothesized that CXCR6 use by SIVsmm may target virus to CD4+ T cells that are relatively expendable for immune homeostasis. In this study, I aimed to define expression patterns of CXCR6 on SM and RM CD4+ T cell subsets. As anti-human CXCR6 reagents do not cross-react with SM or RM, I generated a monoclonal antibody against primate CXCR6 and showed that it detects this molecule from multiple primate species. I used flow cytometry to define patterns of CXCR6 and CCR5 expression on resting and stimulated CD4+ T cells from SIV-uninfected SM and RM. CXCR6 expression was enriched on effector memory CD4+ T cells (Tem), but restricted on Tcm as well as naïve CD4+ T cells from both RM and SM. Also, CXCR6 and CCR5 expression define distinct CD4+ T cell populations in both species, with few resting cells expressing multiple coreceptors. Upon stimulation, the proportion of CXCR6+ CD4+ T cells increased in both SM and RM; this is distinct from CCR5+ CD4+ T cells, which only increase in proportion in RM. These findings suggest that CXCR6 targets SIVsmm to CD4+ Tem cells, which may be less critical for immune homeostasis. These studies also pave the way for analysis of CXCR6 expression in tissues, in order to more fully define cell targeting determined by use of this coreceptor.

Introduction

Over 40 different species of African primates have been identified as endemically infected with species-specific simian immunodeficiency viruses (SIVs) (1). These “natural hosts”, which include the sooty mangabey (SM, *Cercocebus atys*) infected with SIVsmm, generally do not progress to immunodeficiency despite high viral loads and rapid turnover of infected cells (2-5). This provides stark contrast to the progression to AIDS observed among infected “non-natural hosts,” such as humans infected with HIV-1 or macaques infected with SIVmac. Macaques (including rhesus, cynomolgus and pig-tailed) are Asian monkeys that do not naturally carry SIV; SIVmac emerged in the laboratory as the result of accidental cross-species transmission of SIVsmm from sooty mangabeys, and infection of macaques only occurs experimentally (6). Since SIVmac infection of macaques recapitulates many features of HIV-1 infection in humans, such as widespread CD4+ T cell loss and chronic activation of the immune system, it is the best model for HIV infection and pathogenesis to date (7).

Comparative analyses of SIV-infected rhesus macaques (RM, *Macaca mulatta*) and SM have permitted detailed interrogation of correlates of disease progression, given the similarities between the infecting viruses but the drastic differences in infection outcome (8). Such studies have revealed differences in infection and subsequent disruption of anatomic and cellular compartments between these two species. First, SIVmac infection of RM is marked by high viral burden in lymph nodes, as well as lymph node fibrosis and inflammation (9). In contrast, infected SM have much lower viral burden and lack inflammation in the lymph nodes during chronic infection, and lymph node structure is maintained (9). Differences have also been found in the gut, the main site of HIV/SIV replication (10, 11). SIVmac infection of macaques results in drastic loss of gut CD4+ T cells, concomitant with a loss of barrier integrity and translocation of microbial products into circulation, which is thought to be a key driver of the chronic immune activation observed in non-natural host infection (12-15). The disruption of the gut barrier is at least partly attributed to the loss of CD4+ Th17 cells, which secrete cytokines to maintain gut immunity and

integrity (16, 17). While SIVsmm infection also causes acute CD4+ T cell loss in the gut, the CD4+ T cell populations largely rebound after acute infection, Th17 cells and gut barrier integrity are maintained, and microbial translocation does not occur (16, 18).

SIV (and HIV) replicates in CD4+ T cells, including those with activated or memory phenotypes (11, 19, 20). Memory CD4+ T cells originate from naïve T cells (Tn) that have been exposed to cognate antigen, and consist of several distinct types (21-23): stem cell-memory (Tscm), central memory (Tcm), and effector memory (Tem). Tscm and Tcm largely reside in secondary lymphoid tissues, such as the lymph node, have regenerative potential and maintain memory T cell homeostasis. Tem reside mainly in peripheral tissues and have lower replicative potential, a shorter life span, and enact effector functions in response to re-exposure to an antigen. Comparative studies of SM and RM have revealed distinct patterns of infection among memory CD4+ T cells between the two species. In SM, Tcm and Tscm CD4+ T cells, as well as T follicular helper cells (Tfh), which are critical for germinal center reactions in the lymph node, are infected less frequently than the same subsets in RM as measured by cell-associated viral DNA (9, 24-26). It is hypothesized that restriction of infection of these subsets in SM limits damage to the anatomic niches where they are found, namely the lymph node, as well as protects the host's ability to maintain memory CD4+ T cell homeostasis and replenish lost cells. Recent reports have also identified protection of Tscm and Tcm from infection in cohorts of viremic, non-progressing HIV-1 infected humans (27, 28). In RM, it is thought that infection and loss of Tscm and Tcm result in disruption of immune homeostasis and overall loss of CD4+ T cells. Furthermore, the consequent critical need for homeostatic CD4+ T cell proliferation likely contributes to chronic immune activation and immune damage. (Reviewed in (29)).

The main mechanism implicated in protection of Tfh, Tscm and Tcm CD4+ T cells in SM is the infrequent expression of the entry coreceptor CCR5 (9, 24, 25, 30). However, this raises the question as to how SM are able to support such high viral loads in the face of few CCR5+ target

cells. Our lab has shown SIVsmm and several other natural host viruses can use CXCR6 as an entry coreceptor in addition to CCR5, both *in vitro* and in primary lymphocytes *ex vivo*, which may define additional, previously unrecognized SIVsmm target cells (Chapter 2) (31, 32). SIVmac, in contrast, is restricted to use of CCR5 in infection of rhesus macaque lymphocytes (32, 33). This is due to an N terminus polymorphism in rhesus macaque CXCR6, S31R, which renders the molecule a poor coreceptor for SIVmac (34). To understand which cells are truly targets for SIVsmm, as well as to address the validity of CCR5 expression restriction as a mechanism of protection, it is necessary to define expression of CXCR6 on SM CD4+ T cell subsets.

In this study I examined CXCR6 expression on peripheral blood mononuclear cells (PBMC) CD4+ T cell subsets of natural host sooty mangabeys. I hypothesized that CXCR6 expression would be infrequent on critical CD4+ T cell subsets such as Tn and Tcm, but enriched on the replenishable Tem subset. In parallel I examined expression of the canonical coreceptor CCR5. For comparison, I examined expression on PBMC subsets from RM, although since SIVmac cannot use rmCXCR6, it is mainly CCR5 expression that defines potential target cells in this host. To define CXCR6 expression, we developed a novel anti-nonhuman primate CXCR6 antibody, clone 20D8, because currently available anti-human CXCR6 antibodies do not cross-react with SM and RM CXCR6. I found that CXCR6 was enriched on CD4+ Tem, and was expressed on a distinct population from CCR5-expressing cells in both species. These data support a model where CXCR6 use targets SIVsmm to a unique population of Tem, and likely contributes to the ability to maintain high viral loads while sparing critical CD4+ T cell subsets that is observed in SIVsmm infection of SM.

Results

Development of the anti-primate CXCR6 antibody 20D8

Studies of primate CXCR6 expression have been hindered by the fact that anti-human CXCR6 antibodies do not recognize monkey CXCR6 molecules, including that of SM and RM, when expressed via transfection of 293T cells (Figure 4.1C) or on SM or RM lymphocytes (data not shown). Therefore, we generated an anti-primate CXCR6 antibody. Mice were first immunized using DNA, with three doses of a mammalian expression plasmid containing the gene for smCXCR6 or rmCXCR6. This was followed by five intraperitoneal injections of sublethally irradiated murine B78H1 cells that had been transduced to express smCXCR6 or rmCXCR6 (Figure 4.1A). This immunization strategy ensured that the mice were immunized against CXCR6 in the native transmembrane conformation. Two mice were immunized with each species' CXCR6. I then used a cell-based ELISA (cELISA) to screen hybridoma supernatants to identify antibody clones able to recognize CXCR6 in its native conformation. I screened over 1400 hybridomas by cELISA, and isolated CXCR6-reactive clones. One clone, 20D8 (from a mouse immunized with smCXCR6) showed the greatest specific reactivity.

I then further characterized clone 20D8 by evaluating its ability to detect CXCR6 from various species, and also reactivity against other coreceptors. Transfection of 293T cells followed by flow cytometry revealed that 20D8 recognizes CXCR6 from all primates tested thus far, including SM, RM, African green monkey (AGM), as well as human and chimpanzee (Figure 4.1A-B). 20D8 is specific for CXCR6, as it does not recognize sooty mangabey CCR5, CXCR4, GPR15 or APJ (Figure 4.1A-B and data not shown). In contrast, currently available anti-CXCR6 antibodies (such as K041E5; Figure 4.1.D) detect only human and the closely related chimpanzee CXCR6. This is the first reagent that detects CXCR6 from multiple primate species.

CXCR6 is expressed on SM and RM CD4+ lymphocytes and defines a population distinct from CD4+ CCR5+ T cells.

I then used this antibody to define CXCR6 expression on resting SM and RM peripheral blood mononuclear cells (PBMC). The staining panel for flow cytometry included markers to define memory subsets of CD4+ and CD8+ T cells (CD95, CD28, CCR7 and CD45RA) as well as expression of the coreceptor CCR5.

In both species, CXCR6 was expressed on both CD4+ and CD8+ T cells. Also in both species, CCR5 and CXCR6 were expressed largely on distinct cell populations, with mainly single-positive and few double-positive T cells (Figure 4.2A). This result is in contrast to studies of human cells, where frequent CCR5 and CXCR6 coexpression has been reported (35).

I then quantified expression as the percent positive cells for either or both coreceptors for six SM and six RM (Figure 4.2B-C). This showed that CCR5-CXCR6+ cells formed a distinct population of an average of 2% of SM CD4+ T cells. In contrast, CCR5+CXCR6+ cells only averaged 0.3% of SM CD4+ T cells, while CCR5+CXCR6- cells averaged 1.5% SM CD4+ T cells (Figure 4.2B). For RM, CCR5-CXCR6+ cells constituted 5.7% of the CD4+ T cells, while CCR5+CXCR6+ cells averaged 1.6 % of CD4+ T cells, and CCR5+CXCR6- cells averaged 1.4% CD4+ T cells (Figure 4.2C).

A distinct CXCR6+CCR5- population was also observed among CD8+ T cells in the 6 SM and 6 RM, and was particularly large in RM, averaging 33.7% of CD8+ T cells, as opposed to 6.6% in SM. (Figure 4.2B-C). Therefore, RM CD4+ and CD8+ T cells had a greater proportion of CXCR6+ cells than did the same T cell subsets in the SM. RM CD4+ T cells also expressed slightly more CCR5 than did SM CD4+ T cells, although CCR5 expression on RM CD4+ T cells measured here was not as frequent as previously reported (24, 30).

CXCR6 expression is enriched on effector memory CD4+ T cells

Restriction of infection of central memory CD4+ T cells (Tcm) has been associated with the lack of disease progression in natural hosts as well as HIV-1 viremic, nonprogressing humans, and low infection of these subsets correlates with low expression of CCR5 on CD4+ Tcm that likely protects them from infection (24, 26, 28). Given that CXCR6 is a robust natural host SIVsmm coreceptor like CCR5, I anticipated that its expression would likewise be restricted on Tcm. Thus, I also defined CXCR6 expression on memory CD4+ T cell subsets (Figure 4.3). CXCR6 expression was infrequent among on CD4+ Tcm cells (1.0% of CD45RA- CCR7+ CD4+ T cells) and naïve CD4+ T cells (Tn) (0.5% of CD45RA+, CCR7+, CD28+, CD95- CD4+ T cells), but enriched on effector memory CD4+ T cells (Tem) (5.9% of CD45RA-, CCR7- CD4+ cells) (Figure 3). This expression pattern is consistent with reported SIVsmm infection patterns of SM memory CD4+ T cells (24, 25). Furthermore, CXCR6 restriction on Tcm and enrichment on Tem was found for both RM and SM, showing that this pattern of expression is not necessarily specific to natural hosts (although CXCR6+ Tem were more frequent in RM, where 22% of Tem expressed CXCR6).

The selected memory markers did permit staining of stem-cell memory CD4+ T cells (Tscm) (CD45RA+, CCR7+ CD28+, CD95+). However, I identified very few of these cells in these PBMC samples, preventing meaningful analysis of this subset. That being said, the few cells collected had very little CXCR6 expression (data not shown).

The frequency of CXCR6+ cells increases in response to mitogen stimulation

I then measured changes in CCR5 and CXCR6 expression patterns upon mitogen stimulation. Previous studies have shown that the proportion of CCR5+ cells does not increase upon stimulation of SM PBMC, but does increase upon stimulation of RM PBMC (24). To identify any changes in the proportion of CXCR6+ cells following stimulation, I stained SM and RM PBMC for

CD4 and coreceptor expression prior to stimulation (Day 0), and then at days 5, 7 and 9 post stimulation with concanavalin A and IL-2.

For both SM and RM, the average proportion of CXCR6+ cells at 5 days post stimulation had increased from baseline. This increase was from an average of 1.6% to 5.6% for SM, and from an average of 8.1% to 16% among RM (Figure. 4.4). The frequency of CXCR6+ CD4+ T cells then decreased slowly over the remaining time points. In contrast, the proportion of CCR5+ cells first decreased in response to stimulation for both species, in agreement with published findings (24). By day 9 post-stimulation, the proportion of CCR5+ cells remained below baseline for SM CD4+ T cells (Baseline: 3.3%; Day 9: 0.9%), but increased dramatically for RM CD4+ T cells (Baseline: 5.6%; Day 9: 34.7%). These data demonstrate that the proportions of CXCR6+ and CCR5+ CD4+ T cells change distinctly in response to stimulation within each species, and suggest that expression of these coreceptors may be regulated differently.

Figures

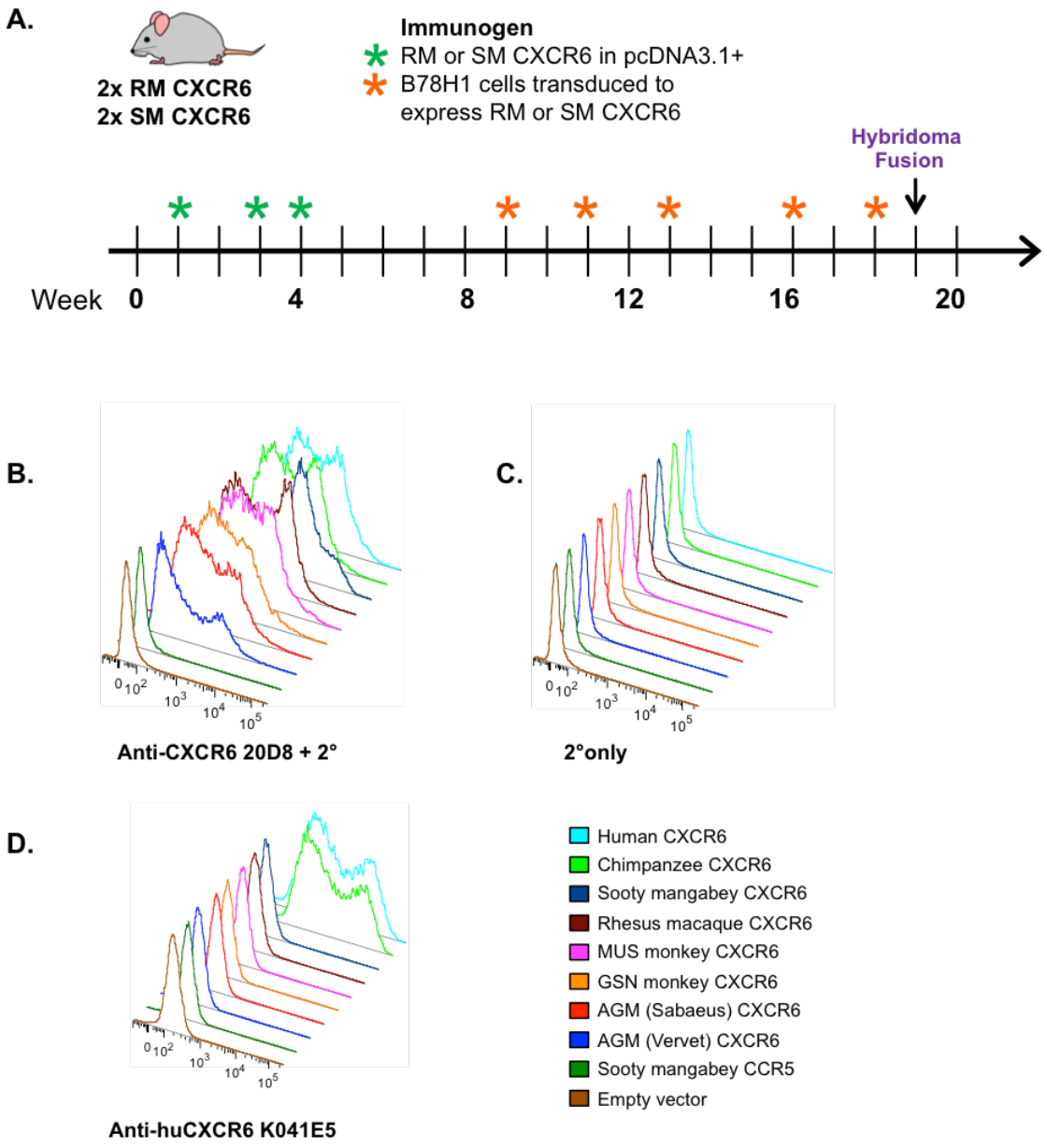

Figure 4.1: Generation of anti-CXCR6 antibody 20D8. A. Balb/c mice were immunized with 3 doses of mammalian expression plasmid containing the genes for either rhesus macaque (RM) or sooty mangabey (SM) CXCR6, followed by 5 doses of sublethally-irradiated B78H1 cells expressing RM or SM CXCR6. B. 293T cells were transfected with expression plasmids containing one of eight primate CXCR6 genes, SM CCR5 or empty vector. 48 hours later, cells were stained with mouse anti-primate CXCR6 antibody 20D8 + a goat anti-mouse secondary (B), secondary only (C) or a commercially available anti-human CXCR6 antibody (D).

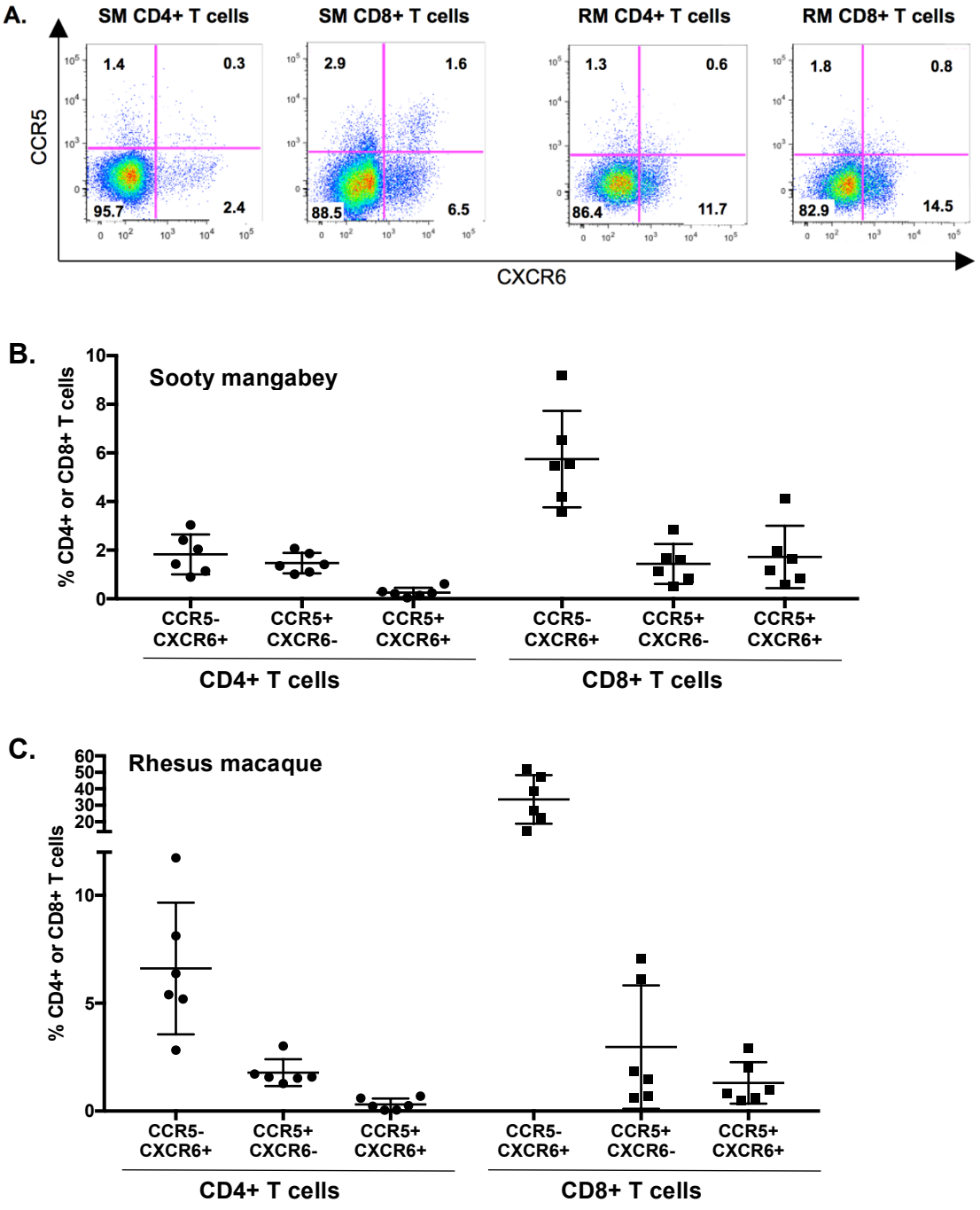

Figure 4.2: CXCR6 and CCR5 are expressed largely on distinct sooty mangabey and rhesus macaque T cell populations. A. Resting SM CD4+ T cells (far left) and CD8+ T cells (mid left), as well as RM CD4+ T cells (mid right) and CD8+ T cells (far right) were stained for both CCR5 (y-axis) and CXCR6 (x-axis). Numbers in the quadrants are the percent of CD4+ or CD8+ T cells expressing the respective combination of coreceptors. One representative animal is shown. B, C. Summary of CCR5 and CXCR6 single- and co-expressing cells as the percent of total CD4+ and CD8+ T cells from 6 SM (B) or 6 RM (C). Reported values represent percent of cells that stained positive for CXCR6 using 20D8 + a secondary antibody minus the percent of cells that stained positive using the secondary alone.

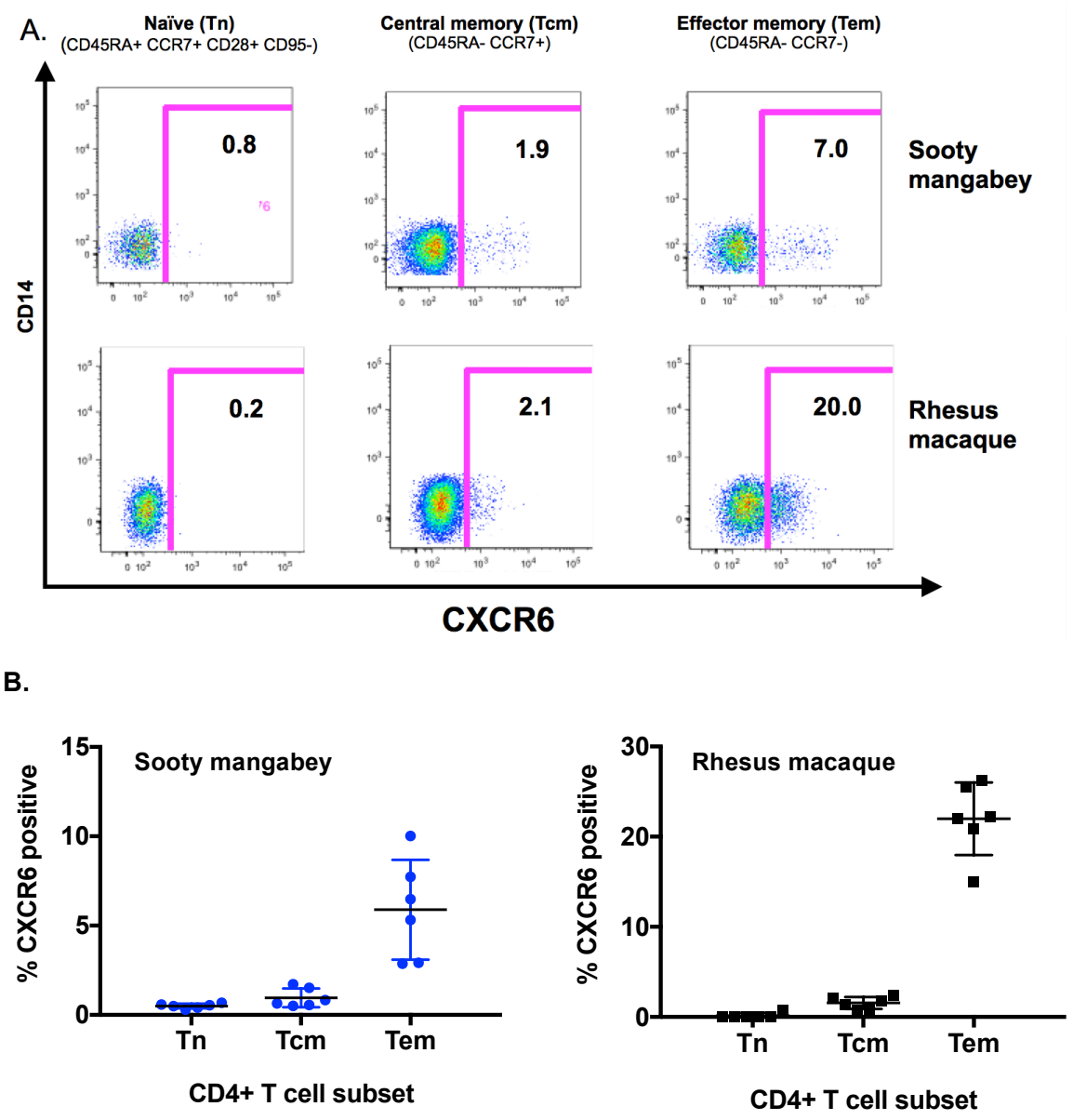

Figure 4.3: CXCR6 expression is enriched on effector memory CD4+ T cells (Tem) Resting sooty mangabey (SM) and rhesus macaque (RM) peripheral blood mononuclear cells were stained using antibodies to define CXCR6 expression of CD4+ memory subsets: naive (Tn: CD45RA+ CCR7+ CD28+ CD95-), central memory (Tcm: CD45RA- CCR7+) and effector memory (Tem: CD45RA- CCR7-). A. CXCR6 expression (x-axis) of memory subsets of one representative sooty mangabey (top panels) and one rhesus macaque (bottom panels). CD14, included in the panel to exclude monocytes in gating, is shown on the y-axis as it permits clear visualization of CXCR6+ cells. B. The proportion of CXCR6+ cells in CD4+ Tn, Tcm, and Tem subsets for 6 SM and 6 RM. Values displayed represent percent of cells that stained positive for CXCR6 using 20D8 + a secondary antibody minus the percent of cells that stained positive using the secondary alone.

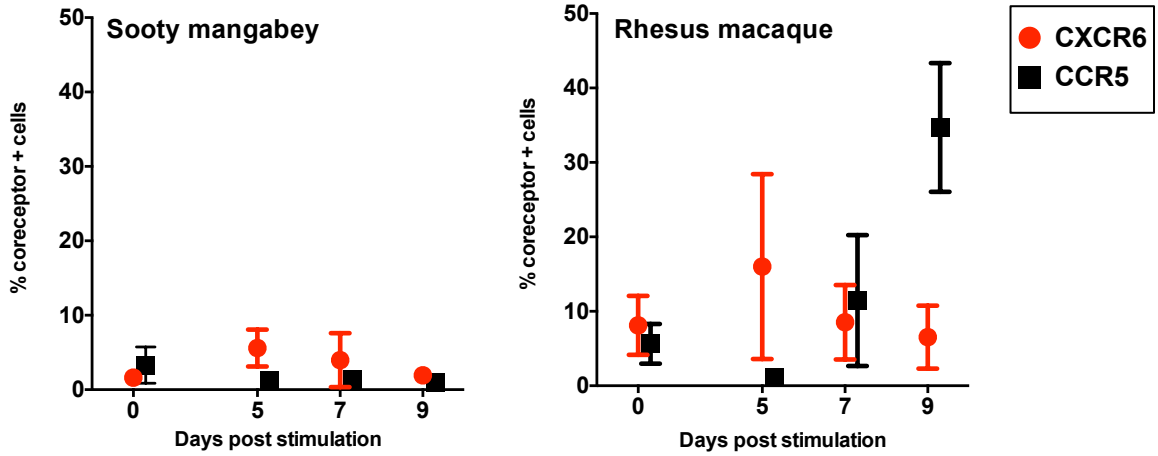

Figure 4.4: The proportion of CXCR6 positive cells increases in response to stimulation for both sooty mangabey and rhesus macaque CD4+ T cells. Cryopreserved sooty mangabey (SM) and rhesus macaque (SM) peripheral blood mononuclear cells were thawed, rested overnight, and then stained (Day 0) or stimulated with Concanavalin A and IL-2. Approximately ½ cells were removed and stained per timepoint (Day 5, 7 and 9) for CD4 and coreceptor expression. Coreceptor expression of SM CD4+ T cells (n=5) is shown in the left panel and RM CD4+ T cells (n=7) are shown on the right.

Discussion

Natural hosts of SIV, such as the sooty mangabey (SM), do not progress to AIDS despite high plasma viremia. One mechanism that likely contributes to this infection outcome is distinct targeting of SIV among CD4+ T cell subsets. Specifically, the protection of certain critical CD4+ T cell subsets and anatomic niches from infection likely preserves immune system function in natural hosts. Conversely, targeting of infection in these hosts to more replenishable, expendable CD4+ T cell subsets results in high viral loads. Our group recently showed that CXCR6 is widely used by SIVs that infect natural hosts, which are known to express very low CCR5 levels, including SIVsmm infection of SM PBMC. Here, I showed that CXCR6 follows an expression pattern on natural host SM memory CD4+ T cells that is consistent with such a model: naïve (Tn) and central memory (Tcm) CD4+ T cells, which are required for maintaining CD4+ T cell homeostasis, had very limited CXCR6 expression, while effector memory (Tem), which are replenishable, were enriched in CXCR6 expression. A similar expression pattern was found in the non-natural host RM, although this is not likely to impact SIV targeting in this species since SIVmac does not use rmCXCR6 for entry, and RM express high levels of CCR5. Furthermore, CXCR6+CCR5- cells formed a distinct subset of CD4+ T cells in RM and SM. For SM, this identifies a previously unacknowledged target cell population in this host that likely contributes to the high viral loads observed in SM despite infrequent CCR5 expression. While we only examined CXCR6 expression here in SM, this pattern may be relevant to other natural hosts whose endemic viruses also use species-matched CXCR6 efficiently, such as African green monkeys infected with SIVagm.

Between RM and SM, CXCR6 expression patterns across memory subsets were generally conserved, namely restriction of expression on Tcm and enrichment on Tem. While this suggests that similar mechanisms regulate expression in both species, subtle differences were noted. A smaller proportion of SM CD4+ Tem cells expressed CXCR6 than did RM CD4+ Tem cells (SM: 5.8% CXCR6+ Tem; RM: 22% CXCR6+). This could reflect evolutionary pressure on SM to

moderate the frequency of this SIVsmm coreceptor and respective CXCR6+ target cells. RM has faced no such pressure, as it is not endemically infected with SIV, CXCR6-using or otherwise.

With the development of the anti-primate CXCR6 antibody 20D8, many additional studies can be performed to investigate the contribution of CXCR6 use to natural host SIV cell targeting and infection outcome. First, as the bulk of SIV replication (and pathology in non-natural host infection) occurs in the lymph node and the gut (10), it will be necessary to define CXCR6 expression patterns in these relevant tissues by flow cytometry or immunohistochemistry. I hypothesize that CXCR6 expression would be infrequent in the lymph node, particularly on Tfh cells, corresponding to the low viral burden observed in this compartment in SM (9). I hypothesize that CXCR6 expression would be higher in the gut, where the bulk of virus replication occurs; however, CXCR6 expression would likely be minimal on Th17 cells that contribute to the maintenance of gut barrier integrity and immunity (16). Th17 cells are maintained in natural host infection, and therefore are unlikely to be SIVsmm targets. Akin to the data regarding peripheral CD4+ T cell subsets shown here, a comparison between SM and RM will offer clues as whether CXCR6 expression is differentially regulated by natural and non-natural hosts in these compartments.

Although characterization of nonhuman primate CXCR6 is limited to the expression data shown here, studies of other species may offer insight into the function and expression of CXCR6 in general. Studies in humans and rats suggest that CXCR6 defines extra-lymphoid homing T cells and is largely excluded from lymph nodes (36, 37). A similar finding in natural hosts would suggest a low frequency of CXCR6+ target cells in this tissue and be consistent with the low virus burden observed in lymph nodes of SM and AGM. In humans, CXCR6 expression has been described primarily on memory CD4+ T cells, particularly Th1 cells, although expression on Th17 cells and naïve T cells has been reported as well (35, 36, 38, 39). Here, expression of CXCR6 on effector memory, but not naïve CD4+ T cells was observed in both SM and RM. CXCR6 can also

be found on human cells that lack CD4 and are therefore not virus targets, such as CD8+ T cells, which was also observed for SM and RM in this study, and NKT cells (35, 36, 40). Human CXCR6 mRNA has been isolated from the placenta, small intestine, thymus and spleen (41); protein expression has been found in the liver, but otherwise is not well defined (42). (The function of CXCR6 and its ligand CXCL16 are discussed in Chapter 5.) Direct investigation of CXCR6 expression in natural hosts will be required to identify the frequency and location of CD4+CXCR6+ target cells of SIV. Given that SIV has been endemic among natural hosts for hundreds of thousands of years or more, it is possible that the expression pattern of this coreceptor in natural hosts vary from that of humans due to host evolution with the virus.

Studies of human lymphocytes have found that CXCR6 and CCR5 are frequently coexpressed on the same population of cells (35). However, in this study of SM and RM lymphocytes, CXCR6 and CCR5 expression largely defined two populations of single-coreceptor expressing CD4+ and CD8+ T cells. This suggests that CXCR6 expression patterns in humans may not reflect those of nonhuman primates, and supports the need for direct studies of natural host tissues to identify CXCR6-expressing target cells.

Additional CD4-expressing cells for which CXCR6 expression ought to be defined include CD4+ T regulatory cells (Tregs), which reduce the effector activity of other immune system cells, and macrophages. Whether natural host Tregs serve as target cells is unclear; a recent study identified frequent infection of this subset in SIVmac infection of RM (43), but CD4+ Treg infection in natural hosts remains undefined. The role of Tregs in natural host infection is likewise uncertain; studies of AGM suggest Treg activity contributes to control of immune activation (44), but studies of SM did not find such a contribution (45). Measuring CXCR6 expression on CD4+ Tregs will inform whether these cells can serve as SIVsmm targets. SIV infection of macrophages has generally been studied in the context of CD4+ T cell depletion. These studies have shown that macrophages can serve as SIV targets in infection of RM (46), but are likely an insignificant

target population in infection of SM (20). A lack of CXCR6 expression by macrophages could be a contributing factor.

In addition to SIVsmm, other natural host viruses can use CXCR6 for entry: SIVagmSab (*ex vivo*, Chapter 3) and SIVagmVer (*in vitro*, species-matched coreceptor)(33), which infect sabaeus and vervet AGM, respectively, and SIVrcm (*in vitro*, human coreceptor)(47, 48), which infects red-capped mangabeys (RCM). Identifying which cells express CXCR6 in these species will define the breadth of the restriction of CXCR6 expression to CD4+ Tem observed here for SM. To investigate vervet AGM CXCR6 expression patterns prior to the generation of anti-CXCR6 20D8, Riddick *et al* quantified CCR5 and CXCR6 mRNA in naïve and memory T cells of vervet AGM as well as RM (33). Surprisingly, they found high CXCR6 mRNA levels in naïve CD4+ T cells in both species; this contrasts with the finding here of little CXCR6 expression on naïve CD4+ T cells in RM. However, RNA expression does not necessarily predict protein expression; if expressed at the protein level it would suggest that naïve T cells could be targets for SIVagmVer, which would be unexpected. Thus, it will be necessary to stain vervet AGM PBMC to define surface expression of CXCR6, as well as correlate CXCR6 mRNA and surface expression levels within a variety of species to determine the relationship between these two values and if post-transcription regulatory pathways exist for expression of this coreceptor.

Both AGM and RCM have evolved specific mechanisms that help protect cells from SIV infection that are not found or are less common in SM. For AGM, CD4+ T cells downregulate CD4 upon entry into the memory pool. This occurs independently of infection, and these CD4- cells are still able to carry out helper functions (49-51). In contrast, RCM have evolved a genetic mechanism that is hypothesized to protect CD4+ T cells, as a CCR5-null allele is common among this species, resulting in homozygous animals that lack CCR5 on the surface of their CD4+ cells. While such a mechanism exists in SM (52), it is much more common in RCM, where 73% of genotyped RCM (n=15) were homozygous for this allele (48). Furthermore, SIVrcm cannot enter

cells using CCR5, but instead can use CXCR6 and CCR2b (*in vitro*, human receptor)(47, 48). These additional CD4+ cell protection mechanisms found in AGM and RCM have possibly exerted distinct pressure on CXCR6 expression in these hosts, and staining for CXCR6 will elucidate whether this resulted in distinct CXCR6 expression patterns from SM in these natural hosts.

While published data from our lab demonstrates a clear role for CXCR6 in SIVsmm infection (31, 32, 52), the relationship between CCR5 and CXCR6 and cell targeting *in vivo* remains to be definitively established. The development of the anti-NHP CXCR6 antibody 20D8 now allows such experiments. To identify coreceptor entry pathways used by SIVsmm *in vivo*, cell-associated viral DNA could be measured in PBMC from SIV+ SM sorted by double, single or no CCR5 and CXCR6 expression to define the proportion of infected cells among each subset. These studies could be complimented by *ex vivo* experiments investigating the relative permissiveness of CCR5 and CXCR6 expressing cells, where SM PBMC would be infected with SIVsmm and stained for coreceptor expression and the presence of virus (either by staining for the capsid protein p27 or by using a GFP reporter virus).

CCR5 and CXCR6 were largely expressed on distinct CD4+ T cells in both RM and SM. Furthermore, expression patterns of these coreceptors on PBMC diverged in response to mitogenic stimulation, with SM and RM CXCR6 expression peaking earlier, RM CCR5 expression peaking later, and SM CCR5 expression remaining low throughout. Together, these data suggest that distinct mechanisms regulate the expression of CXCR6 as compared to CCR5, and CCR5 in RM as compared to SM. These data also suggest that regulation of expression of these coreceptors in monkeys is distinct from that in humans, where coordinate CCR5 and CXCR6 expression has been reported (35). Further studies are warranted to discern how this regulation occurs, such as whether such differences emerge at transcription due to distinct promoter elements, or later, such as protein regulation due to production of their respective cytokines.

Also, this analysis showed changes in the frequency of coreceptor expressing cells over time, but did not distinguish between increases due to upregulation of coreceptor expression and death of non-coreceptor expressing cells. Future studies require marking dividing cells with a dye such as carboxyfluorescein succinimidyl ester (CFSE) and measuring coreceptor expression of divided cells in order to determine if activated, replicating cells upregulate CXCR6. Lastly, in this study, PBMC were stimulated with concanavalin A to induce robust activation. However, a more biologically relevant stimulation could be using antibodies against CD3 and CD28**.** Such experiments will be facilitated by the recent generation of a conjugated version of 20D8.

In addition to *ex vivo* activation, changes in the frequency of the CXCR6+ CD4+ T cell population likely change in response to *in vivo* activation; namely, in response to SIVsmm infection. An important experiment to address this would define coreceptor expression in one cohort of RM and SM by measuring the frequency of CXCR6+ cells prior to experimental SIV infection, then at several time points in both the acute and chronic phases, as has been done for CCR5 expression in these species (24). However, given that current regulations prevent the experimental infection of SM, cells from distinct populations of uninfected and infected primates would need to be compared instead, which would identify general changes in the population of CXCR6+ cells due to SIV infection.

One caveat of this study is that the observed frequency of CCR5 positive cells was lower than previously reported studies, particularly for RM (24, 30). This may be due to the fact that the available samples for this study were cryopreserved CD4+ T cells, and freezing is known to reduce the frequency of CCR5 expression (53). To more accurately measure frequency of CCR5+ cells in comparison to CXCR6+ expressing cells, a study using whole blood, where CCR5 expression is most robust, is warranted. However, it is unlikely that such analysis would alter the observation that CXCR6 expression is generally on a population of CD4+ T cells distinct from those expressing CCR5.

One feature of natural host infection observed in both SM and AGM is a low frequency of mother to infant transmission (MTIT). Studies in both species have found this low rate corresponds to a low frequency of CCR5+CD4+ target cells (54-56). In order to fully understand this phenotype, the frequency of CXCR6+CD4+ cells in infant and juvenile animals must also be measured. A recent study of MTIT in SM by Chahroudi *et al* measured mRNA of CXCR6 and did find a low frequency of CXCR6 mRNA in infant SM (56), suggesting that CXCR6 surface expression in this cohort is likely low as well.

In summary, these data identify effector memory CD4+ T cells in peripheral blood as CXCR6-expressing target cells for SIVsmm infection of sooty mangabeys. The finding that CXCR6 expression is restricted on naïve and central memory CD4+ T cells, as is CCR5 expression, suggests that these cells are protected from infection and use of CXCR6 targets virus towards more differentiated and likely expendable cell populations in natural hosts. The future studies described here, made possible by the generation of the novel anti-primate CXCR6 antibody 20D8, will elucidate the degree to which CXCR6 contributes to cell targeting and maintenance of immune function observed in natural hosts.

Materials and Methods

Generation of anti-primate CXCR6 monoclonal antibody 20D8

To generate a CXCR6-expressing cells as an immunogen, a plasmid was made with GFP fused to the C-terminus of RM CXCR6 and SM CXCR6 (rmCXCR6-GFP or smCXCR6-GFP). Proper translation and expression of the construct was ensured by testing the ability of the CXCR6-GFP gene products to permit SIVsmm entry. The CXCR6-GFP genes were then inserted into the lentiviral vector pELNS. Cells of the murine line B78H1 were transduced with the vectors, and GFP-high cells were selected for immunization of mice.

Balb/c mice were immunized against SM CXCR6 or RM CXCR6 by three hydrodynamic tail vein injections of pcDNA3.1+ containing SM or RM CXCR6 (15 ug plasmid each) given one, three and four weeks after a pre-immunization bleed. Five weeks later, mice began a series of five bi-weekly intraperitoneal injections of smCXCR6-GFP or rmCXCR6-GFP high-expressing B78H1 cells that had been sublethally irradiated (10×10^6 cells per injections 1-3, 50×10^6 cells per injections 4 and 5). One week after the final immunization, spleens were harvested and hybridomas were generated by fusing splenocytes with the Sp2/0 myeloma cell line.

Hybridoma supernatants were screened for production of CXCR6 reactive antibody by a 293T cell-based ELISA (cELISA; modified from Atanasiu *et al*, JVI 2016, (57)). 293T cells were transfected with an expression vector containing smCXCR6, rmCXCR6 or empty vector. 48 hours later, cells were lifted with 2mM EDTA and resuspended in 3%BSA in PBS+ Mg^{2+} and Ca^{2+}. 1e5 cells were plated/well in a 96 well plate and incubated 30 min at room temperature. Plates were spun, buffer aspirated and incubated for 1 hour with hybridoma supernatant at 4°C. Plates were spun and cells washed twice with PBS + Mg^{2+} and Ca^{2+}. Cells were resuspended in a 1:100 dilution of goat anti-mouse horseradish peroxidase (HRP) antibody in 3% BSA and incubated for 1 hr at room temperature. Cells were washed with PBS twice, then fixed with 3% PFA. Plates were spun, PFA aspirated and cells washed with 20mM ph4.5 sodium citrate. Plates were spun,

citrate buffer removed and HRP substrate was added. Color was allowed to develop for 15 minutes and hybridoma supernatant reactivity with cells expressing CXCR6 vs. cells not expressing CXCR6 was determined visually. Over 1400 hybridomas were screened, and one was identified (20D8) from an smCXCR6-immunized mouse that was reactive by cELISA with cells expressing smCXCR6, but not cells transfected with empty vector.

To determine the breadth of primate CXCR6 recognition by 20D8, 293T cells were transfected with pcDNA3.1+ containing human, chimpanzee, mustached monkey, greater spot-nosed monkey, RM, SM, sabaeus AGM, or vervet AGM CXCR6, or SM CCR5 or empty pcDNA3.1+ vector. (Genes not cloned in this paper were previously cloned by us or collaborators.) 48 hours later, cells were lifted and stained with unconjugated 20D8 followed by APC goat anti-mouse IgG 2° (Poly4053, BioLegend), the 2° alone or anti-human CXCR6 AF647 (K041E5, BioLegend).

CXCR6 expression on sooty mangabey and rhesus macaque PBMC

PBMC were isolated from six SM, 5 of which were homozygous for wild-type CCR5, and one of which was heterozygous for the CCR5 Δ2 mutation (52). Cells were thawed and rested overnight in RPMI supplemented with 10% FBS, 1% L-glutamine and 1% pen/strep prior to staining.

SM PBMCs were thawed and rested overnight in RPMI supplemented with 10% FBS, 1% pen/strep and 1% L-glutamine. Cells were first stained with the anti CXCR6 clone 20D8, followed by a goat-anti mouse AF488 secondary (Poly4053, BioLegend). The cells were then washed and stained with Aqua Live/Dead Dye (Invitrogen) and the remaining surface antibodies: anti-CD3 APC-Cy7 (clone SP34-2, BD Pharmingen), anti-CD4 PECy5.5 (clone S3.5, Invitrogen), anti-CD8 BV570 (clone RPA-T8, BioLegend), anti-CCR7 (clone G043H7, BioLegend), anti-CD95 PECY5 (clone DX2, BD Biosciences) anti-CD28 ECD (clone CD28.2, Beckman Coulter), anti-CCR5 PE (clone 3A9, BD Pharmingen), anti-CD45RA PE Cy7 (clone 5H9, BD Pharmingen), anti-CD20 BV650 (clone 2H7, BD Horizon), anti-CD16 BV650 (clone 3G8, BioLegend), anti-CD14 BV605

(M5E2, BioLegend). Cells stained with the entire surface panel but with the AF488 secondary antibody only (and not unconjugated 20D8) were stained in parallel to define non-specific staining by the secondary. The gate for CXCR6 expression was then drawn on the cells stained with the secondary antibody only. As slight non-specific staining by the secondary was observed, the reported percent positive cells for CXCR6 staining were calculated by subtracting the percent of AF488 positive cells in the secondary only condition from the anti-CXCR6 20D8 plus secondary condition. Samples were run on an LSRII and data analyzed using FlowJo software (v. 9.9).

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CHAPTER 5

Discussion

Natural hosts of SIV do not develop immunodeficiency despite high viremia, and this mutually satisfactory relationship between virus and host is likely the product of hundreds of thousands of years of coevolution (1). Understanding the mechanisms by which these nonhuman primates (NHP) are able to maintain high level viremia yet not develop disease will further elucidate the incompletely understood mechanisms of pathogenesis in primate lentivirus hosts that do progress to AIDS, such as HIV-1 infected humans.

Of the many mechanisms that have been implicated in immunodeficiency of non-natural hosts, a common thread is infection and loss of certain critical CD4+ T cells that are maintained in natural hosts. Preservation of these subsets, such as central memory CD4+ T cells (Tcm) and Th17 cells, paired with infection of more expendable CD4+ T cells, likely supports viremia without progression to disease. Thus, an essential question to answer to understand SIV/HIV pathogenesis is what defines target cells of natural hosts, and how this differs in infection in non-natural hosts. Earlier work from my lab indicated that use of the coreceptor CXCR6 by SIVsmm to enter primary T cells from its natural host, the sooty mangabey (SM), distinguished this infection from pathogenic infections such as HIV-1 and SIVmac, which are largely restricted to use of species-matched CCR5 and do not use CXCR6 for entry (2-4). In this thesis, I aimed to expand upon this finding by defining the breadth of CXCR6 use among additional natural host SIVs, investigating the coreceptor use patterns and relationship to pathogenesis within the virus lineage that gave rise to the HIV-1 pandemic, and identifying SIVsmm target cells defined by expression of this coreceptor in SM.

I have presented data showing that SIVagmSab (Chapter 2) and SIVmus (Chapter 3) can use the species-matched coreceptor CXCR6 in addition to CCR5 *in vitro*, and verified use of this entry pathway in SIVagmSab infection of sabaeus lymphocytes (Chapter 2). When added to published work from our lab and others demonstrating species-matched CXCR6 use by SIVsmm *in vitro* and *ex vivo* (3, 4), and by SIVagmVer *in vitro* (5), my data support the conclusion that CXCR6

use is a common feature of natural host SIVs. This feature is in stark contrast to HIV-1, SIVmac and SIVcpz (Chapter 3) that cannot use CXCR6 in addition to CCR5 and are pathogenic in their respective hosts. This recurrent association between CXCR6 use and lack of pathogenesis suggests that infection of CXCR6-expressing target cells contributes to this phenotype in natural hosts.

The finding that SIVmus can use species-matched CXCR6 for entry, while SIVcpz cannot, has implications beyond bolstering the relationship between lack of pathogenesis and CXCR6 use. A virus of the lineage to which SIVmus belongs gave rise to the envelope gene of SIVcpz (6, 7); therefore, use of CXCR6 was seemingly lost in the emergence of SIVcpz, and restriction to CCR5 as a coreceptor is associated with the emergence of pathogenesis in the new chimpanzee host. This restriction to CCR5 use from CXCR6 and CCR5 use parallels observations made when comparing the non-natural host virus SIVmac to its natural host virus ancestor SIVsmm.

Finally, I provided the first analysis of CXCR6 expression on nonhuman primate (NHP) peripheral blood cells, and found that CXCR6 expression is enriched on effector memory CD4+ T cells (Tem) and largely defines a separate population of CD4+ T cells than does CCR5 expression in both SM and rhesus macaques (RM). These data suggest that CXCR6 expression defines a previously unrecognized population of SIV target cells in SM. Collectively, the data in this thesis suggest that entry through CXCR6 (in addition to CCR5) by natural host SIVs (which express low levels of CCR5) is a key feature of natural host viruses that may permit high levels of viremia without immunodeficiency.

Differential targeting by natural and non-natural SIV/HIV

Many studies have demonstrated that natural and non-natural host infections have distinct patterns of tissue and cell targeting. Natural host infections are characterized by low viral burden in lymph nodes, including among T follicular helper cells (Tfh) (8-11), low frequency of infection of

central memory (Tcm) and stem cell memory (Tscm) CD4+ T cells (12, 13), and maintenance of Th17 cells in the gut (14). In contrast, non-natural host infections have high viral burden, inflammation and progressive fibrosis in lymph nodes, including infection of Tfh, (15, 16), higher frequency of Tcm and Tscm infection, which likely disrupts CD4+ T cell homeostasis, as well as drastic Th17 cell loss, which likely contributes to microbial translocation and chronic immune activation (17-19). As receptor and coreceptor expression is the primary determinant of HIV and SIV target cells, virus coreceptor use likely influences these differences in cell targeting and therefore pathogenicity.

Early studies of natural hosts quickly identified restricted SIV cell targeting as a possible mechanism to maintain immune system function despite viremia, defining limited CCR5 expression as a common feature of natural hosts that occurred independent of infection status (20). Detailed studies of SM showed that this restriction is particularly profound in memory CD4+ T cell subsets with greater regenerative potential, including Tcm and Tscm (12, 13). While restriction of CCR5 expression likely contributes to protection of certain CD4+ T cell subsets, it raises the question of how natural host infections are able to maintain high viral loads in the face of limited target cell frequency and the observed high turnover of infected cells (21). The identification of CXCR6 as a robust coreceptor for SIVsmm (3, 4) and SIVagmSab (Chapter 2) suggests that CXCR6 expressing cells are also SIV targets, and could define additional, previously unrecognized SIV target cells.

By developing anti-NHP CXCR6 antibody 20D8, I was able to address which peripheral CD4+ T cells express CXCR6 and therefore are targets of SIVsmm infection of SM. As described in Chapter 4, I found that CXCR6 expression was restricted on undifferentiated (naive, Tn) and less differentiated memory CD4+ T cell subsets (Tcm). This expression pattern corresponds with data suggesting that protection of Tcm from infection is paramount to maintaining immunocompetence in SM as well as rare viremic non-progressing HIV-1 infected humans (12, 22). In contrast,

CXCR6 expression was enriched on effector memory CD4+ T cells, which are considered more expendable and may be able to support viremia without putting a strain on memory CD4+ T cell homeostasis (23, 24). Furthermore, the identification of CXCR6+CCR5- CD4+ T cells identifies a novel population of SIVsmm target cells that when added to the infrequent population of CCR5+CD4+ T cells increases the total proportion of CD4+ T cells that can serve as targets and support high viremia.

MODEL: Divergent entry pathways between natural and non-natural hosts of SIV

Considering that entry is a key determinant of SIV target cells, I propose a model of natural host SIV infection whereby use of CXCR6 (and possibly other alternative coreceptors), concomitant with a low frequency of CCR5+ cells, targets natural host SIVs towards more replenishable CD4+ T cell subsets (Figure 5.1). Infection of such CXCR6-expressing replenishable cells would permit replication to high titers without causing the destruction of T cell homeostasis and lymph node and gut architecture that characterizes pathogenic infection. Due to their enriched CXCR6 expression (Chapter 4) and limited role in maintaining the memory CD4+ T cell pool (25-27), CD4+ Tem cells likely represent one such replenishable subset. In support of this, the frequency of infection CD4+ Tem, but not Tcm, was found to correlate with plasma viral load in sooty mangabeys (8). In contrast, in non-natural host infections, where CCR5 but not CXCR6 is used for entry, a higher frequency of CCR5+ CD4+ T cells targets the virus to various compartments, including the lymph node, gut, and more critical CD4+ memory T cell subsets such as Tcm. Consequently, gut and lymph node integrity is lost and T cell homeostasis is disrupted, driving chronic immune activation, prolonged CD4+ T cell homeostatic proliferation and the progression to immunodeficiency. These non-natural host viruses include HIV-1, SIVcpz and SIVmac, all of which are the results of cross-species transmission events, suggesting that cross-species transmission to a naïve host may trigger this altered targeting.

To investigate this model, a necessary next step is to define CXCR6 expression (and CCR5 coexpression) in the major sites of SIV replication: the gastrointestinal tract and the lymph node. From the model, I hypothesize that CXCR6 expression would be enriched in the gut, where most natural host SIV replication occurs (28, 29), but only on cell subtypes that would not contribute to disruption of barrier integrity when infected and lost. In contrast, I hypothesize that CXCR6 cells would be present at a low frequency in the lymph node, which carries reduced viral burden in natural hosts (8, 9). Studies of sooty mangabey lymph node infection have demonstrated that SIVsmm replication occurs largely outside the follicles (8). Therefore, I would hypothesize that any CXCR6 expression present is likewise excluded. Furthermore, T follicular helper CD4+ cells (Tfh) that reside in the lymph node are also infected less frequently in natural hosts (8), suggesting CXCR6 expression would be restricted on this subset as well. Prior to performing these studies, some insight into possible CXCR6 tissue distribution patterns can be gleaned from analysis of murine and human studies, which largely agree with these predictions, as studies in humans and rats suggest that CXCR6 characterizes extra-lymphoid homing T cells (30, 31). In humans, CXCR6 mRNA has been isolated from the placenta, small intestine, thymus and spleen (32).

That being said, it is quite possible, if not likely, that patterns of CXCR6 expression differ between NHP and humans. Studies of CXCR6 expression in human peripheral lymphocytes have described frequent coexpression of CCR5 and CXCR6 (33). This is distinct from my finding of largely segregated CCR5 and CXCR6 expressing cells in SM (and RM) lymphocytes (Chapter 4). This suggests that monkey expression patterns may be distinct from those found in humans. Furthermore, human studies have found CXCR6 expression on tonsil Th17 cells (34). From our model, I hypothesize that Th17 cells, which are maintained in natural host infection and therefore unlikely to be target cells, would not be enriched in CXCR6 expression. Defining CXCR6-expressing cells in natural host tissues by flow cytometry or immunohistochemistry will test these

predictions. Staining SM, RM and human tissue in parallel will provide insight as to whether CXCR6 expression in tissues is regulated differently between natural and non-natural hosts.

Defining the phenotype of CXCR6-mediated targeting

If CXCR6 expression in RM mirrors that of SM in tissues as it does in the blood, infecting macaques with a virus restricted to use of rmCXCR6 would help define the degree to which targeting mediated by CXCR6 contributes to infection outcome. Such a virus could be generated by serially passaging SIVmac on a mixture of engineered SupT1 cells where the proportion of cells expressing rmCXCR6 greatly exceeds those expressing rmCCR5, followed by passaging on cells expressing rmCXCR6 only, thus allowing replication but prompting evolution to use rmCXCR6 (akin to generation of CD4-independent SIVmac variants, (35)). Given that other viruses can use rmCXCR6 *in vitro* ((5) and data not shown), imposing use of rmCXCR6 use onto SIVmac is likely biologically feasible. If such passaging is not sufficient to eliminate CCR5 use, the virus could be further modified to ablate CCR5 binding (if possible), or, infections could proceed in the presence of the CCR5-blocker maraviroc. This CXCR6-restricted infection could mimic natural host infection by forcing the virus into more replenishable CXCR6-expressing subsets, such as Tem, and protecting cells such as Tcm that don't express CXCR6 in RM. I hypothesize that such an infection in rhesus macaques would have high viremia due to sufficient CXCR6+ target cells, but less pathogenicity than a typical SIVmac infection due to lack of infection of critical subsets such as Tcm that infrequently express CXCR6. Any residual pathology could be due to additional mechanisms to modulate the immune system in the presence of SIV infection that non-natural hosts have not been pressured to evolve, and this macaque model could be used to define such mechanisms.

While data clearly show use of CXCR6 for entry by SIVsmm and SIVagmSab *ex vivo* (4) (Chapter 2), many questions remain regarding the use of these coreceptors *in vivo*, and the relative contribution each plays in natural host SIV infection. To determine the relationship between

CXCR6 expression and *in vivo* infection of SM or AGM, CD4+ T cells from chronically infected animals could be sorted into four groups based on single, double or no CCR5 and CXCR6 expression and cell-associated viral DNA quantified. This would identify which coreceptor-expressing subsets are more frequently targeted by SIV *in vivo*. (Reduced viral loads of CCR5-null sooty mangabeys suggest that CCR5+ CXCR6- cells do serve as targets in these animals. (2)) Previous studies have identified decreased cell-associated viral DNA in sooty mangabey Tcm, as compared to sooty mangabey Tem and rhesus macaque Tem (12); sorting by both memory markers and coreceptor expression would reveal the contribution of CXCR6 use to this difference.

The CXCR6 expression data shown here (Chapter 4) was performed on cells from SIV negative sooty mangabeys. Monitoring CXCR6 expression over the course of an SIV infection would indicate if coreceptor expression is actively modulated or changes as a consequence of infection, thus altering what cells serve as targets. For CCR5, previous studies have shown that the proportion of CCR5+ CD4+ T cells increases slightly in acute infection of sooty mangabeys, but less so than the drastic increase observed in rhesus macaque infection (12). In chronic infection of SM, the proportion of CCR5+ CD4+ T cells is reduced (36). However, current regulations against experimental infection of SM make it impossible to carry out a time course study in experimental SIVsmm infection. However, such a study could be performed in African green monkeys (AGM) after defining CXCR6 expression in this host, or by comparing coreceptor expression levels between populations of chronically infected and uninfected sooty mangabeys.

Use of coreceptors in addition to CXCR6

While CCR5 and CXCR6 were the most robust coreceptors in vitro for both SIVsmm and SIVagmSab, blocking entry through CCR5 and CXCR6 was insufficient to completely block replication of both viruses (4) (Chapter 2). We think this is likely due to the inability of maraviroc and CXCL16 to completely block entry through CCR5 and CXCR6, respectively. However, it

could also be due to the use of coreceptors in addition to CCR5 and CXCR6. In particular, GPR15 was used by both viruses *in vitro*, albeit more efficiently by SIVagmSab. GPR15 has no known ligand or small molecule inhibitor, so we have been unable to test use of this coreceptor directly. However, advances in gene-editing technologies may permit future studies where use of these three coreceptors is interrogated further by gene deletion from primary lymphocytes. Staining of lymphocytes from several SM and RM demonstrated GPR15 expression on CD4+ T cells, which was largely independent of CCR5 and CXCR6 expression (data not shown). This finding is congruous with studies of human coreceptor expression, where CXCR6 and CCR5 were expressed by Th1 cells (30, 37) and GPR15 was expressed by Th2 cells (38). However, there is evidence that GPR15 may not function as a coreceptor on primary cells even if it functions in transfected cells: GPR15 is robustly expressed on rhesus macaque CD4+ T cells, and SIVmac uses rmGPR15 efficiently *in vitro* in transfected cells, but does not use it to enter RM CD4+ T cells *ex vivo* or appear to use it *in vivo* (39-41). This suggests that at least for SIVmac, expression of CD4 and GPR15 is not sufficient to render a CD4+ T cell a target. A possible explanation could be if Th2 cells were intrinsically less permissive to SIVmac infection. Alternatively, there may be currently unappreciated differences between GPR15 structure or post-translational modification when expressed *in vitro* vs. endogenously, therefore underscoring the importance of confirming pathway use by blocking entry in lymphocytes.

Alternative coreceptor use by untested SIVs

SIVsmm, SIVagm, and SIVmus represent three distinct lineages of natural host SIVs that diverged at least hundreds of thousands of years ago, suggesting that CXCR6 use by natural host SIVs is longstanding, in addition to widespread (1, 42). Other distinct but untested viruses include SIVmnd, SIVolc and SIVlho that infect mandrills, olive colobus monkeys and l'Hoest's monkeys, respectively. Defining the coreceptor usage patterns of these SIVs akin to studies in this thesis would flesh out the breadth of CXCR6 uses among natural host SIVs.

Cross species transmission, coreceptor restriction and the emergence of pathogenesis

The lack of pathogenesis of SIV infection of natural hosts is thought to be the product of prolonged coevolution between virus and host. Thus, the emergence of pathogenesis when an SIV infects a naïve host is not unexpected. Two major examples of this pattern exist: One, SIVsmm infection of rhesus macaques resulted in SIVmac, the main animal model for HIV-1 infection of humans (43). Two, a virus of the lineage to which SIVmus belongs crossed into chimpanzees (along with SIVrcm that contributed the 5' half of the genome) giving rise to pathogenic SIVcpz (44, 45). SIVcpz then crossed the barrier into humans, giving rise to HIV-1, which is even more pathogenic in its respective host than SIVcpz is believed to be (46, 47).

Of note, cross-species transmission events (both experimental and in the wild) do not always result in immunodeficiency. For example, experimental infection of RM with SIVagmSab results in control of the virus (48). Also, SIVagmSab DNA was isolated from a Patas monkey infected in the wild, and a follow-up experimental infection of Patas monkeys with SIVagmSab resulted in a natural host phenotype (49, 50). Patas monkeys are characterized by a low frequency of CCR5+ CD4+ T cells, suggesting that their coreceptor expression patterns and therefore virus targeting may parallel natural hosts of SIV even though there presently is no identified circulating SIV in this species.

For SIVmac and SIVcpz/HIV-1, a common outcome of their origin in cross-species transmission is restriction to use of CCR5 as a coreceptor from use of both CCR5 and CXCR6. Yet, the origin of this phenotype differs between these two cross-species transmission events. For SIVmac, the inability to use rmCXCR6 efficiently for entry originates from the sequence of the coreceptor, as the R31 residue encoded in the N-terminus renders rmCXCR6 a poor coreceptor for SIVmac (51). *In vitro*, SIVmac can use CXCR6 of other species for entry (4, 51), and this is consistent with the correlation between the ability to use CXCR6 and the lack of a restrictive V3 loop Pro

residue (Chapter 3). However, I found that the inability to use CXCR6 by SIVcpz is conferred by the virus, not the host, as it cannot use CXCR6 of any species for entry and this *env* encodes the CCR5-restrictive V3 loop Pro residue, which is also true for HIV-1.

The contribution of restriction to CCR5 use to pathogenesis likely lies in the fact that CCR5 is much more highly expressed on critical CD4+ cell subsets in non-natural hosts than natural hosts, defining these cells as targets, unlike the limited CCR5 expression on such subsets observed in natural hosts. The infrequent expression of CCR5 observed in natural hosts is hypothesized to have evolved as a mechanism to coexist with the virus; of course, humans and macaques species have not had prolonged virus-mediated pressure to acquire this mechanism. However, a cohort of highly viremic nonprogressing children was recently identified in Africa, and a correlate of this phenotype was low infection of CD4+ Tcm, concomitant with reduced CCR5 expression (22). Use of CXCR6 and other coreceptors by their viruses has not yet been determined. Studies of *P.t. verus* chimpanzees, which do not harbor an endemic SIV, have found an intermediate level of CCR5 on their CD4+ T cells (20). This suggests that pressure in addition to lentivirus coreceptor use can limit CCR5 expression. It is unknown if similar CCR5 expression is also found in *P.t. troglodytes* and *P.t. schweinfurthii* that do carry SIVcpz.

While the host-driven inability of SIVmac to use CXCR6 is quite simple, a more complicated question is why SIVmus would have lost use of CXCR6 upon cross-species transmission into chimpanzees, particularly because use thereof is widespread among SIVs and is associated with an expanded range of target cells. Loss of CXCR6 use as SIVcpz emerged could have been a) a coreceptor-independent consequence of adapting to a new host; b) a stochastic event associated with lack of selection pressure to retain CXCR6 use; or c) a necessary, coreceptor-dependent step in the evolution of the virus.

To address the first option, the observation that SIVmus cannot use chimpanzee CD4 for entry suggests that the barrier towards cross-species transmission may have been high for *envs* of this lineage (Chapter 3). It's possible that in the adaptation to use cpzCD4, restriction to use of CCR5 by *env* occurred in tandem. This could have happened incidentally, due to high selection pressure on *env* in general, or perhaps a necessary consequence of generating Env that could bind chimpanzee CD4 and still maintain a viable structure. An experiment to address this could be to generate replication competent SIVmus (and other SIVs of that lineage) and passage it on chimpanzee CD4+ T cells, or a cell line expressing chimpanzee CD4 and coreceptor. Such passaging could promote affinity for the chimpanzee CD4, and *in vitro* analysis of coreceptor usage could determine whether the resulting virus had restricted coreceptor usage as a result of the changes needed to adapt to chimpanzee CD4.

A second explanation could be that use of CXCR6 by the SIVcpz forerunner was no different than use of CCR5, and therefore lost without selection pressure as SIVcpz emerged. This would be most plausible if CCR5 and CXCR6 were frequently coexpressed on chimpanzee lymphocytes, as they are on human cells, therefore rendering entry through CXCR6 a redundant pathway that offered no selective advantage for the virus. Staining of chimpanzee cells for coreceptor expression would inform this possibility. Similarly, if chimpanzee cells infrequently express CXCR6, pressure to retain use of this coreceptor would have been minimal. However, chimpanzee cells are not available for research studies to test these points directly.

A third possibility is that restriction to CCR5 explicitly was a necessary event for SIVcpz to emerge. It's possible that an increase in affinity to cpzCCR5 was required for efficient entry, and use of cpzCXCR6 was lost as a consequence. However, I found that SIVmus could readily enter through cpzCCR5, suggesting that the need to adapt to cpzCCR5 was not a source of evolutionary pressure. To test this thoroughly, however, it would be useful to test efficiency of use

more directly, for example by titrating cpzCCR5 *in vitro*, akin to the SIVagmSab experiments in Chapter 2.

Selection for use of CCR5 over CXCR6 could also occur due to post-entry events; this would require a fitness advantage for viruses that entered via cpzCCR5 over cpzCXCR6. This could occur through two possible mechanisms: a) CCR5+ cells are distinct from CXCR6+ cells and are better at supporting viral replication, independent of coreceptor engagement; or b) binding of CCR5 by Env and subsequent signaling could promote a cellular environment that supports virus replication better than CXCR6-Env interactions. (This second mechanism could occur whether CCR5 and CXCR6 are coexpressed or not.) Given the sequence divergence of these two chemokine receptors, distinct downstream signaling events are plausible and will be discussed in more detail later in this section. Again, staining chimpanzee CD4+ T cells would be required to determine whether or not CCR5 and CXCR6 are coexpressed in this host. If they are differentially expressed, the susceptibility of distinct populations of coreceptor expressing cells could be measured by infecting lymphocytes with GFP reporter viruses while staining for coreceptor expression and measuring relative infectivity of CCR5 or CXCR6 expressing populations.

Lastly, the pressure against CXCR6 use could have been driven by the chimpanzee immune system, rather than requirements for virus replication in a new host. In humans and macaques, adaptive immune pressure against CXCR4-using viruses has been observed (52, 53), and a similar pressure could be exerted on CXCR6-using viruses in chimpanzees. It is not clear if or why such pressure would exist in chimpanzees but not in natural host monkeys. However, this would be difficult to investigate as experimental infection of chimpanzees is prohibited.

An additional challenge to studying SIVcpz restriction to CCR5 use is that the originating cross-species transmission event is believed to have occurred one time, unlike SIVsmm into humans that has occurred at least eight times, and SIVcpz into humans that has occurred four times (54).

Multiple cross-species transmissions allow comparison of requirements for transmission, particularly when the resulting viruses vary in fitness in their new host (55). However, no comparison exists for the emergence of SIVcpz. The occurrence of only a single transmission event could reflect a high barrier to cross species transmission. However, SIVgsn/mus/mon also are less prevalent in their hosts than other viruses that have crossed the species barrier, so fewer relative exposures have likely been made (56). Also, recombination between SIVrcm and SIVmus could have been necessary for emergence of SIVcpz, with the presumably low likelihood of coinfection with two distinct but compatible viruses.

CXCR6-using HIV-1 variants are rarely identified (57). Coreceptor use of HIV-1 beyond CCR5 and CXCR4 is infrequently investigated, so extensive analysis of CXCR6 use is lacking. However, the CXCR6-incompatible V3 loop Pro is strongly conserved among HIV-1 variants (58). Thus, I speculate that *env* of HIV-1 (and SIVcpz) has evolved such that the barrier to regaining use of CXCR6 as a coreceptor is high. The data demonstrating the inability to confer CXCR6 use to SIVcpz supports this (Chapter 3) but further attempts to do so would be warranted to draw this conclusion. Thorough analysis of CXCR6-using HIV-1 variants could elucidate determinants of CXCR6 usage by HIV-1, as could passaging HIV-1 and/or SIVcpz to promote adaptation to CXCR6 as previously described. Alternatively, there could be human-specific selection pressure against CXCR6-using HIV-1 variants, such as immune pressure (as is the case for CXCR4-using variants, (52, 53)) or cellular factors that restrict their replication that don't exist in natural hosts. Investigation of the contribution of CXCR6 use by HIV-2, which can use CXCR6 *in vitro* (59), would inform the human pressures on CXCR6-using viruses.

Properties of the entry coreceptor CXCR6

All SIV coreceptors identified thus far, including CCR5 and CXCR6, are 7 transmembrane G protein coupled receptors (7TMRs). While these molecules are quite distinct at the amino acid level (many sharing no more than 25% amino acid identity with each other) they do share several

structural and amino acid level features. As their name suggests, these molecules cross the plasma membrane seven times, and all have several Tyr residues in the N terminal domain. For CCR5, it is known that sulfation of several of these Tyr is essential for interaction with the HIV envelope (60). Studies of CCR5 and CXCR4 have revealed that this N-terminal domain interacts with the bridging sheet and the base of the V3 loop of Env, while the second extracellular loop (ECL2) interacts with the V3 loop of Env (61). Although CXCR6 and other alternative coreceptors have not yet been analyzed, this two-part mechanism is likely conserved. Future experiments involving swapping domains between CXCR6 and a 7TMR that is not used as a coreceptor could easily parse out which CXCR6 domains are necessary for coreceptor use. The fact that a single amino acid change (R31) in the N-terminus of rmCXCR6 renders it a poor coreceptor for SIVmac suggests that the N-terminus is an important determinant of CXCR6 use, at least for SIVmac (51). However, the N-terminus of CXCR6 varies distinctly between musCXCR6 and cpzCXCR6, yet SIVmus can use both for entry (albeit musCXCR6 more robustly) (Chapter 4) (Figure 5.2). Furthermore, SIVmus entry through musCXCR6 is not seemingly hindered by the same R31 that makes rmCXCR6 a poor coreceptor for SIVmac (Chapter 4). Therefore, requirements for use of this domain might vary between viruses, or specific regions of the N-terminus might be more important for CXCR6 use or CXCR6 structure than others.

CXCR6 also has several features that distinguish it from other 7TMRs such as CCR5. Structurally, many 7TMRs have four extracellular Cys residues, which form disulfide bonds between the N-terminus and ECL3, and ECL1 and ECL2. In contrast, CXCR6 lacks Cys residues in the N-terminus and ECL3, thus limiting the coreceptor to one disulfide bond and likely resulting in a more flexible structure (Figure 5.2). As described in Chapter 3, Envs that use CXCR6 also have a structural signature; the V3 loop that interacts with the coreceptor encodes Ala, Ser or Thr, instead of the rigid Pro observed in SIVcpz and HIV-1 that is associated with the inability to use CXCR6. Further studies are warranted to determine how these features contribute to CXCR6-Env interactions.

Secondly, most 7TMRs signal through a DRY domain in the second intracellular loop that interacts with G proteins; CXCR6 has a DRF motif instead, which allows association with a broader range of G proteins and may promote adhesion of CXCR6-expressing cells to cells expressing the CXCR6 ligand CXCL16 on their surface (62, 63). This could result in distinct post-entry events due to coreceptor-mediated signaling by Env. Data suggests that HIV-1 Env-mediated signaling through CCR5 is not necessary for entry (64-66), while HIV-1 Env binding to CXCR4 promotes cytoskeleton remodeling to permit infection of resting cells (67). Irrespective of the requirement for entry, HIV-1 and SIVmac Env-coreceptor interactions do induce intracellular signaling (68-70), and engagement of CCR5 has been suggested to promote virus replication by augmenting CD4+ T cell activation (70). Given the distinct intracellular signaling motifs between CXCR6 and CCR5, its possible that SIV entry via CXCR6 initiates distinct signaling cascades, with distinct cellular consequences, than does entry via CCR5. So far, studies of CXCR6 signaling have identified that CXCL16 binding only weakly induces chemotaxis, unlike robust chemotaxis observed for CCR5 (31, 63, 71). Analysis of the phosphoproteome post entry through these two coreceptors could elucidate effects of distinct coreceptor use and suggest whether entry via one or the other altered cellular activity in a way that would impact virus replication. Such a study would be aided by the generation of identical SIVs that only varied in their capacity to use either CCR5 or CXCR6 for entry.

Studies of humans and rats have suggested that CXCR6 serves as an extralymphoid homing receptor; in humans, CXCR6 is rarely coexpressed with the lymphoid homing marker CCR7 (30), and rat lymph node CD4+ T cells rarely express CXCR6 (31). Likewise, I found that RM and SM cells lacking CCR7 (Tem in this study) were enriched for CXCR6 expression, while cells expressing CCR7 (Tn and Tcm) had little to no CXCR6 expression (Chapter 4), although tissues have yet to be analyzed. Stimulation of CD4+ T cells has demonstrated that most CXCR6+ cells have a Th1 phenotype (30). Additionally, many studies have identified an expansion of CXCR6+

cells at sites of inflammation in humans, such as rheumatoid arthritis (72) and colitis (73), as well as in cancer of tissues such as the liver (74), prostate (75) and gut (76), suggesting that CXCR6+ cells are immune effectors. In particular, expression of CXCR6, as well as its ligand CXCL16, is often associated with increase tumor invasion and metastasis (77). The ligand CXCL16 is unique in that it exists in both membrane-bound and soluble forms, and CXCL16-expressing cells include antigen presenting cells and epithelial cells (78-80). Studies of human molecules suggest that CXCR6-CXCL16 binding mediates retention of CXCR6+ cells at effector sites (63, 81, 82). Thus, CXCL16 expression could also contribute to localization of CXCR6+ target cells in natural hosts, and CXCL16 expressing cells ought to be defined as well.

In Conclusion

Analyses of SIVagmSab and SIVmus described in this thesis, in addition to published data regarding SIVsmm and SIVagmVer, support a paradigm of CXCR6 use as a common feature of natural host viruses. In contrast, pathogenic SIVcpz cannot use CXCR6, like HIV-1, suggesting that use of this coreceptor was lost upon cross-species transmission and the emergence of SIVcpz. While transfer from the low-CCR5 environment of natural hosts to the higher-CCR5 environment of non-natural hosts would have enabled a virus to be CCR5-restricted yet fit, further studies will be necessary to elucidate the reasons that CXCR6 use was lost. Nevertheless, a consequence of this coreceptor transition would be targeting of virus to distinct CD4+ cell populations, with distinct outcomes on immune function and homeostasis. Future experiments will delineate the precise consequences of SIV entry through CXCR6, particularly in regard to target cells and anatomic niches defined by this coreceptor, as well as virus-mediated signaling events. As CCR5 gene deletion of CD4+ T cells of HIV-1 infected patients to confer resistance becomes a possible therapy (83, 84), it is conceivable that the ability to use other coreceptors (beyond CXCR4) might be acquired. Thus, fully understanding virus- and host-mediated determinants of target cells is essential. Furthermore, African humans are regularly exposed to SIV+ nonhuman primates, and understanding the barriers to cross-species transmission will allow full

comprehension of the risk of interaction with various primates (56). Therefore, continued studies of the contribution of CXCR6 use to natural host SIV infection are critical to maximize our understanding of HIV pathogenesis and cross-species transmission.

Figures

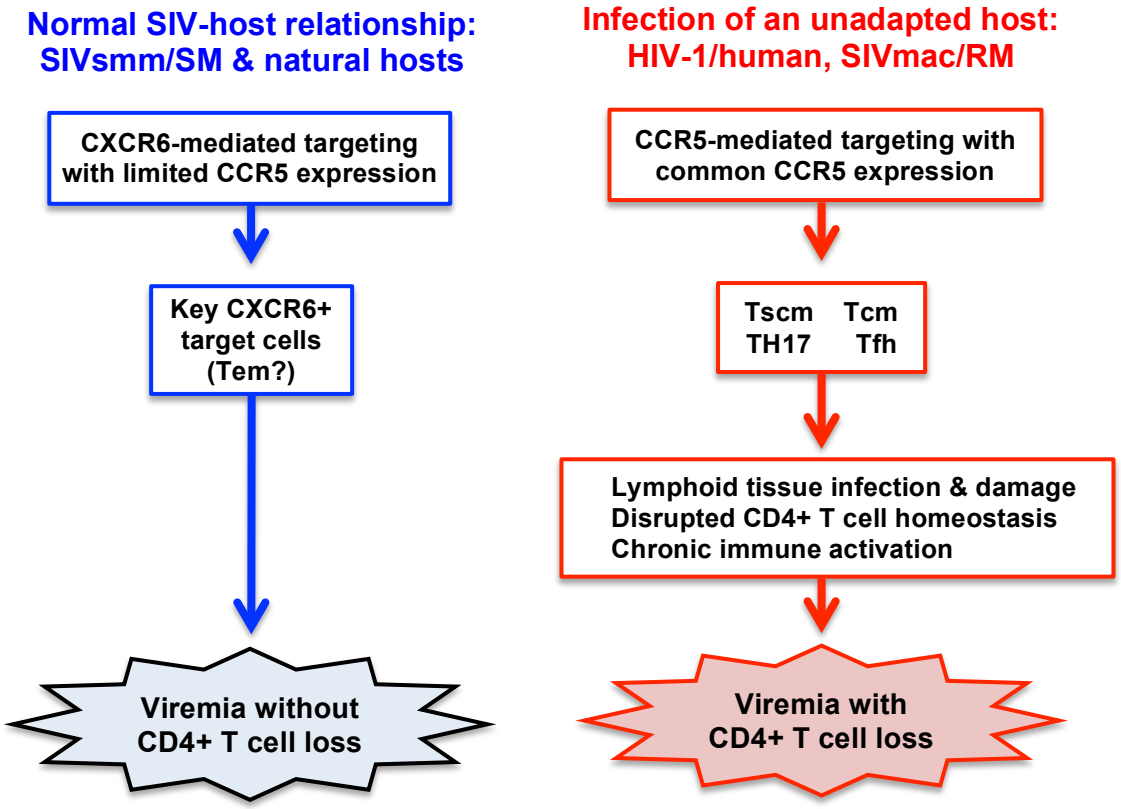

Figure 5.1: Model for divergent coreceptor entry pathway use in natural and non-natural HIV/SIV hosts that may contribute to distinct infection outcomes. Left: Use of CXCR6 in the setting of restricted CCR5 expression in natural hosts targets SIV to CD4+ T cells that support viremia without CD4+ T cell loss and immunodeficiency. Right: Use of CCR5 in pathogenic SIV and HIV infections where CCR5 expression is plentiful results in targeting of CD4+ T cell subsets that compromise the immune system and cause CD4+ T cell loss and immunodeficiency.

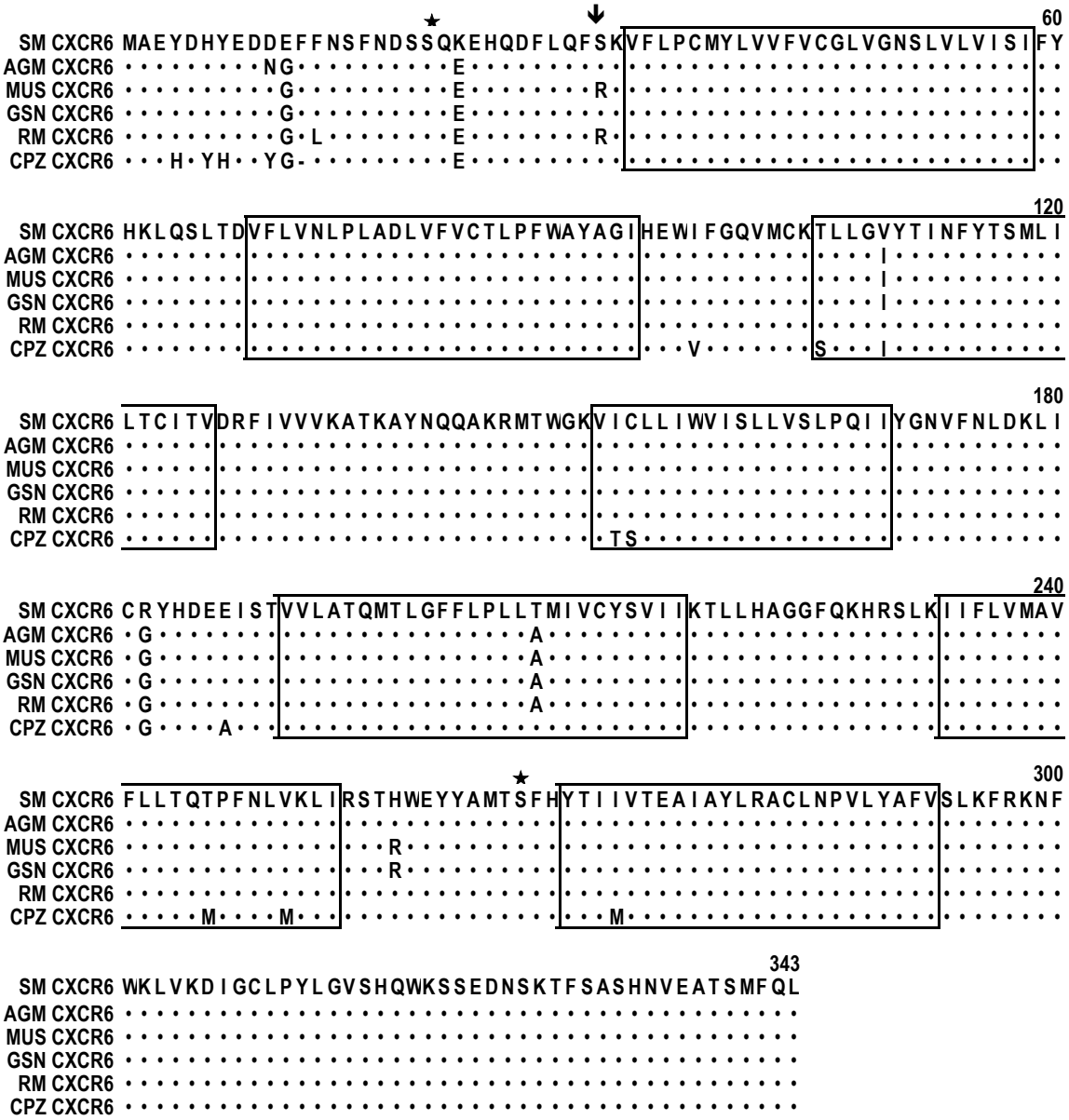

Figure 5.2 Sequence alignment of CXCR6 from SM, AGM, MUS, GSN, RM and CPZ. Highlighted features include: N terminal Tyr residues (residues 4, 7, or 6,10 (CPZ only)); predicted N-linked glycosylation site (residue 17); residue 31 that codes for an Arg in RM and MUS instead of the more common Ser and renders RM CXCR6 a poor coreceptor (🡻); extracellular Cys residues that form a disulfide bond (residues 103 and 181); and the G protein signaling motif DRF (residues 127-129). ★ indicates the approximate location of Cys residues often found in other 7TMRs, including CCR5, that can form a second disulfide bond but which are lacking in CXCR6. Boxes represent transmembrane domains predicted by the TMPRed program. The AGM CXCR6 allele shown here has been isolated from both sabaeus and vervet AGM.

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