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A Novel CCR5 Mutation in Sooty Mangabeys Reveals SIVsmm Infection of CCR5-null Natural Hosts: Examining the Potential Roles of Alternative Entry Pathways in HIV and SIV Infection

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Abstract

Natural hosts of SIV, such as sooty mangabeys (SM), maintain high levels of virus replication, but do not typically develop CD4+ T cell loss and immunodeficiency. Understanding the virus/host relationship in natural hosts will enable better understanding of pathogenic HIV infection of humans. Host cell targeting in vivo is an important determinant of pathogenesis, and is defined mainly by expression of coreceptors used by the virus for entry, in conjunction with CD4. Established dogma holds that, with rare exceptions, SIV uses CCR5 for entry. However, SM and other natural hosts express extremely low CCR5 levels on CD4+ T cells. I identified a novel SM-CCR5 mutant allele containing a two base-pair deletion (CCR5-d2) that results in a non-functional protein. Using PCR screening and direct sequence confirmation in a large captive SM colony, I found an allelic frequency of 26% for CCR5-d2, along with 3% for a previously-described CCR5-d24 null allele. Notably, SM-CCR5-d2 was also present in West African wild-living SM. Approximately 8% of captive SM were homozygous for CCR5-null alleles. Surprisingly, SIVsmm infection was not significantly less prevalent in SM lacking functional CCR5 compared to CCR5-expressing animals, and CCR5-null animals displayed high-level viremia. Primary PBMC from SM were permissive for SIVsmm replication in both the absence and presence of CCR5 in vitro. Pseudotype virions carrying Envs from both CCR5-null and wild-type infected SM used CXCR6 and GPR15 efficiently for entry in transfected cells, suggesting likely alternative entry molecules. In preliminary studies using human cells, I found that CD4+ T cell expression of CXCR6 and GPR15 was predominantly on central and effector memory subsets, although expression patterns on SM cells will require antibodies that can detect SM proteins. This finding challenges the long-standing notion that CCR5 is the exclusive coreceptor used for SIV entry in vivo, and reveals that SIVsmm can infect target cells in vivo using non-CCR5 entry pathways. Since CD4/coreceptor interactions determine viral tropism and cell targeting, these results suggest that infection in natural host SM may involve target cells defined by the distribution and use of alternative coreceptors CXCR6 and GPR15. Combined with restricted expression of CCR5 on critical target cells previously described, alternative coreceptor-dependent targeting may allow virus replication in more expendable cells without loss of immune cell homeostasis. We hypothesize that SM natural hosts may have evolved to restrict expression of CXCR6 and GPR15, in addition to CCR5, to dispensable cell subsets, thus allowing for high viral replication without causing disease. Future studies comparing CXCR6 and GPR15 expression profiles in natural and non-natural hosts will be of importance in determining the role of alternative coreceptors in natural hosts in vivo.

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DEDICATION

I dedicate this thesis to my grandparents, Jean T. Cooke and Arthur A. Cooke, and to my

mother, Josephine C. Riddick. I would not be the person I am today without their

strength, support, wisdom and unconditional love.

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ABSTRACT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I

GENERAL INTRODUCTION

Epidemiology

In 1983, two laboratories identified human immunodeficiency virus (HIV) as the virus that causes acquired immunodeficiency syndrome (AIDS) (7, 82). Over the past 30 years, HIV has become a leading cause of death worldwide especially in regions of sub-Saharan Africa (1). As of 2010, an estimated 34 million people are living with HIV worldwide, which has increased 17% from the number of infected individuals in 2001. This increase in HIV prevalence reflects new infections as well as an increase in the life expectancy of HIV-infected individuals due to more effective therapeutics and a significant rise in access to antiretroviral therapy (ART). Although there is an increase in the total number of people living with HIV over the past decade, the estimated number of new HIV cases (2.7 million) was 21% lower in 2010 than the number of new infections in 1997, at the peak of the HIV epidemic (1). However, there is still no cure or vaccine available against HIV, which is a major issue since a large proportion of people living with or at risk for infection, in developing countries, still do not have access to treatment or care.

HIV is transmitted through the transfer of bodily fluids (i.e. blood, semen, vaginal fluid, and breast milk) from an infected individual to an uninfected recipient. The major routes of HIV transmission are unprotected sex, contaminated needles, during birth (perinatal), and through breast milk (25, 47, 92) Notably, the main route of transmission differs from region to region. Historically, in the U.S., HIV infection predominantly affected intravenous drug users and men who have sex with men (MSM). Although, these groups remain at high risk for infection, heterosexual transmission accounts for a growing number of HIV cases in the U.S., especially among women. The HIV epidemic

in Eastern Europe is primarily driven by intravenous drug users, while the HIV epidemic in Sub-Saharan Africa and parts of the Caribbean are mainly the result of heterosexual contact.

HIV disease pathogenesis

HIV infects CD4+ T lymphocytes and macrophages, which play critical roles in both the innate and adaptive arms of the immune system. A typical HIV infection consists of three stages: the acute (primary) infection, the chronic infection (clinical latency), and the onset of AIDS. The acute phase generally lasts several weeks with symptoms ranging from asymptomatic to flu-like in infected individuals. During this stage, an inverse correlation between plasma viremia and peripheral blood CD4+ T cell counts is observed, in which CD4+ T cell counts decrease concomitant with peak viremia (24). Early in infection, memory CD4+ T lymphocytes are preferential targets of HIV, likely due to their moderate to high CCR5 expression and activated state. The majority of activated memory CD4+ T cells reside in the gut associated lymphoid tissue (GALT). HIV infection leads to a rapid and massive depletion of these cells, which is sustained throughout the course of infection (13, 89). Towards the end of the acute phase, viral loads decline to a set level and peripheral blood CD4+ T cell counts partially rebound as demonstrated in **Figure 1-1**.

The chronic phase of infection is characterized by a prolonged asymptomatic period lasting for many years. Throughout this period of clinical latency, viral loads gradually increase while CD4+ T cell counts slowly decline (35). Notably, infected persons remain infectious during this period (59, 80). At the end stage of disease (AIDS),

CD4+ T cell counts in the blood are extremely low likely due to direct viral killing of infected cells, an increased rate of apoptosis of infected and bystander (uninfected) cells, and cell-mediated killing of infected cells (96). When CD4+ T cell counts decrease below a critical threshold, cell-mediated immunity is severely compromised and the host becomes more susceptible to opportunistic infections (72, 83).

The development and use of antiretroviral therapy (ART) has dramatically reduced HIV-related mortality and morbidity in middle to high-income countries where access to ART is available. In the late 1980's, antiretroviral (ARV) drugs became available for use by HIV-infected patients. However treatment with a single ARV drug led to drug resistant strains of the virus given that HIV mutates at a high rate due to its rapid and persistent replication combined with the lack of polymerase proofreading activity (50, 51). In the mid 1990's, combination therapy was developed, which defends against HIV resistance by suppressing HIV replication at multiple stages in the virus life cycle. Highly active antiretroviral therapy (HAART) consists of a combination of three or more antiretroviral drugs that maximally suppress HIV replication. With proper use, HAART suppresses HIV viral loads below detectable levels reducing the likelihood of HIV transmission by infected individuals. However, inconsistent use of HAART can lead to multi-drug resistant strains, which considerably limits future treatment options. Unfortunately, access to drug treatments are not uniformly available in low and middle-income countries, where treatment needs are the greatest.

While the majority of HIV-infected persons ultimately progress to AIDS if left untreated, a small percentage of HIV-infected individuals appear to naturally control the infection, called long term non-progressors (LTNP). There is no universally accepted

4

definition for LTNP, but in general these infected individuals maintain a normal range of

CD4 T cell counts in the peripheral blood without the use of antiretroviral therapy, and do

not progress to AIDS after prolonged periods of observation.   LTNP may have

detectable viral loads that typically do not exceed more than 2,000 copies/ml. A subset

of LTNP called elite controllers (EC) have undetectable viral loads (< 50 copies/ml) and

naturally maintain normal CD4 T cell counts in the blood, without the use of

antiretroviral therapy (28).
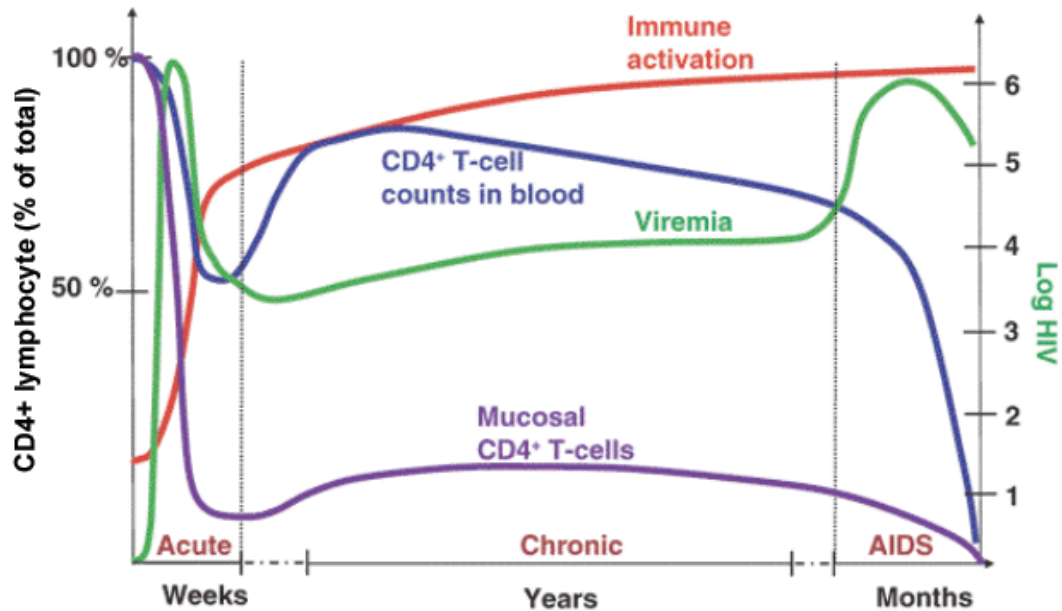
Figure 1-1. Viral and cellular parameters of disease progression during HIV-1 infection.
Acute infection is characterized by a peak in viral load and a rapid increase in immune activation.
During this phase, mucosal CD4 T cells are severely depleted while a transient decrease in
peripheral blood (PB) CD4 T cells is observed, which partially rebounds towards the early stage
of chronic infection. During chronic infection, viral loads are maintained at a viral set point and
immune activation remains elevated while mucosal CD4 T cell counts remain low and CD4 T
cells in the periphery begin to steadily decline. When PB CD4 T cells decrease below a critical
threshold, this defines the onset of AIDS, which is characterized by an increase in viral
replication and severe immunodeficiency, resulting in opportunistic infections. Schematic was
adapted from *Grossman, Z. et al. Nature Medicine 2006.*

SIV: origins of HIV-1 and HIV-2

Through comparative sequence analysis, the origins of HIV have been traced to specific strains of SIV found in African non-human primates (NHP). HIV-1 is the result of cross-species transmission of a strain of simian immunodeficiency virus (SIV) found in chimpanzees, SIVcpz (56, 58, 101). Hahn et al. determined that all examined HIV-1 isolates from groups M, N and O were only closely related to lineages of SIVcpz found in the chimpanzee subspecies, *Pan troglodyte troglodyte,* inferring that on at least three separate occasions, SIVcpz crossed into the human population (40). Chimpanzees were originally categorized as a natural host of SIV, however recent studies revealed that SIV-infected chimpanzees exhibited increased mortality and morbidity compared to uninfected animals, and they are now designated as intermediate hosts of SIV (60). Studies indicate that SIVcpz contains a recombinant genome with regions similar to two strains of SIV, SIVrcm found in red-capped mangabeys (RCM; *Cercocebus torquatus*) and SIVgsn found in greater-spot nosed monkey (*Cercopithecus nictitans*) (5). HIV-2 and SIVmac are also the result of two separate cross-species transmission events of SIVsmm, a strain of SIV found in sooty mangabeys (SM), into the human and rhesus macaques (RM) populations, respectively (19, 20, 41, 42, 52). Currently, over 40 different species-specific SIV strains have been identified in African NHP, thus the possibility of another zoonosis into the human population is plausible. The phylogenetic relationship between SIVcpz/HIV-1 and SIVsmm/HIV-2 is depicted in **Figure 1-2.**

SIV is endemic in African NHP and infected natural hosts live a normal lifespan and remain asymptomatic, despite high viral loads (95). In stark contrast, SIV-infected non-natural hosts (i.e. Asian macaques) experience an AIDS-like disease that results in

death due to opportunistic infections. The pathogenic potential of SIV was first observed

when lymphoma outbreaks occurred in captive Asian macaques (RM and stump-tailed

macaques), which had been in contact with SIV-infected SM prior to the outbreaks.

Inadvertently, this virus was serially transmitted to other macaques giving rise to the

well-established SIVmac strain (27). SIV-infected RM exhibit a disease course similar to

that of HIV-infected humans, therefore SIV-infected RM serve as a valuable animal

model to study HIV infection.
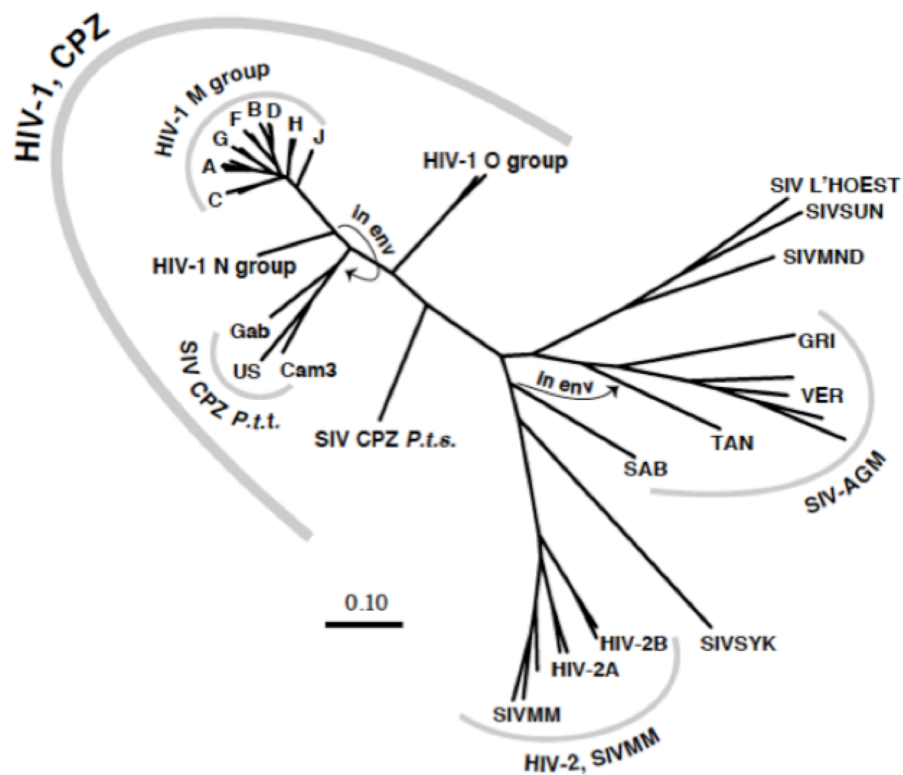
Figure 1-2. Phylogenetic relationship among HIV and SIV strains. This phylogenetic tree was generated based on the similarity of *pol* gene sequences between various strains of HIV and SIV. This tree clearly demonstrates that HIV-1 (groups M, N and O) is closely related to SIVcpz *pol* sequences while HIV-2 is most closely related to SIVsmm. Notably, HIV-1 N group and SIVagm$_{SAB}$ have recombined genomes. Based on *pol* gene relatedness, the HIV-1 N group is more closely related to the HIV-1 M group, as shown. However, based on *env* gene phylogenies, the HIV-1 N group clusters within the SIVcpz (P.t.t) clade. SIVagm$_{SAB}$ is recombinant with other SIVagm strains and SIVsmm. Due to a recombination event in the *pol* gene of SIVagm$_{SAB}$, this branch is placed between SIVagm and SIVsmm, however the placement of SIVagm$_{SAB}$ branch does not reflect true evolutionary history. Small arrows indicate the branch position of recombinant viruses (HIV-1 N group and SIVagm$_{SAB}$) based on an *env* phylogenetic tree reconstruction. *Theoretical Biology and Biophysics Group, Los Alamos National Laboratory, 1999.*

Chronic Immune Activation

Chronic immune activation is a key feature that distinguishes non-pathogenic from pathogenic infection. Typical features of HIV-associated chronic immune activation include a higher frequency of activated T and B cells, increased lymphocyte turnover, high activation-induced cell death and high levels of pro-inflammatory cytokines and chemokines in serum from infected individuals. Interestingly, the acute phase of SIV infection in natural and non-natural hosts is characterized by strong innate and adaptive immune responses to the virus. However, natural hosts downregulate this response early in the chronic phase and maintain a low immune activation state throughout infection.

Both viral and host factors appear to contribute to chronic immune activation. The role of virus-induced immune activation is demonstrated by the fact that ART reduces immune activation in HIV-infected persons (32). However, viral factors alone cannot account for HIV/SIV-associated immune activation since SIV-infected natural hosts exhibit high levels of viral loads while immune activation during the chronic phase of infection is relatively low (15, 96). Viral factors associated with immune activation likely include HIV/SIV-induced innate and adaptive immune responses and re-activation of latent viruses, while host factors associated with immune activation may include mucosal immune dysfunction and homeostatic proliferation.

Numerous HIV/SIV-specific viral factors may contribute to chronic immune activation including: (i) gp120-mediated signaling through CD4 and/or coreceptor, (ii) specific antiviral adaptive immune responses elicited by HIV/SIV-specific T and B cells (68), and (iii) stimulation of the innate immune response via pattern recognition receptors

(toll-like receptors) that recognize components of bacterial pathogens (which may have entered the bloodstream as a result of microbial translocation) and HIV-derived ligands (HIV single-stranded RNA) (4, 12). Additionally, during the late stage of disease, reactivation of latent viruses may contribute to chronic immune activation. This can occur in the setting of a compromised immune system where latent herpes viruses (i.e. cytomegalovirus [CMV] and Epstein-Barr virus [EBV]) frequently reactivate, which may stimulate large numbers of T cells specific for CMV and/or EBV viral antigen (73).

During early stages of infection, studies suggest that damage to the gastrointestinal (GI) immune system may increase systemic immune activation during the chronic phase of pathogenic HIV/SIV infection. During acute infection, mucosal CD4 T cells are severely depleted, likely since a large proportion of these cells express the HIV co-receptor, CCR5 (45, 79). Mucosal CD4 T cells are primarily comprised of Th1 and Th17 cells, which produce and secrete interferon-gamma (IFN-g) and interleukin-17 (IL-17), respectively. Profound depletion of Th17 cells is associated with a breakdown in the mucosal barrier resulting in translocation of microbial products (i.e. LPS and flagellin) from the intestinal lumen to systemic circulation (12, 33). Aberrant circulation of microbial products may cause broad immune activation through TLR-mediated stimulation of immune cells as well as triggering bystander activation of circulating lymphocytes. Evidence demonstrating a causal relationship between microbial translocation and immune activation is seen in SIV-infected natural hosts where lack of microbial translocation in these animals is associated with attenuated chronic immune activation (6, 12, 16, 97).

Another factor that potentially affects chronic immune activation is homeostatic proliferation. Homeostatic proliferation is the ability of T cells to divide in the absence of activation, and is aimed at repopulating particular T cell pools. During acute HIV infection, there is severe and persistent depletion of mucosal CD4 T cells in the gut as well as transient depletion of CD4 T cells in the blood. The adaptive immune system responds by proliferation of HIV/SIV-specific T cells in response to encountering viral antigen, however antigen-independent homeostatic proliferation also occurs in an attempt to replenish the continuously depleting CD4 T cell pool. A clear relationship between intestinal CD4 T cell depletion and systemic/intestinal CD4 T cell proliferation was demonstrated in HIV-infected and uninfected individuals (44, 98).

Divergent host responses to SIV infection

 As previously mentioned, SIV infection of natural hosts is non-pathogenic while HIV/SIV infection of non-natural hosts results in a pathogenic infection, characterized by chronic immune activation, immunodeficiency and ultimately death as a result of opportunistic infections. However, the mechanisms underlying the absence of disease in infected natural hosts are poorly understood. Schematics highlighting similarities and differences in key parameters of pathogenic and non-pathogenic SIV/HIV infections are depicted in **Figure 1-3**.

There are a number of key features that differ between SIV-infected natural and non-natural hosts, however one shared feature is high levels of virus replication. Originally, researchers hypothesized that absence of disease in infected natural hosts may be due to non-pathogenic or less fit virus. However, subsequent studies indicated that

lack of disease progression in natural hosts is not associated with low levels of viral replication (15, 53, 84, 96). Many studies have confirmed that infected natural hosts exhibit viral loads equal to or greater than viral loads seen in SIV-infected non-natural hosts (15, 84, 94).

While both non-pathogenic and pathogenic infections result in high viral loads, pathogenic infections result in CD4+ T cell loss in the blood, and progression to AIDS, which is extremely rare in infected natural hosts (14, 76). However, studies have demonstrated rare instances in which SIV-infected SM experienced severe depletion of peripheral blood CD4+ T cells, either naturally or experimentally, yet these animals did not progress to AIDS (69, 70). This observation suggests that natural hosts have developed at least two levels of protection in response to SIV infection: i) protection from loss of CD4+ T cells during SIV infection (possibly due to mechanisms which maintain homeostasis of the immune system), and ii) protection from AIDS when CD4+ T cells are lost (69, 70).

As previously mentioned, a key feature that differs between infected natural and non-natural hosts is chronic immune activation. Immune activation during the acute phase of infection is exhibited in both pathogenic and non-pathogenic infections, however studies indicate that infected natural hosts downregulate immune responses through mechanisms not fully understood, during early chronic infection (10, 48, 57). Differential downregulation of innate immune responses can be observed in SM and African green monkeys (AGM) natural hosts, which downmodulate type I interferon responses within four to six weeks post-infection, whereas SIV-infected RM exhibit persistently high interferon responses throughout the chronic phase of infection (48).

13

This is only one such example where natural hosts have evolved mechanisms to attenuate chronic immune activation.

Intriguingly, depletion of gut memory CD4+ T cells occurs in both SIV-infected natural and non-natural hosts (45, 79), however microbial translocation is only observed in pathogenic SIV infections. Microbial translocation, which likely results from a breach in the gut epithelial barrier, is thought to be a major driver of chronic immune activation (12, 33). Studies suggest that the sparing of CD4+ Th17 cells in the gut of infected natural hosts may contribute to intact gut immunity and the absence of microbial translocation in these hosts (11). Notably, an association between circulating microbial products and immune activation was emphasized in a study where a single dose of LPS was administered to chronically SIVagm-infected AGM, which led to an increase in proliferation and activation of CD4+ T cells, as well as an increase in viral loads (78). However, whether induced-immune activation in these animals would have progressed to disease is unknown since this was a short-term study.

Another major difference between natural and non-natural hosts is the level of CCR5 expression on CD4+ T cells. SIV natural hosts express extremely low levels of CCR5 on CD4+ T cells from the blood, lymph nodes and mucosal tissues compared to CCR5 levels on CD4+ T cells of non-natural hosts (77). Interestingly, both natural and non-natural hosts exhibit similar levels of CCR5 expression on CD8+ T cells, which calls to question if low levels of CCR5 expression on natural host CD4+ T cells is an evolutionary adaptation in response to SIV. Furthermore, several natural host species (i.e. SM, AGM, mandrills, and sun-tailed monkeys) exhibit extremely low CCR5

14

expression levels on CD4+ T cells demonstrating convergent evolution of the natural host immune system in response to endemic lentiviruses.

It has been speculated that low CCR5 levels on CD4+ T cells may be beneficial to the host for a number of reasons, such as: (i) a reduction in target cells for virus replication, and (ii) decreased homing of activated CD4+ T cells to sites of inflammation. The latter point suggests that low CCR5 levels may also contribute to the low chronic immune activation state seen in infected natural hosts, since fewer potential target cells are recruited to sites of inflammation, which are likely sites of active virus replication. Additionally, low CCR5 levels on CD4+ T cells has been hypothesized to contribute to non-pathogenicity in natural hosts (75), creating a paradox since infected natural hosts exhibit robust viral replication. My work addresses in part, mechanisms by which high viral loads can be sustained in the face of restricted CCR5 expression on natural host target cells.

a HIV/SIV infection in non-natural hosts

PB CD4

Immune activation

Viral load

MALT CD4

Acute   Chronic   AIDS

b SIV infection in natural hosts

PB CD4

Viral load   AGMs

SMs

MALT CD4

Immune activation

Acute   Chronic   No AIDS
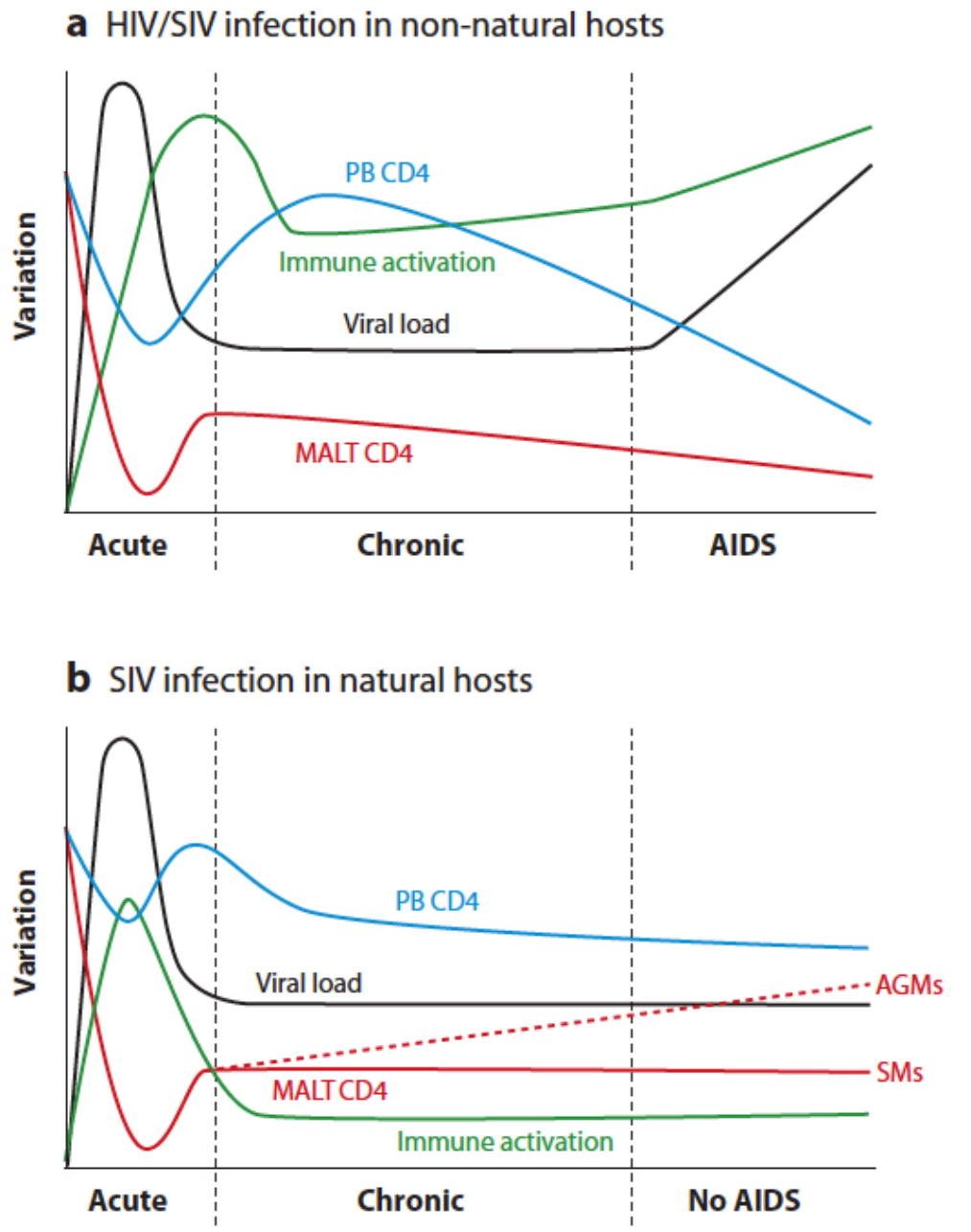
Figure 1-3. Comparison of disease course in HIV/SIV-infected natural and non-natural hosts. A) Viral and cellular parameters of HIV/SIV infection in non-natural hosts were described in detail in Fig. 1-1. B) In SIV-infected natural hosts, viral loads peak during acute infection then decrease to a viral "set point" that persists throughout the lifetime of the infected animal. Peripheral blood CD4+ lymphocytes transiently decrease during the acute infection then rebound and are maintained throughout the infection while CD4 T cells in the MALT are severely depleted and do not rebound to pre-infection levels. Additionally, generalized immune activation in SIV-infected natural hosts increases during acute infection but is downregulated during the chronic phase of infection. *Paiardini, M. et al. Ann. Rev. Med. 2009.*

16

HIV/SIV life cycle

HIV/SIV are members of the Lentivirus genus within the Retroviridae family. Lentiviruses contain a positive-sense, single-stranded RNA genome that is enveloped in a lipid bilayer derived from the host cell. HIV/SIV particles contain two copies of their RNA genome enclosed in a capsid core, which is surrounded by a lipid bilayer, studded with the viral envelope glycoprotein (Env) (43). HIV and SIV genomes are comprised of nine genes. The structural genes (*gag*, *pol* and *env*) encode proteins required for the production and assembly of new virion particles, while the accessory genes (*tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* [SIVcpz/HIV-1] or *vpx* [many SIVs/HIV-2]) have regulatory functions, which affect the synthesis and processing of viral RNA as well as viral infectivity. Long terminal repeats (LTR) flank the viral genome and play an integral role in the insertion of the viral genome into the host DNA.

HIV/SIV utilizes CD4 and a co-receptor (chemokine or chemokine-like receptor) for entry into target cells (21, 26, 29, 36, 62, 86). Steps involved in virus entry will be discussed in detail in the following section. Once inside the cell, HIV/SIV usurp cellular machinery for viral propagation, requiring only three additional enzymes, reverse transcriptase (RT), protease and integrase, which are packaged in the virion capsid and deposited into target cells upon viral entry. Reverse transcriptase is a RNA-dependent DNA polymerase, which is extremely error-prone since it lacks proof-reading activity. RT reverse transcribes the viral RNA genome into a complementary DNA copy, and then degrades the RNA strand of the RNA/DNA hybrid with its RNase H activity. Cellular polymerases synthesize the complementary strand resulting in a double stranded DNA copy of the viral genome. Next, viral DNA is transported to the nucleus where viral

integrase process the LTRs and catalyze the insertion of the proviral DNA into the host genome. One characteristic that distinguishes lentiviruses from other retroviruses is their ability to replicate in non-dividing cells. Integrated proviral DNA is transcribed and translated generating structural polyproteins, which are processed and used to assemble new virion particles, as well as copies of full-length viral genomes that are incorporated into new virions (46). The final steps of the life cycle include budding from the cell surface and maturation of virion particles, which requires the cleavage of polyproteins into mature matrix protein by viral proteases.

HIV/SIV entry

HIV/SIV entry into target cells is generally believed to occur via a receptor-mediated, pH-independent pathway (23). Several chemokine receptors and chemokine receptor-like proteins mediate HIV/SIV entry in vitro (31), however CCR5 and CXCR4 are thought to play a dominant role in HIV entry in vivo, whereas it is generally accepted that CCR5 alone mediates SIV entry in vivo (88). HIV/SIV entry mechanisms are extremely similar; therefore only HIV entry will be discussed in detail.

The viral protein required for target cell entry is the envelope glycoprotein, gp160, a 160 kDa polyprotein, which is cleaved by cellular proteases to yield two subunits: (i) gp120, the surface subunit and (ii) gp41, the transmembrane subunit (2, 102, 105). Gp120 and gp41 are non-covalently linked and functionally exist as a trimer of dimers on the viral particle (49, 64, 66, 103, 107, 108). Each trimer is referred to as an envelope spike; on average HIV virions are studded with ten envelope spikes (43). The gp120 subunit is localized on the surface of the viral particle where it can interact with

cellular receptors. This subunit is composed of five constant regions (C1-C5), which contain critical domains for binding to entry receptors, as well as five variable regions (V1 – V5) (71, 106) that can withstand high sequence variability allowing them to act as immunological shields, protecting the conserved, more critical regions, from antibody neutralization. In another effort to evade the humoral immune response, gp120 is heavily glycosylated obscuring much of its surface from antibody recognition (65).

In order for HIV to gain entry into a target cell, the gp120 subunit must first bind the cellular receptor, CD4 (26, 62). This interaction causes conformational changes in gp120 resulting in the formation and exposure of the coreceptor binding site (87). The third variable loop (V3) of gp120 is critical for coreceptor binding. V3 is composed of three structural regions: (i) the base, which sits closest to the core, (ii) the flexible stem region, and (iii) the highly conserved tip region. Studies indicate that the base of V3 interacts with the N-terminus of CCR5, while the tip and stem regions of V3 interact with CCR5 extracellular loops (ECL) (54, 55). **Figure 1-4** shows the crystal structure of trimeric gp120 bound to the CD4 receptor. In this model, the V3 loop of each gp120 monomer is extended towards the cell membrane (55). Upon coreceptor binding, subsequent conformational changes occur in gp41 exposing the hydrophobic fusion peptide, which quickly inserts into the target cell membrane (38, 39). Gp41 contains two heptad repeat regions (HR1 and HR2). Once triggered, HR2 domains in each gp41 subunit fold back on HR1 domains forming a complex (18, 104), the 6-helix bundle, which brings the viral and target cell membranes into close proximity promoting fusion through mechanisms that are not fully understood (67). Once fusion occurs, the viral

19

capsid core, containing the viral genome, is released into the target cell. HIV receptor

engagement and entry steps are depicted in **Figure 1-5**.
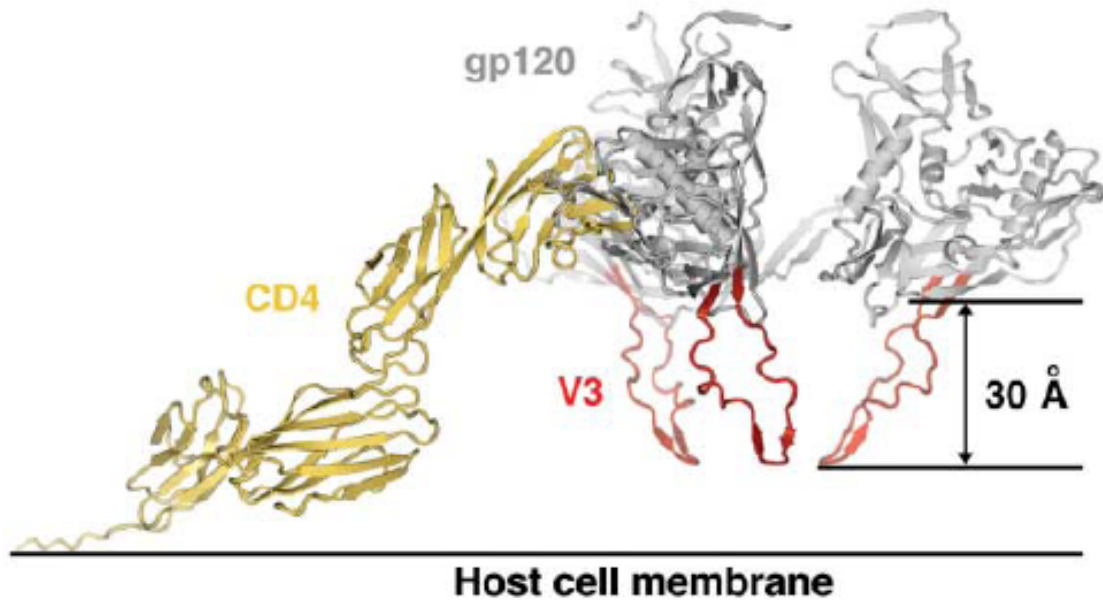
Figure 1-4. Crystal structure of gp120 trimer with exposed V3 domains conjugated to CD4.
Trimeric model of gp120 where the core (grey) and V3 loops (red) are superimposed onto the
structure of a four-domain CD4 molecule (yellow). In the CD4-triggered state, the V3 domains
protrude 30 angstrom away from the viral envelope and towards the cellular membrane. Image
obtained from *Kwong, P. et al. Science 2005.*
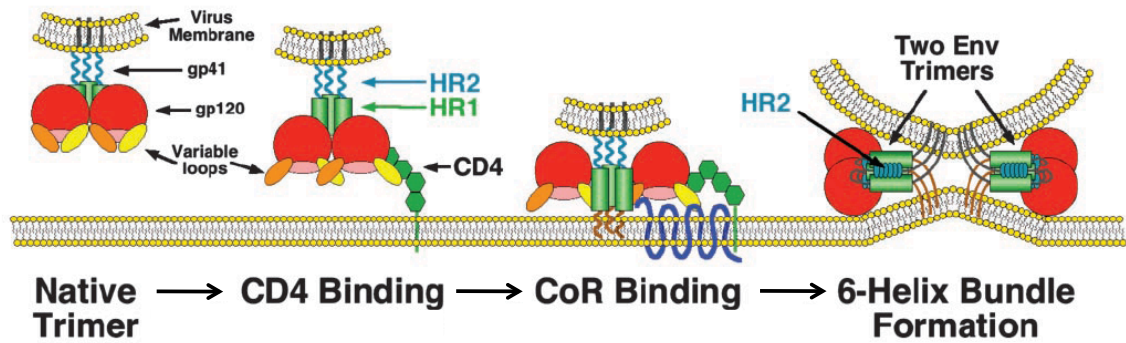
Figure 1-5. Model of HIV/SIV entry. A trimeric HIV/SIV Env is shown attached to the viral membrane. Once the virus comes into close proximity with the cellular membrane, the surface unit of Env (gp120) binds to the cellular receptor, CD4. Upon binding, conformational changes occur in Env allowing gp120 to bind to the coreceptor. Gp120/coreceptor engagement causes subsequent conformational changes exposing the fusion peptide of gp41, which inserts into the cellular membrane. Formation of the 6-helix bundle brings the viral and cellular membranes into close proximity, which promotes membrane fusion, ultimately resulting in viral entry. *Adapted from Moore, J.P., Doms, R.W. PNAS 2003.*

HIV/SIV entry coreceptors and tropism

Chemokine receptors are critical for HIV/SIV entry into target cells. These receptors are members of a large protein family of G protein-coupled receptors (GPCR). Physiologically, chemokine receptors interact with their cognate ligand, which activates signaling pathways, ultimately resulting in cell migration. Chemokine receptors are composed of seven transmembrane helices, which form three extracellular loops, three intracellular loops, an N-terminus exposed to the extracellular space, and a C-terminus exposed to the cytosol. Chemokine receptors contain a conserved DRYLAVHA sequence ("DRY motif") in the second intracellular loop that has been implicated in G-protein interaction and signaling. In addition to the role chemokine receptors play in cellular immunity, these receptors have been extensively studied over the past decade due to their integral role in HIV/SIV entry.

Various *in vitro* studies indicate that two particular regions of CCR5 and CXCR4 are critical for HIV entry, the N-terminal domain and extracellular loop two (ECL2). Studies suggest that tyrosine resides in the N-terminal domain of CCR5 are post-translationally sulfated; these modifications are critical for high affinity interactions between the coreceptor and CD4-triggered HIV-1 envelopes (22, 34, 93). As previously mentioned, the V3 loop plays a central role in coreceptor binding and tropism. Based on NMR and crystallographic data, Kwong et al. demonstrated that regions of the V3 base interacted with sulfated tyrosines located in the N-terminus of CCR5 (54). Additionally, the use of chimeric receptors and site-directed mutagenesis of ECLs revealed that ECL2 also plays a critical role in viral entry (9, 17, 30, 81, 86). Taken together, these studies,

as well as others, implicate the importance of CCR5 N-terminus and ECL2 in HIV-1 gp120 binding and entry.

Receptor expression is a principal determinant of viral tropism. Early characterization of HIV entry revealed two viral phenotypes: (i) syncytia-inducing or T cell-tropic viral isolates that infected primary lymphocytes and lymphocyte cell lines, but not primary macrophages, and (ii) non-syncytia-inducing or M-tropic viral isolates, which infected primary macrophages and primary lymphocytes, but not transformed T cell lines (3, 90, 91, 99). Subsequent studies revealed that differences in target cell tropism by M-tropic and T-tropic viral isolates was attributed in large part to the cellular expression of CCR5 and CXCR4 on macrophage and T cell lines, respectively. This observation lead to the current classification system where HIV viruses are categorized based on entry coreceptor use: (i) R5 viruses use CCR5 for entry, (ii) X4 viruses use CXCR4, and (iii) R5X4 or dual-tropic viruses use both CCR5 and CXCR4 (8).

Studies indicate that R5-tropic viruses are commonly transmitted during HIV infection. These viruses predominate during early infection and exist throughout all stages of disease. Interestingly, in 50% of HIV infections, R5-tropic viruses acquire the ability to use CXCR4. Acquisition of CXCR4 use shifts viral tropism from predominantly CCR5-expressing memory CD4+ T cells to CXCR4-expressing naïve CD4+ T cells (74, 100), which has been associated with accelerated disease progression (63).

In contrast to HIV, SIV strains use CCR5 but not CXCR4. However, a number of additional molecules function *in vitro* for SIV entry. Based largely on studies using human molecules, these include CXCR6 (STRL33/Bonzo), GPR15 (BOB), GPR1,

23

CCR2, CCR3, CCR4, CCR8 and APJ. As discussed in Chapter 2, CCR2 is thought to be utilized by SIVrcm in RCM, a large percentage of which lack CCR5 due to a genetic polymorphism. Otherwise, however, alternative coreceptors have been considered largely irrelevant to infection *in vivo*. For many years, alternative coreceptor use by SIV was considered an *in vitro* phenomenon, however a number of studies report SIV replication in T cell lines lacking CCR5, suggesting non-CCR5 entry pathways can mediate SIV entry (37, 61). My work in Chapter 2 indicates that alternative entry pathways mediate SIVsmm transmission and replication *in vivo* (85).

Goals of this thesis

It is well established that SIV infection of natural hosts (African NHP) results in a non-pathogenic infection, while HIV/SIV infection of non-natural hosts (humans and Asian NHP) causes pathogenic infections. It appears that natural hosts have developed several mechanisms to counter the potential pathogenic effects of SIV infection such as attenuated chronic immune activation, "protection" of critical cell subsets required for maintenance of the immune system, and the ability to regulate CCR5 expression on specific cell subsets.

The goal of this thesis is to better understand the underlying mechanisms that contribute to the non-pathogenic nature of SIV infection in natural hosts. Natural hosts exhibit significantly lower CCR5 expression on their CD4+ T cells compared to CCR5 expression on CD4+ T cells from non-natural hosts. However both infected natural and non-natural hosts exhibit robust virus replication. This raises the question where does robust virus replication come from in infected natural host species since these animals

exhibit such low CCR5 expression on CD4+ T cells? My work shows that in natural host

SM, non-CCR5 entry pathways can support SIV transmission and replication *in vivo*. I

then showed that CXCR6 and GPR15 efficiently mediate SIV entry *in vitro.* Based on

these findings, we hypothesize that alternative coreceptor expression in natural hosts is

restricted to expendable cell subsets that allow robust virus replication without causing

disease.

Next, we sought to study the expression profiles of CXCR6 and GPR15 on human

and SM primary cells. I first set out to examine the expression profile of CXCR6,

GPR15 and CCR5 on various human cell populations since a SM-CXCR6 antibody is not

currently available. This work will serve as a framework for future studies comparing

CXCR6 and GPR15 expression patterns in SM, which we hypothesize evolved to restrict

expression to dispensable cell types.

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26

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CHAPTER II

A NOVEL CCR5 MUTATION COMMON IN SOOTY MANGABEYS REVEALS SIV$_{SMM}$ INFECTION OF CCR5-NULL NATURAL HOSTS AND EFFICIENT ALTERNATIVE CORECEPTOR USE *IN VIVO*

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Abstract

In contrast to HIV infection in humans and SIV in macaques, SIV infection of natural

hosts including sooty mangabeys (SM) is non-pathogenic despite robust virus replication.

We identified a novel SM CCR5 allele containing a two base pair deletion ($\Delta2$) encoding

a truncated molecule that is not expressed on the cell surface and does not support SIV

entry *in vitro*. The allele was present at a 26% frequency in a large SM colony, along

with 3% for a CCR5$\Delta24$ deletion allele that also abrogates surface expression. Overall,

8% of animals were homozygous for defective CCR5 alleles and 41% were

heterozygous. The mutant allele was also present in wild SM in West Africa. CD8+ and

CD4+ T cells displayed a gradient of CCR5 expression across genotype groups, which

was highly significant for CD8+ cells. Remarkably, the prevalence of natural SIVsmm

infection was not significantly different in animals lacking functional CCR5 compared to

heterozygous and homozygous wild-type animals. Furthermore, animals lacking

functional CCR5 had robust plasma viral loads, which were only modestly lower than

wild-type animals. SIVsmm primary isolates infected both homozygous mutant and

wild-type PBMC in a CCR5-independent manner *in vitro*, and Envs from both CCR5-

null and wild-type infected animals used CXCR6, GPR15 and GPR1 in addition to CCR5

in transfected cells. These data clearly indicate that SIVsmm relies on CCR5-independent

entry pathways in SM that are homozygous for defective CCR5 alleles and, while the

extent of alternative coreceptor use in SM with CCR5 wild type alleles is uncertain,

strongly suggest that SIVsmm tropism and host cell targeting *in vivo* is defined by the

distribution and use of alternative entry pathways in addition to CCR5. SIVsmm entry

through alternative pathways *in vivo* raises the possibility of novel CCR5-negative target

cells that may be more expendable than CCR5+ cells and enable the virus to replicate

efficiently without causing disease in the face of extremely restricted CCR5 expression

seen in SM and several other natural host species.

Author Summary

SIV causes AIDS in macaques, like HIV-1 does in humans, but not in its natural host

species such as sooty mangabeys (SM). It is therefore important to understand infection

in natural hosts, including the mechanisms and cellular targets of infection. SIV

replication in SM is thought to exclusively use CCR5 as its entry coreceptor, which

mediates viral entry in conjunction with CD4 and is the main determinant of target cell

tropism. However, other molecules also function as SIV coreceptors *in vitro*. We

discovered that inactivating mutations in the CCR5 gene are common among SM and,

furthermore, homozygous mutant animals lacking functional CCR5 still become infected

and have high viral loads. *Ex vivo*, SM lymphocytes can be infected independently of

CCR5, and several alternative entry coreceptors are used by SIV from both CCR5+ and

CCR5-null animals. Thus, SIV infection in SM is mediated by other coreceptors in

addition to CCR5, suggesting that these molecules together may determine tropism and

cell targeting *in vivo*. These results provide new insight into an important model of

nonpathogenic natural host infection, and identify a novel role for alternative entry

pathways suggesting a potentially broader range of target cells *in vivo* than currently

recognized.

Introduction

HIV-1 emergence into the human population resulted from cross-species transmission of SIVcpz from chimpanzees (*Pan troglodytes*), which itself resulted from transmission and subsequent recombination of SIVs infecting primates on which chimpanzees prey (4, 24).   Similarly, both simian AIDS caused by SIVmac/smm in rhesus macaques (RM; *Macaca mulatta*) and HIV-2 infection of humans originated from cross-species transmission of SIVsmm from naturally infected sooty mangabeys (SM; *Cercocebus atys*) (3, 31, 55). In marked contrast to pathogenic infections leading to AIDS in non-natural hosts, infection in natural host species including SM is typically non-progressive (36, 67, 69). Importantly, the benign nature of SM infection *in vivo* is not due to overall restricted viral replication, as both nonpathogenic natural host and pathogenic nonnatural host infections are characterized by robust viremia (11, 32, 59, 68). This observation indicates that immunodeficiency virus replication and pathogenesis are not inextricably linked. Thus, understanding natural host infection has become a high priority for identifying key features of infection *in vivo* that regulate pathogenesis and, potentially, identify opportunities to modulate disease apart from or in addition to suppressing overall virus replication through pharmacologic or immune mechanisms.

HIV and SIV entry into target cells is initiated by binding of the viral envelope glycoprotein (Env) to cell surface CD4, followed by structural changes that enable interactions with a seven transmembrane G protein coupled cell surface receptor that then triggers fusion. HIV-1 isolates use CCR5 or CXCR4 or both, and *in vitro* use other molecules infrequently. The restricted expression of CCR5 mainly on memory CD4+ T cells, but broader expression of CXCR4 on both memory and naïve subsets, is thought in

part to underlie the accelerated disease progression seen in individuals in whom CXCR4-using HIV-1 variants emerge late in the course of infection (6, 38, 65, 72). In contrast, SIV strains use CCR5 almost universally and very rarely use CXCR4. Sooty mangabeys express very low levels of CCR5 on their CD4+ T cells, a mechanism by which replication might be regulated *in vivo* and restrict transmission and pathogenesis (52, 53). However, most strains of SIV use a number of other alternative coreceptors in *in vitro* assays, such as CXCR6 (STRL33), the orphan receptors GPR1 and GPR15, and several others (18, 21, 61). Despite the efficient use of such alternative entry pathways by SIVmac and SIVsmm isolates in transfected cells, infection and cell targeting *in vivo* is generally thought to be dependent on CCR5 (57). Notably, however, the proportion of CD4+ T cells depleted and/or infected at a given time in macaques and mangabeys may exceed the proportion of cells with detectable CCR5 expression, raising the possibility that other pathways in addition to CCR5 might be utilized (28, 45).

Although both natural and non-natural host infections result in high level virus replication, several distinguishing features provide probable clues as to possible causes for the distinct outcomes. It is long believed that in addition to CD4+ T cell destruction, pathogenesis involves an inability to effectively replenish these populations (29, 46). In pathogenic rhesus macaque infection, damage to the CD4+ T central memory (Tcm) subpopulation appears to play a central role in the inability of infected animals to replenish CD4+ T effector and effector memory (Tem) cells depleted by infection (48). It has been recently found that cell-associated viral loads in CD4+ Tcm are considerably lower in SM than RM, despite equivalent or higher Tem infection levels, which might enable better immune cell homeostasis in infected SM (50). Another difference is the

presence of chronic generalized immune activation in infected humans and RM, whereas natural hosts display generalized immune activation during acute infection that then rapidly resolves (7, 35, 44, 68). Chronic generalized immune activation may contribute to accelerated T cell turnover and ultimate depletion, and is believed to result in large part from translocation of gut microbial products due to gastrointestinal barrier damage during acute infection (9). However, vigorous virus replication and extensive CD4+ T cell depletion in gut mucosal tissue occur in both natural and non-natural hosts (10, 28, 54, 73). A potentially important difference is Th17 CD4+ T cells, which play a critical role in defense against bacteria at mucosal sites, and are lost in HIV-1 and SIVmac rhesus macaque infection but spared in infected natural hosts (8, 12, 22, 58). Thus, the factors regulating CD4+ T cell subset targeting *in vivo* may be central to defining the outcome of infection in natural or non-natural hosts.

Interestingly, evolution appears to have favored mutations in the CCR5 gene that abrogate surface expression of this molecule. An allele containing a 32 base pair frameshift deletion in human CCR5 that abrogates cell surface expression (CCR5Δ32) is present at a frequency of 10% in the Caucasian population, resulting in about 18% heterozygous and 1% homozygous individuals. CD4+ cells from individuals homozygous for CCR5Δ32 are resistant to infection by CCR5-using HIV-1 isolates *in vitro* but permissive for strains that can use CXCR4, and the essential role for CCR5 in HIV-1 transmission and infection is demonstrated by the finding that individuals homozygous for CCR5Δ32 are highly resistant to infection (43, 63). Furthermore, heterozygous individuals can be infected but show lower viral loads and slower disease progression, in association with lower levels of CCR5 expression (17, 33). Red-capped

mangabeys (RCM; *Cercocebus torquatus*) are the natural host of SIVrcm, and a 24 base pair in-frame deletion (CCR5Δ24) that also abrogates surface expression is present at an allelic frequency of 87% (14). As a result, ≥70% of RCM are homozygous for the mutation and do not express CCR5, and the one known exception to exclusive CCR5 dependence by SIV *in vivo* is SIVrcm, which uses CCR2b for entry and cannot use CCR5. The same CCR5Δ24 mutant allele was also reported in two different populations of SM, which are closely related to RCM, but with a low allelic frequency of 4% and no animals homozygous for the allele were found (14, 51).

In this study, we identified a novel 2 base pair deletion and frameshift mutation in SM CCR5 (CCR5Δ2) that results in a lack of surface expression and coreceptor function. The mutation is present at a 26% allele frequency in the large Yerkes National Primate Research Center (YNPRC) SM colony, and together with the previously-described Δ24 allele results in 8% of animals lacking functional CCR5. However CCR5-null SM are susceptible to natural and experimental SIVsmm infection and exhibit robust viral replication. This is the first clear evidence for *in vivo* alternative coreceptor use by SIVsmm in its natural hosts, which provides an explanation for the efficient use of alternative coreceptors by the SIVsmm/mac family of viruses. This data also suggests that cell targeting and tropism in sooty mangabeys is linked to expression and use of both CCR5 and additional alternative entry pathways, and identifies a third example of convergent evolution resulting in nonfunctional mutant CCR5 alleles among primate species.

Materials and Methods

Ethics Statement

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

Animals and primary cells

These studies utilized blood cells from animals housed at the YNPRC or TNPRC. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using standard density gradient separation methods. For genomic analysis, approximately 0.8×10^6 PBMC were lysed in DNA lysis buffer (100 mM KCl; 0.1% NP40; 20 mM Tris pH 8.4; 0.5 mg/ml proteinase K; 200ul total volume) and used as a template for PCR amplification. For infection studies, cryopreserved PBMC were thawed under standard conditions and maintained at 10^6 cells/ml in RPMI supplemented with 10% FBS, 1% glutamine and 1% penicillin-streptomycin, stimulated for 3 days with 5 μg/ml of phytohemagglutinin (PHA; MP Biomedical), infected and then maintained in the same media in the presence of IL-2 (50 U/ml; Novartis). Analysis of wild SM genotypes was carried out on purified DNA derived from fecal specimens collected in Cote d'Ivoire, which were previously characterized by mitochondrial DNA sequence analysis to represent 33 distinct individuals (64).

Cloning SM CCR5 genes

Full-length SM-CCR5 genes were amplified by PCR from SM genomic DNA using high fidelity DNA polymerase (Phusion; Finnzymes) and primers based on conserved 5' and 3' regions of published SM CCR5 coding sequences (forward: 5'-ATG GAC TAT CAA GTG TCA AGT CCA ACC-3'; reverse: 5'-TCA CAA GCC AAC AGA TAT TTC CTG CTC C-3'). PCR reactions contained Phusion polymerase (1 unit) in HF buffer, primers (0.5 uM), dNTPs (0.2 mM) and 200 ng of purified genomic DNA as template in a 50 ul reaction volume. Thermocycling conditions: initial denaturation at 98°C for 45 seconds, followed by 20 cycles of 98°C for 10 seconds, 71°C for 30 seconds and 72°C for 90 seconds, with a final extension step of 72°C for 10 minutes. PCR amplicons were column purified (QIAquick PCR Purification kit; Qiagen) and then used in a second PCR reaction employing primers that incorporated a *HindIII* restriction site at the 5' end of the coding region and a *BamHI* restriction site at the 3' end of the CCR5 coding sequence (forward: 5'-GCT GCT ATA AGC TTC CAC CAT GGA CTA TCA AG-3'; reverse: 5'-AGC GAG CGG ATC CTC ACA AGC CAA CAG ATA-3'; restriction sites underlined). Thermocycling conditions for the second PCR reaction were: an initial denaturation step at 98°C for 45 seconds, followed by 5 cycles of 98°C for 10 seconds, 71°C for 45 seconds and 72°C for 60 seconds, followed by 15 cycles of 98°C for 10 seconds, 76°C for 45 seconds and 72°C for 60 seconds, and final extension at 72°C for 10 minutes. CCR5 amplicons were cloned into the expression plasmid pcDNA3.1+ (Invitrogen) using *HindIII* and *BamHI,* and screened by restriction analysis followed by sequence confirmation. Sequences of the smCCR5Δ2, smCCR5Δ24 and smCCR5 wild-type genes cloned here have been deposited in Genbank (accession numbers HM246694, HM246695 and HM246693, respectively)

CCR5 genotyping of SM

A two-step PCR-based genotyping assay was developed that identifies CCR5 wild-type and CCR5Δ2 alleles based on differential primer annealing, using genomic DNA from lysates of SM PBMC. Genomic DNA was subject to PCR amplification using the first round primers described above, to amplify the entire CCR5 coding region, in reactions that contained Platinum Taq polymerase (1 unit; Invitrogen), 1.5 mM $MgCl_2$, primers (0.5 uM each), dNTPs (0.2 mM), 1-3 ul DNA lysate and Taq buffer in 25 ul reaction volumes. Thermocycling conditions were: initial denaturation at 98°C for 45 second followed by 10 cycles of 94°C for 20 seconds, 68°C for 45 seconds, 72°C for 60 seconds and final extension at 72°C for 10 minutes. The product of this reaction (2.5 ul) was then used as a template for two separate second-round amplifications, each of which used a common downstream primer but different upstream primers specific for the wild-type and Δ2 alleles, respectively (forward CCR5 wild-type: 5'-ATC ACT TGG GTG GTG GCT-3'; forward CCR5Δ2: 5'-ATC ACT TGG GTG GTG CGT-3'; common downstream: 5'-GGT GTT CAG GAG AAG GAC AAT GTT G-3'). The second round PCR reaction used the same conditions as the first reaction. Products of the wild-type and Δ2 amplification reactions were visualized by 2% agarose gel electrophoresis and ethidium bromide staining, which demonstrated a 325 base pair product following amplification with wild-type or Δ2 primer pairs, or both for heterozygotes (Figure S1A).

Each animal was also screened for the CCR5Δ24 deletion allele with a two-step PCR-based assay. Products of the first round PCR reaction described above, were subjected to nested amplification with inner primers (forward: 5'-GGC TAT CGT CCA TGC TGT GT-3'; reverse: 5'-GAC CAG CCC CAA GAT GAC TA-3') and thermocycling conditions as follows: initial denaturation at 94°C for 45 seconds, followed by 25 cycles of 94°C for 20 seconds, 59°C for 45 seconds, 72°C for 60 second, and a final extension at 72°C for 10 minutes. Products were visualized by 3% agarose gel electrophoresis and ethidium bromide staining, yielding a 205 base

pair product from the Δ24 allele, which was easily distinguishable from the 227-229 bp product from the wild-type and CCR5 Δ2 alleles (Figure S1B).

As a secondary confirmation of genotypes established by PCR screening, direct bulk sequencing was carried out on PCR amplified genomic DNA. Amplification was done using the outer primer set as described above, except that Phusion high fidelity polymerase was used for 30 cycles. Products were column-purified and sequenced. Genotypes were verified by manual inspection to confirm the presence of uniform sequences for homozygous animals, or detection of expected frameshifts resulting in overlapping sequences for heterozygous animals (Figure S1C).

For fecal-derived samples, 0.5 ug of purified DNA (of which only a fraction reflected host-derived DNA) was PCR amplified for 35 cycles using first round outer primers as described above, and then subjected to nested amplification for 30 cycles using the inner primer set described above. Products were then subjected to direct sequence analysis.

Pseudotype luciferase reporter virus infections and coreceptor use analysis

SIVsmm Env-mediated entry was analyzed using luciferase-expressing reporter viruses pseudotyped with envelope glycoproteins of interest. Pseudotype viruses were generated by co-transfecting 293T cells with a plasmid encoding the NL4-3-based *env*-deleted luciferase-expressing virus backbone (pNL-luc-E$^-$R$^+$) (16) along with expression plasmids encoding SIVsmm, SIVmac, HIV-1 or VSV-g envelope glycoproteins. Cells were transfected overnight using Fugene (Roche) and washed the next day to remove residual transfection reagent. Supernatants were collected 2 days later, clarified by centrifugation and stored at -80°C until use. Pseudotype viruses were quantified based on HIV-1 Gag p24 antigen ELISA (PerkinElmer) and virion infectivity measured on U87 cells stably expressing CD4 and CCR5. Inocula were then standardized on the basis of infectivity in U87/CD4/CCR5 cells (1×10^6 relative light units; RLU).

The ability of pseudotype viruses to use different coreceptors was assayed in target 293T

cells expressing CD4 and the coreceptor of interest. Target cells were prepared by co-transfection with expression plasmids carrying CD4 and the desired co-receptor (1ug of each plasmid) using Fugene. Cells were re-plated one day post-transfection at $2x10^4$ cells/well in 96-well plates and then infected the following day with pseudotype reporter viruses using equivalent inocula ($1x10^6$ RLU) by spin inoculation for 2 hours at 1200G. Three days later cells were lysed (0.5% Triton X-100 in PBS) and infection quantified on the basis of luciferase production in target cells, determined by adding an equal volume of luciferase substrate (Promega) and measuring luciferase activity in RLU with a luminometer.

SIV envelope clones and infectious viruses

SIV envelopes used in pseudotype infections were generated from plasma of SIVsmm-infected CCR5 wild-type (FFv) and Δ2 homozygous (FNp) SM by single genome amplification (SGA) using methods and protocols previously described and cloned into pcDNA3.1 using Topo TA (Invitrogen) (41, 60). Infectious SIVsmm strains M923 and M951 were isolated as previously described (25) and stocks were prepared in primary SM PBMC.

FACS analysis of wild-type and mutant CCR5 surface expression

293T cells were transfected with wild-type or mutant forms of CCR5 using Fugene according to manufacturer's instructions. One day later cells were detached by incubation in PBS containing 2 mM EDTA, washed in FACS buffer (PBS containing 1% FBS and 0.1% sodium azide), and stained with the CCR5 monoclonal antibody, clone 3A9-[APC] (BD Pharmingen) or isotype-matched control. Cells were analyzed using a FACS Caliber flow cytometer (BD Biosciences) and FloJo software (Tree Star, Inc.) to determine CCR5 surface expression.

SIV infections of primary SM PBMC

PHA-stimulated SM PBMC were plated in 96-well plates at 2.5×10^5 cells/well, incubated for 1 hour in the presence or absence of the CCR5 antagonist maraviroc (15 uM; Pfizer), and then infected with SIVsmm strains (M923 and M951) by spin inoculation (1200xg for 2 hours) followed by overnight incubation. The next day cells were washed in PBS and maintained in media containing IL-2 (50 U/ml), with or without maraviroc (15 uM). Cell supernatants were collected periodically for 3 weeks and replication measured by SIV Gag p27 antigen in cell supernatant by ELISA (Advanced BioScience Laboratories).

Analysis of SM virological and immunological parameters

Clinical data on infected and uninfected SM housed at YNPRC has been reported previously for surveys carried out in 2004-5 and 2006-7 and 2008-9 (70). CD3, CD4 and CCR5 expression on PBMC were analyzed by FACS and plasma viral loads were measured by real-time PCR as described (68, 70). For purposes of analysis, any undetectable viral loads were set at 75 copies, the lower limit of detection. In cases where animals seroconverted during the period of observation, data from prior to infection was included with uninfected animals, while data from after infection was included with infected animals. If multiple measurements were available for individual animals for any parameters, mean values were utilized.

Statistical Analysis

Virological and immunological data were compared between genotype groups using ANOVA or Kruskal-Wallis test followed by the Dunn's multiple comparison test for multiple groups. The two-sample proportions test and Chi-Square test were used for comparison between independent groups. Statistical tests were performed using Prism 4.0 software and OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version 2.3. Data were considered significant when P-value was below 0.05.

Results

Identification of a novel mutation in the sooty mangabey CCR5 gene

We amplified the CCR5 coding sequence from SM genomic DNA, cloned it into an expression vector and analyzed several clones by sequence analysis. Cloning from four independent PCR reactions amplifying smCCR5 from one animal (FVq) resulted in the identification of two distinct alleles in each of the PCR amplifications (**Figure 2-1**). One was a wild-type allele, similar to published smCCR5 sequences (smCCR5 wt), and the other was a novel allele containing a two base pair deletion at nucleotides 466 and 467 of the coding sequence (smCCR5Δ2), which corresponds to the fourth transmembrane domain (TM4) of the smCCR5 protein (**Figure 2-1A**). This deletion causes a frameshift that results in a predicted protein with 110 missense amino acids prior to termination at residue 265 (**Figure 2-1B**). In addition to this deletion, the smCCR5Δ2 allele contains two nucleotide substitutions compared with the wild-type allele identified. The first is a 436T>G substitution resulting in a L146V amino acid change, which is also found in several published wild-type smCCR5 sequences. The second nucleotide substitution is 538C>T, but is masked in CCR5Δ2 due to the frameshift.

In addition to premature truncation of the protein, the mutation results in several charged amino acids predicted within TM4, as well as loss of both disulfide bonds (between the N-terminus and third extracellular loop (ECL), and between the first and second ECLs, respectively) that maintain secondary structure. These features suggest that the mutant protein is unlikely to be configured in a manner to allow proper membrane placement and either normal signaling or SIV/HIV entry coreceptor function.

There are several other mutations in primate CCR5 genes, including the well-

studied 32 base pair frameshift mutation in human CCR5 (CCR5Δ32) (17, 43, 63) and a 24 base pair deletion (CCR5Δ24) that is common in RCM and also reported at low frequency in SM (14, 51). Therefore, we examined the relationship between this SM CCR5Δ2 deletion and the Δ24 RCM/SM and human Δ32 deletions (**Figure 2-1A & B**). The smCCR5Δ2 deletion occurs in the same region of TM4, and overlaps the Δ24 deletion, which is characterized by multiple G-T-G repeats. In contrast, the human CCR5Δ32 deletion occurs approximately 90 bases downstream of smCCR5Δ2, within the second extracellular loop of the protein. Interestingly, the SM and RCM Δ24 alleles also contain the 436T>G and 538C>T substitutions seen smCCR5Δ2, which result in amino acids identical to those in human CCR5 at those respective sites (valine at position 146 and serine at position 180; **Figure 2-1B**).

Because the CCR5Δ24 mutation was previously reported to be present in animals at YNPRC (51), animals were screened for its presence by PCR and several Δ24 carriers were identified (**Figure 2-S1**). We therefore generated a clone of the CCR5Δ24 coding region by PCR of genomic DNA from one heterozygous animal. Sequence analysis of this CCR5Δ24 clone was similar to the sequence previously described (14, 51), except for a non-coding 1026G>T substitution.

```
SMCCR5 WT   401  TGTTTGCTTT  AAAAGCCAGG  ACAGTCACCT  TTGGGTTGGT  GACAAGTGTG  450
SMCCR5 Δ2   401  ..........  ..........  ..........  .....G....  ..........  450
SMCCR5 Δ24  401  ..........  ..........  ..........  .....G....  .G.-------  443
RCMCCR5Δ24  401  ..........  ..........  ..........  .....G....  .G.-------  443
HuCCR5 WT   401  ..........  ..........  ......G...  .....G....  ..........  450
HuCCR5 Δ32  401  ..........  ..........  ......G...  .....G....  ..........  450

SMCCR5 WT   451  ATCACTTGGG  TGGTGGCTGT  GTTTGCCTCT  CTCCCAGGAA  TCATCTTTAC  500
SMCCR5 Δ2   451  ..........  .....--C..  ..........  ..........  ..........  498
SMCCR5 Δ24  444  ----------  -------...  ..........  ..........  ..........  476
RCMCCR5Δ24  444  ----------  -------...  ..........  ..........  ..........  476
HuCCR5 WT   451  ..........  ..........  .......G..  ..........  ..........  500
HuCCR5 Δ32  451  ..........  ..........  .......G..  ..........  ..........  500

SMCCR5 WT   501  CAGATCTCAG  AGAGAAGGTC  TTCATTACAC  CTGCAGCCCT  CATTTTCCAT  550
SMCCR5 Δ2   499  ..........  ..........  ..........  .......T..  ..........  548
SMCCR5 Δ24  477  ..........  ..........  ..........  .......T..  ..........  526
RCMCCR5Δ24  477  ..........  ..........  ..........  .......T..  ..........  526
HuCCR5 WT   501  ..........  ....A .A..  ..........  .......T..  ..........  550
HuCCR5 Δ32  501  ..........  ....A .A..  ..........  .......T..  ..........  550

SMCCR5 WT   551  ACAGTCAGTA  TCAATTCTGG  AAGAATTTCC  AGACATTAAA  GATAGTCATC  600
SMCCR5 Δ2   549  ..........  ..........  ..........  ..........  ..........  598
SMCCR5 Δ24  527  ..........  ..........  ..........  ..........  ..........  576
RCMCCR5Δ24  527  ..........  ..........  ..........  ..........  ..........  576
HuCCR5 WT   551  ..........  ..........  ..........  ..........  ..........  600
HuCCR5 Δ32  551  .----------  ----------  ----------  -----....  ..........  568
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B.

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                                                      TM1
SMCCR5-wt   MDYQVSSPTY  DIDYYTSEPC  QKINVKQIAA  RLLPPLYSLV  FIFGFVGNIL  VVLILINCKR  60
SMCCR5-Δ2   ..........  ..........  ..........  ..........  ..........  ..........
SMCCR5-Δ24  ..........  ..........  ..........  ..........  ..........  ..........
RCMCCR5Δ24  ..........  ..........  ..........  ..........  ..........  ..........
HuCCR5-wt   .......I.  ..N.....  ..........  ..........  ..........  M.  .I....
HuCCR5-Δ32  .......I.  ..N.....  ..........  ..........  ..........  M.  .I....

                   TM2                                           TM3
SMCCR5-wt   LKSMTDIYLL  NLAISDLLFL  LTVPFWAHYA  AAQWDFGNTM  CQLLTGLYFI  GFFSGIFFII  120
SMCCR5-Δ2   ..........  ..........  ..........  ..........  ..........  ..........
SMCCR5-Δ24  ..........  ..........  ..........  ..........  ..........  ..........
RCMCCR5Δ24  ..........  ..........  ..........  ..........  ..........  ..........
HuCCR5-wt   ..........  ....F.  ..........  ..........  ..........  ..........
HuCCR5-Δ32  ..........  ....F.  ..........  ..........  ..........  ..........

                                         TM4                        ▼
SMCCR5-wt   LLTIDRYLAI  VHAVFALKAR  TVTFGLVTSV  ITWVVAVFAS  LPGIIFTRSQ  REGLHYTCSP  180
SMCCR5-Δ2   ..........  ..........  ....V...  ........R.CLS  PRNHLYQI.E  .RSSLHLQLS
SMCCR5-Δ24  ..........  ..........  ....V...  --- -----  ..........  ..........S
RCMCCR5Δ24  ..........  ..........  ....V...  --- -----  ..........  ..........S
HuCCR5-wt   .....V  ..........  ....V...  ..........  K.........  S
HuCCR5-Δ32  .....V  ..........  ....V...  ..........  K.........  S

                     ▽                            TM5
SMCCR5-wt   HFPYSQYQFW  KNFQTLKIVI  LGLVLPLLVM  VICYSGILKT  LLRCRNEKKR  HRAVRLIFTI  240
SMCCR5-Δ2   FSIQ.VSILE  EFPDIKDSHL  GAGPAAACHG  HLLLGNPENS  ASVSKR.EEA  QGCEAYLHHH
SMCCR5-Δ24  ..........  ..........  ..........  ..........  ..........  ..........
RCMCCR5Δ24  ..........  ..........  ..........  ..........  ..........  ..........
HuCCR5-wt   ..........  ..........  ..........  ..........  ..........  ..........
HuCCR5-Δ32  ....IKDSHL  GAGPAAACHG  HL.LGNPKNS  ASVSK*

            TM6                                                 TM7
SMCCR5-wt   MIVYFLFWAP  YNIVLLLNTF  QEFFGLNNCS  SSNRLDQAMQ  VTETLGMTHC  CINPIIYAFV  300
SMCCR5-Δ2   DCLFS.LSLQ  HCPSPEHLPG  ILWPE*
SMCCR5-Δ24  ..........  ..........  ..........  ..........  ..........  ..........
RCMCCR5Δ24  ..........  ..........  ..........  ..........  ..........  ..........
HuCCR5-wt   ..........  ..........  ..........  ..........  ..........  ..........
HuCCR5-Δ32  ..........  ..........  ..........  ..........  ..........  ..........

SMCCR5-wt   GEKFRNYLLV  FFQKHIAKRF  CKCCSIFQQE  ASERASSVYT  RSTGEQEISV  GL*          352
SMCCR5-Δ2   .
SMCCR5-Δ24  ..........  ..........  ..........  ..........  ..........  ..*
RCMCCR5Δ24  ..........  ..........  ..........  ..........  ..........  ..*
HuCCR5-wt   ..........  ..........  ..........  .P......  ..........  ..*
HuCCR5-Δ32  .
```

Figure 2-1. Sequence alignment of wild-type and mutant CCR5 genes.
(A) Partial nucleotide sequence alignment and (B) predicted protein sequence alignment of the wild-type smCCR5 and smCCR5Δ2 alleles (GenBank HM246693 and HM246694), the CCR5Δ24 alleles previously described in sooty and red capped mangabeys (GenBank AF07473 and AAC62474), and human wild-type CCR5 and CCR5Δ32 alleles (GenBank DQ217934 and U66285). Residues identical to the wild-type smCCR5 molecule are indicated by '.' and gaps are shown as dashes. Location of the frameshift resulting from the smCCR5Δ2 deletion in TM4 is indicated by an inverted black triangle while the huCCR5Δ32 frameshift is indicated by an inverted white triangle. Transmembrane domains are indicated by shaded boxes and mis-sense amino acid sequences resulting from mutations are highlighted.

Cell surface expression of mutant smCCR5Δ2

Previous studies have shown that proteins encoded by the mutant human CCR5Δ32 and RCM/SM CCR5Δ24 are not expressed on the cell surface (51, 63). To test whether the smCCR5Δ2 mutant gene gave rise to a protein expressed on the cell surface, we transfected 293T cells with wild-type and mutant SM CCR5 expression plasmids, along with human CCR5, and measured surface expression by flow cytometry. Staining utilized mAb 3A9, which cross-reacts with both human and SM CCR5 and, importantly, recognizes an epitope in the N-terminus that is upstream of the mutation and should be detected if the predicted protein were expressed.

As shown in **Figure 2-2**, surface expression of CCR5 was readily detected on cells transfected with SM or human wild-type CCR5. In contrast, neither the Δ2 nor Δ24 SM mutant CCR5 alleles gave rise to detectable surface expression. We have so far been unable to assess intracellular expression because of high nonspecific intracellular staining with 3A9 and other anti-N-terminal CCR5 antibodies in all cells tested so far (data not shown). From this data, we conclude that the frameshift mutation in smCCR5Δ2 results in a truncated protein that is not expressed on the cell surface. In addition, our observation that the CCR5Δ24 mutant allele is not expressed at the cell surface is consistent with previous reports that this deletion abrogates cell surface expression even though it is not a frameshift (14, 51). We also asked if the mutant protein might have a dominant negative effect on wild-type CCR5 expression, but found no change in CCR5 staining if the wild-type CCR5 plasmid was co-transfected along with the Δ2 or Δ24 alleles (**Figure 2-S2**).
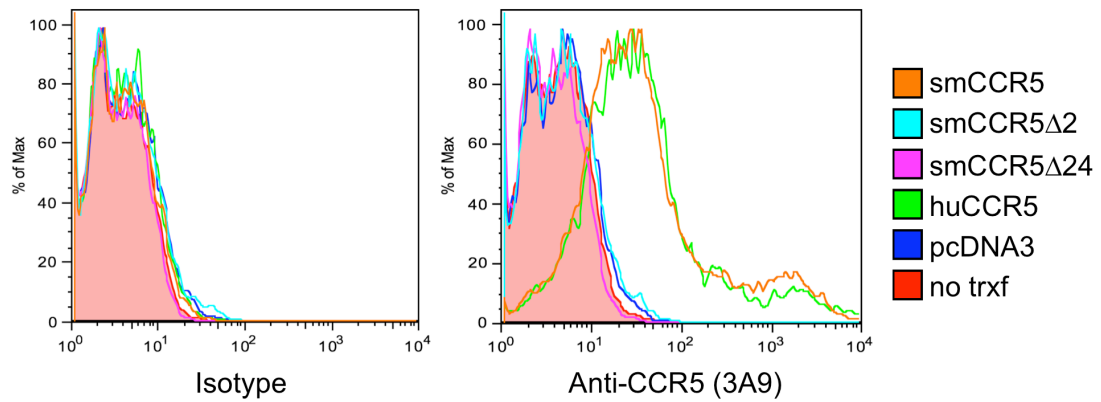
Figure 2-2. Surface expression of wild-type and mutant CCR5 variants *in vitro*.
293T cells were transfected with expression plasmids encoding smCCR5 wild-type (orange), smCCR5Δ2 (light blue), smCCR5Δ24 (magenta) and huCCR5-wt (green), along with empty expression plasmid (dark blue). Untransfected 293T cells (shaded red) serve as controls. Cells were stained with an anti-CCR5 mAb (clone 3A9; APC-conjugated), which recognizes the N-terminal region of both human and sooty mangabey CCR5 (right panel), or an isotype-matched control antibody (left panel).

Prevalence of the smCCR5Δ2 allele in the YNPRC sooty mangabey population

Next we determined the prevalence of the smCCR5Δ2 allele in SM housed at the Yerkes National Primate Research Center (YNPRC), which is the largest captive colony of SM in the world (n=202). All animals were initially screened using a discriminatory PCR assay that specifically identifies the smCCR5 wild-type and Δ2 alleles (**Figure 2-S1A**). Animals were also screened for the Δ24 allele using primers that discriminate Δ24 from Δ2 and wild-type alleles based on amplicon size (**Figure 2-S1B**). Results were then verified by direct bulk sequencing of genomic DNA amplicons that confirmed the presence or absence of homozygous genotypes, or demonstrated frameshifting with sequence overlap in heterozygous animals (**Figure 2-S1C**).

The result from this analysis is shown in **Table 2-1**. Five of six possible genotypes were identified: 50.5% of the SM carried two wild-type CCR5 alleles; 37.6% were heterozygous for wild-type and Δ2 alleles, and 4% of the SM were heterozygous for the wild-type and Δ24 alleles. Notably, 6.4% of the SM were homozygous for the smCCR5Δ2 allele, and 1.5% carried both Δ2 and Δ24 mutant alleles (**Table 2-1**). Thus, nearly 8% of animals carry two CCR5 mutant alleles encoding defective CCR5 proteins. We did not identify any SM that were homozygous for the Δ24 allele.

In this SM population, analysis of allelic frequencies showed 26% of alleles carried the novel Δ2 deletion, 3% carried the Δ24 deletion, and 71% were wild-type. Of note, the 3% frequency we found for the Δ24 allele is similar to the 4% allelic frequency described 12 years ago among SM housed at YNPRC (51). We then used the allelic frequencies to calculate a predicted genotype distribution (**Table 2-1** and **Table 2-S1**). There was close agreement between predicted and observed genotypes (p=ns; Chi-square

test), suggesting that the CCR5 alleles are in equilibrium in this population (of which ~60% are SIV-infected), without evidence of selective pressure favoring or disfavoring any of the genotypes.

Table 2-1. **Genotypic frequencies in Sooty Mangabeys at YNPRC**

Genotype:	n	Observed frequency (%)[1]	Predicted frequency (%)[2,3]
W/W	102	50.5	50.8
W/Δ2	76	37.6	37.1
W/Δ24	8	4.0	3.9
Δ2/Δ2	13	6.4	6.8
Δ2/Δ24	3	1.5	1.4
Δ24/Δ24	0	0	0.1
Total	202		

[1] Genotype distribution among all sooty mangabeys was determined by PCR and direct genomic sequence analysis

[2] Predicted genotype distribution was calculated by Punnett square analysis, based on allelic frequencies (W=0.71; Δ2=0.26; Δ24=0.03) derived from observed genotypes.

[3] Observed and predicted genotype frequencies are not significantly different (Chi-square test)

Prevalence of mutant CCR5 alleles in other captive and wild African sooty mangabey populations

The YNPRC colony is the largest population of SM in the US and the close match between predicted and observed CCR5 genotype distributions suggested an absence of selective pressure for or against any specific genotype. However, we wished to determine the frequency with which these alleles and genotypes were present in other SM populations, so we analyzed genomic DNA obtained from 29 animals housed at the Tulane National Primate Research Center (TNPRC). Of note, many of the monkeys that founded the TNPRC colony originally came from YNPRC in the 1980s, but have been housed and bred separately since then. In this smaller population the Δ2 allele was present at a frequency of 19% and the Δ24 allele had a frequency of 5%. As shown in **Table 2-2** and **Table 2-S2**, the observed genotype frequencies in this population also do not differ from those predicted by allele frequencies.

We also asked whether the CCR5Δ2 allele was present in SM in Africa. For this analysis we amplified CCR5 genes from fecal-derived host DNA samples from 33 wild-living animals in the Tai forest of Cote d'Ivoire (64). Five animals carried both the CCR5Δ2 and wild-type alleles, and in one animal only the Δ2 allele was detected. An additional 2 animals carried both Δ24 and wild-type alleles, while the remaining animals revealed only wild-type sequences. Because this analysis utilized fecal DNA containing limiting quantities of host DNA, we can only be certain that both alleles were captured if animals were found to be heterozygous. Thus, while it is not possible to determine a precise allele frequency, these genotypes suggest a minimum allele frequency of 9% for CCR5Δ2 and 3% for CCR5Δ24 in this population. This result indicates that the Δ2 allele

57

is also present in wild-living SM in Cote d'Ivoire, although likely at a lower frequency than in captive animals at YNPRC.

Table 2-2. **Observed and predicted genotype frequencies in Sooty Mangabeys housed at the TNPRC[1]**

Genotype:	n	Observed frequency (%)[2]	Predicted frequency (%)[3,4]
W/W	18	62.1	57.6
W/Δ2[5]	7	24.1	28.8
W/Δ24	1	3.4	7.8
Δ2/Δ2	1	3.4	3.6
Δ2/Δ24	2	6.9	2.0
Δ24/Δ24	0	0.0	0.3
Total	29		

[1] All animals except one are SIV-infected

[2] Genotype distribution among all sooty mangabeys was determined by PCR and direct genomic sequence analysis

[3] Predicted genotype distribution was calculated by Punnett square analysis, based on allelic frequencies (W=0.76; Δ2=0.19; Δ24=0.05) derived from observed genotypes.

[4] Observed and predicted genotype frequencies are not significantly different (Chi-square test).

[5] Includes one SIV-negative animal.

Expression of CCR5 on SM CD4+ and CD8+ T cells *ex vivo*

Since overexpression studies in transfected cells suggested that neither this common smCCR5Δ2 nor the less common Δ24 proteins are expressed on the cell surface, we examined the relationship between genotypes and CCR5 expression on primary SM CD4+ and CD8+ T cells. To address this point, we analyzed CCR5 expression data that was available from animals housed at the YNPRC collected during periodic surveys between 2004 and 2009, focusing on uninfected animals, and grouped individuals according to their CCR5 genotypes. Since neither Δ2 nor Δ24 CCR5 alleles express following transfection, the two mutations were combined for the purposes of this analysis into a homozygous wild-type, heterozygous, and homozygous deletion allele groups (**Figure 2-3**).

As previously reported (52) and as shown in Figure 3, CCR5 expression is markedly greater on SM CD8+ T cells than CD4+ T cells. In both populations there was a gradation in the percentage of cells staining positive for CCR5 that correlated with CCR5 genotype status. The difference was particularly evident for CD8+ T cells, and the percentage of CCR5+/CD8+ T cells was 9.9%, 5.4% and 0.8% for wild-type, heterozygous and homozygous mutant groups, respectively (Figure 3A; p<0.0001 by Kruskal-Wallis test). This finding indicates that the CCR5Δ2 genotype acts as a determinant of CCR5 expression on CD8+ T cells. There was also a strong linear relationship between wild-type gene dosage and CCR5 expression for CD8 cells (R^2=0.99996; p=0.009 by Pearson's 2-tailed correlation coefficient), consistent with the absence of a dominant negative effect by the mutant alleles in transfected cells (Figure S2). A trend was also evident for CCR5 staining on CD4+ T cells among the three

groups (2.8%, 1.7% and 1.2% in the wild-type, heterozygous and homozygous mutant groups, respectively), which did not reach statistical significance (p=ns by Kruskal-Wallis test) but is consistent with the notion that, although overall very low, CCR5 expression on CD4+ T cells is also regulated by CCR5 genotype.

Representative FACS plots showing CCR5 expression on CD4+ and CD8+ T cells from a homozygous wild-type, heterozygous and homozygous mutant animal are shown in **Figure 2-3B**. We think the low level (~1%) of cells within the CCR5 gate for homozygous mutant animals likely represents background staining, rather than low levels of N-terminal expression given the result of transfection studies (**Figure 2-2**). Unfortunately, antibodies available that are directed at other epitopes in human CCR5 do not recognize SM CCR5.
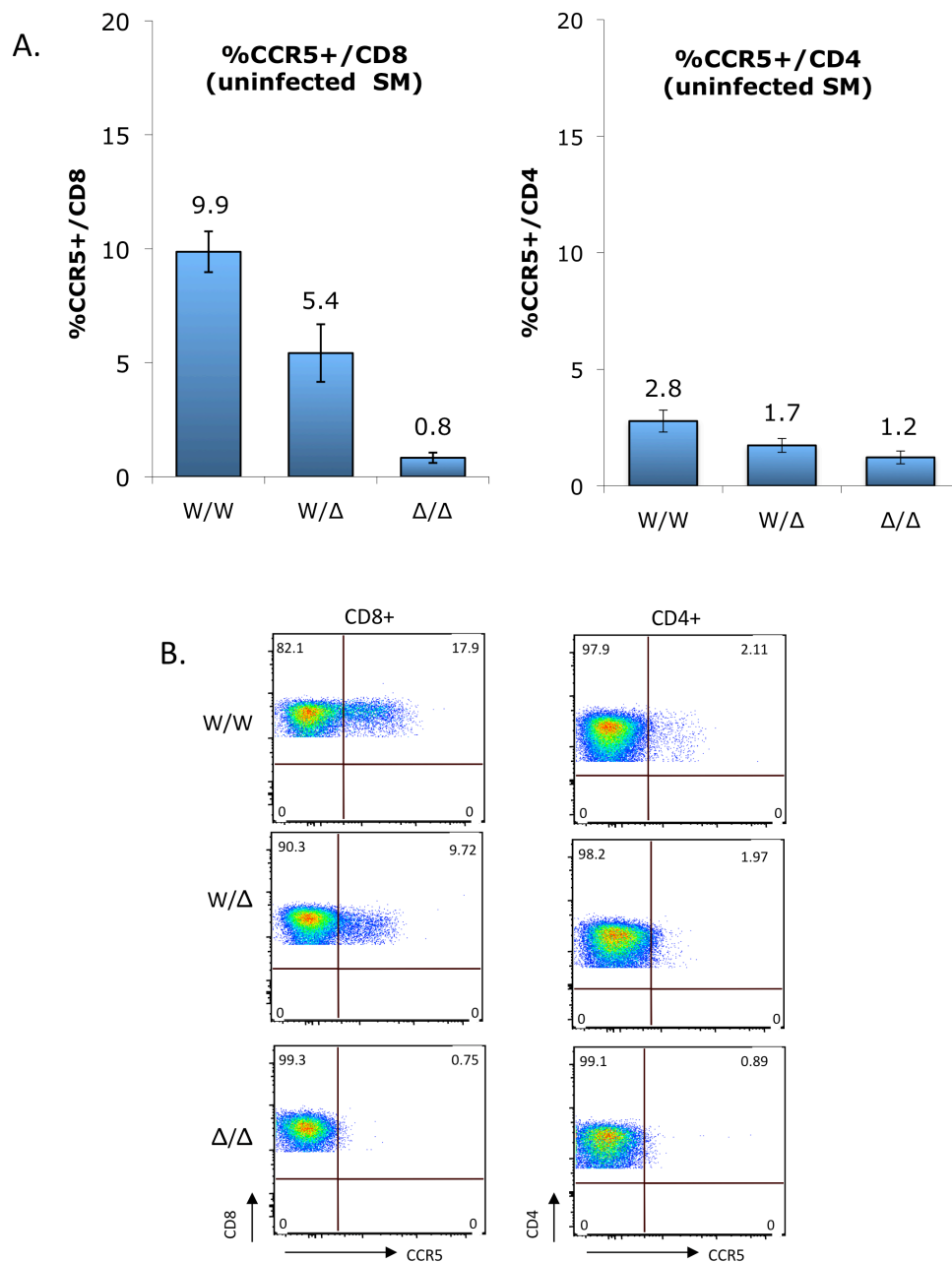
Figure 2-3. CCR5 surface expression on sooty mangabey CD4+ and CD8+ T cells *ex vivo*.
(A) CCR5 staining on CD8+ T cells (left) and CD4+ T cells (right) of uninfected sooty mangabeys was carried out between 2004 and 2009, and analyzed according to genotype groups (mean ± SEM): CCR5 wild-type (W/W; n=38), heterozygous (W/Δ; n=34) and homozygous mutant (Δ/Δ; n=7). Because both Δ2 and Δ24 alleles are functionally null, they were combined for this analysis. CCR5 expression by CD8+ T cells differs significantly between genotype groups (p<0.0001 by Kruskal-Wallis test), while the trend for CD4+ T cells does not reach statistical significance (p=ns). (B) FACS plots showing CCR5 staining on CD4+ and CD8+ T cells from representative homozygous wild-type, heterozygous and homozygous mutant animals.

CCR5-null genotype does not protect sooty mangabeys from SIVsmm infection *in vivo*

In humans, the CCR5Δ32 homozygous genotype provides powerful protection against HIV-1 infection (17, 43, 63). Therefore, we asked if animals homozygous for CCR5-null alleles were infected by SIVsmm. In order to more properly assess natural susceptibility, we restricted this analysis to YNPRC animals that were naturally infected (n=120) and those with documented SIV-negative status (n=72), and excluded animals in the colony known to have been experimentally infected (n=10).

As shown in **Table 2-3**, we found that among SM with the CCR5 homozygous wild-type genotype, 65% were naturally infected while 35% were uninfected, and 62% of CCR5 heterozygous animals were infected and 38% were uninfected. Unexpectedly, among animals homozygous for CCR5-null alleles (n=14), 50% were naturally infected and 50% were seronegative. Thus, animals lacking functional CCR5 genes are susceptible to natural SIVsmm infection. The slightly lower prevalence of SIV infection among animals in the CCR5-null group was not statistically significant (p=ns; Chi-square test). We also compared the distribution of genotypes within the SIV-negative and naturally-infected SIV populations (**Table 2-4**). Similarly, there were no significant differences in genotype distribution between the SIV serostatus groups (p=ns for each genotype group; 2-sample proportions test), although a slightly lower proportion of homozygous CCR5 mutant animals was seen in the infected animals compared with SIV-negative animals (5.8% vs. 9.7%; p=ns). Therefore, the CCR5-null genotype does not prevent natural acquisition of SIVsmm infection. Furthermore, neither heterozygosity nor homozygous null genotype appears to significantly influence SM susceptibility to

SIVsmm infection *in vivo*, and while we cannot absolutely rule out a small effect, any protection that might be afforded by CCR5-null status would be slight. This result stands in marked contrast to the profound protective effect of CCR5Δ32 homozygosity in humans.

We also determined the genotypes of 10 animals at YNPRC that were infected experimentally. Five of these animals were homozygous for the CCR5 wild-type gene, three were heterozygotes (all W/Δ2) and two were homozygous for smCCR5Δ2. Furthermore, all but one of the TNPRC animals studied are SIVsmm-infected, including a mix of both natural infections and experimental inoculation done in earlier decades. Among the infected animals were three homozygous CCR5-null animals, while the one uninfected animal was heterozygous (W/Δ2). Therefore, SM naturally deficient in CCR5 expression are susceptible to experimental as well as natural SIVsmm infection, confirming that non-CCR5 entry pathways can mediate SM natural host infection.

Table 2-3. **Prevalence of naturally-acquired SIV infection among YNPRC sooty mangabeys based on CCR5 genotype.**

Genotype[1]	SIV positive[2] (n)	SIV negative[3] (n)	% SIV infected[4]
W/W	63	34	65
W/Δ	50	31	62
Δ/Δ	7	7	50
Total	120	72	63

[1] CCR5Δ2 and Δ24 alleles were grouped together as defective for expression (Δ)

[2] Includes animals known to be infected naturally in the wild or in captivity, and excludes 10 animals infected experimentally

[3] Includes animals known to be SIV-negative at the time of last survey

[4] SIV prevalence does not differ significantly among the genotype groups (Chi-square test).

Table 2-4. **Genotypic distribution among YNPRC sooty mangabeys based on SIV infection status**

Genotype[1]	SIV positive SM (n=120)[2,4]	SIV negative SM (n=72)[3,4]
W/W	0.525	0.472
W/Δ	0.417	0.431
Δ/Δ	0.058	0.097

[1] CCR5Δ2 and Δ24 alleles were grouped together as defective for expression (Δ).

[2] Includes animals known to be infected naturally in the wild or in captivity, and excludes 10 animals infected experimentally.

[3] Includes animals known to be SIV-negative at the time of last survey

[4] Differences in genotype distribution among animals based on SIV infection status are not statistically significant (2-sample proportions test).

Influence of smCCR5 genotype on plasma viral loads and CD4 counts in SIVsmm-infected SM

We next asked if CCR5 genotype affected plasma viral loads in infected animals. Based on previously collected data, the $\log_{10}$ viral load (mean ± SEM) was calculated for animals in each genotype group; for animals with multiple data points available, their mean viral load ($\log_{10}$) was used (**Figure 2-4**). This analysis showed robust viral loads in all genotype groups, with a modest but statistically significant gradient in VL dependent on the presence of a wild-type CCR5 allele (p=0.005 by Kruskal-Wallis test). Animals possessing two wild-type alleles exhibited the highest viral load (4.83 ± 0.10 $\log_{10}$),

heterozygotes showed an intermediate level (4.65 ± 0.10 $\log_{10}$), and infected animals with the CCR5-null genotype had the lowest VL (4.37 ± 0.15 $\log_{10}$). Thus, there is approximately 0.5 $\log_{10}$ difference in viral load in animals with two wild-type compared with two CCR5-null alleles. The two important points here are that homozygous mutant animals have vigorous viral replication despite lacking functional CCR5, and simultaneously exhibit a small but significant difference in plasma viral load associated with increasing CCR5 gene dosage.

CD4 counts remain stable during chronic SIVsmm infection in the vast majority of animals, but some exceptions have been noted (2, 47), so we next asked if SIV-infected animals exhibit differences in CD4 counts depending on their CCR5 genotype. However, there was no significant difference in CD4+ T cell levels among the genotype groups, whether assessed based on absolute counts (**Figure 2-S3A**; p=ns, Kruskal-Wallis) or on the basis of CD4 percentage (**Figure 2-S3B**; p=ns, ANOVA). These results suggest that CD4+ T cell levels are maintained similarly in SIV-infected SM regardless of CCR5 genotype.
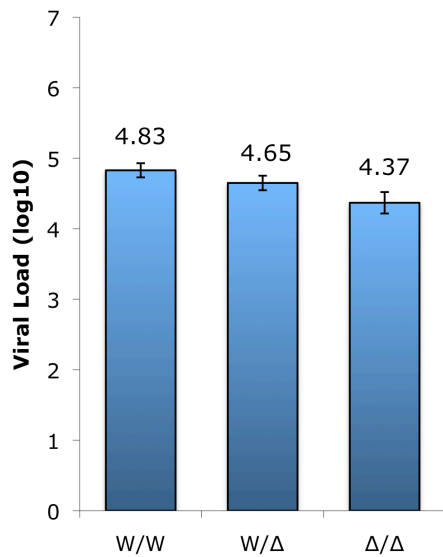
Figure 2-4. SIV plasma viral load measurements in infected sooty mangabeys between genotype groups.
Plasma viral load measurements (VL log$_{10}$; means ± SEM) collected in surveys carried out between 2004 and 2009 of infected animals in the wild-type (n=60), heterozygous (n=49), and homozygous mutant (n=7) genotype groups. The Δ2 and Δ24 alleles were combined for this analysis. The difference in VL between homozygous wild-type and CCR5-null genotype groups (W/W vs. Δ/Δ) is statistically significant (p<0.05; Dunn's multiple comparison test) whereas the differences for the heterozygous group (W/Δ vs. W/W; W/Δ vs. Δ/Δ) does not reach statistical significance (p=ns; Dunn's multiple comparison test).

smCCR5Δ2 does not support SIV entry *in vitro*

We then asked if there was any chance that the smCCR5Δ2 mutant allele, when

expressed along with CD4, could support SIV infection *in vitro*. We considered it

unlikely but thought it was necessary to test directly given the staining patterns by CCR5

mAb 3A9 in Δ2 homozygous primary mononuclear cells (**Figure 2-3**). We also

considered it important to test using SIVsmm from a CCR5Δ2 homozygous infected

animal, in case viral adaptation might have enabled use of an N-terminal region alone.

Therefore, we generated pseudotype virions carrying Env glycoproteins cloned directly

from plasma virus of an SIVsmm-infected CCR5Δ2 homozygous animal (FNp), along

with pseudotypes carrying Envs cloned from plasma of an infected wild-type animal

(FFv). Use of plasma virus ensured that these Envs were derived from actively

replicating virus. Target 293T cells were co-transfected with CD4 plus plasmids

encoding wild-type smCCR5, smCCR5Δ2, human CCR5 or an empty vector as a control,

then infected with the primary SIVsmm pseudotypes. Of note, pseudotype virions

carrying the FNp 5.1 Env were considerably less infectious than the other Envs and very

large amounts of this virus were required to achieve equivalent infectious inocula, which

generally resulted in high levels of background for this Env.

As shown in **Figure 2-5**, wild-type smCCR5 and human CCR5 support infection

of all SIVsmm variants to similar levels. In contrast, the smCCR5Δ2 allele does not

support infection by any of the viruses. Importantly, CCR5Δ2 does not function as a

coreceptor for Env variants from CCR5-null animals. Thus, SIVsmm infections in

CCR5-null animals are mediated through pathways independent of CCR5.
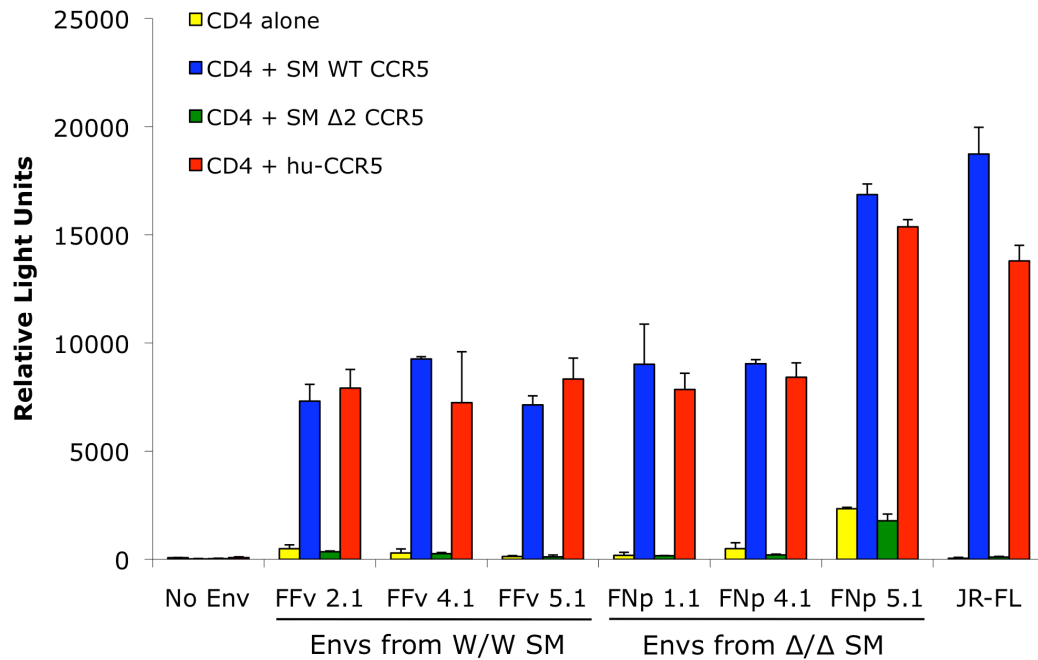
Figure 2-5. Mutant smCCR5Δ2 does not support SIV infection *in vitro*. 293T cells were transfected with CD4 alone (yellow bars) or in combination with wild-type smCCR5 (blue bars), smCCR5Δ2 (green bars) or wild-type human CCR5 (red bars). Target cells were infected with luciferase-expressing pseudotype virions carrying Env glycoproteins that were cloned from plasma of two SIVsmm-infected sooty mangabeys (FFv: W/W SM; FNp: Δ2/Δ2 SM). Pseudotypes carrying the R5-tropic HIV-1 Env JRFL and virions lacking envelope glycoproteins served as controls. Infection was measured by relative light units (RLU) in cell lysates 3 days after infection (mean ± SD).

Use of alternative coreceptors by SIVsmm isolates

The data presented here indicates that SIVsmm must be able to use entry pathways other than CCR5 for replication *in vivo*. It is long known that many SIV strains use a number of alternative coreceptors in addition to CCR5 *in vitro*, although these viruses rarely use CXCR4. We therefore tested the ability of SIVsmm envelopes to mediate infection through human CCR2b, CCR3, CCR8, GPR1, GPR15 (BOB), CXCR6 and CXCR4. This analysis employed the uncultured SIVsmm envelope glycoproteins cloned from plasma of the CCR5-null (FNp) and homozygous wild-type (FFv) infected animals.

As shown in **Figure 2-6**, all SIVsmm envelope glycoproteins tested mediated entry into cells expressing CD4 in conjunction with GPR15 and CXCR6, while GPR1 was also used but less efficiently. In contrast, none of the SIVsmm envelope glycoproteins could use CXCR4 as a coreceptor, nor CCR3 or CCR8 (data not shown). Interestingly, SIVsmm envelope glycoproteins failed to use CCR2b, an entry pathway employed by SIVrcm in RCM that typically lack CCR5 due to a high prevalence of the Δ24 mutation (14). Furthermore, the patterns of alternative coreceptor use were similar for envelope glycoproteins derived from CCR5-null and CCR5 wild-type animals, indicating that alternative coreceptor utilization is a feature shared by SIVsmm regardless of whether the host animal expresses CCR5. While absolute luciferase production varied among experiments, GPR15 and CXCR6 typically supported levels of infection similar to that mediated by CCR5 (**Figure 2-S4**), although transfected targets likely represent maximum levels of potential utilization relative to primary cells that would express these molecules at physiological levels.
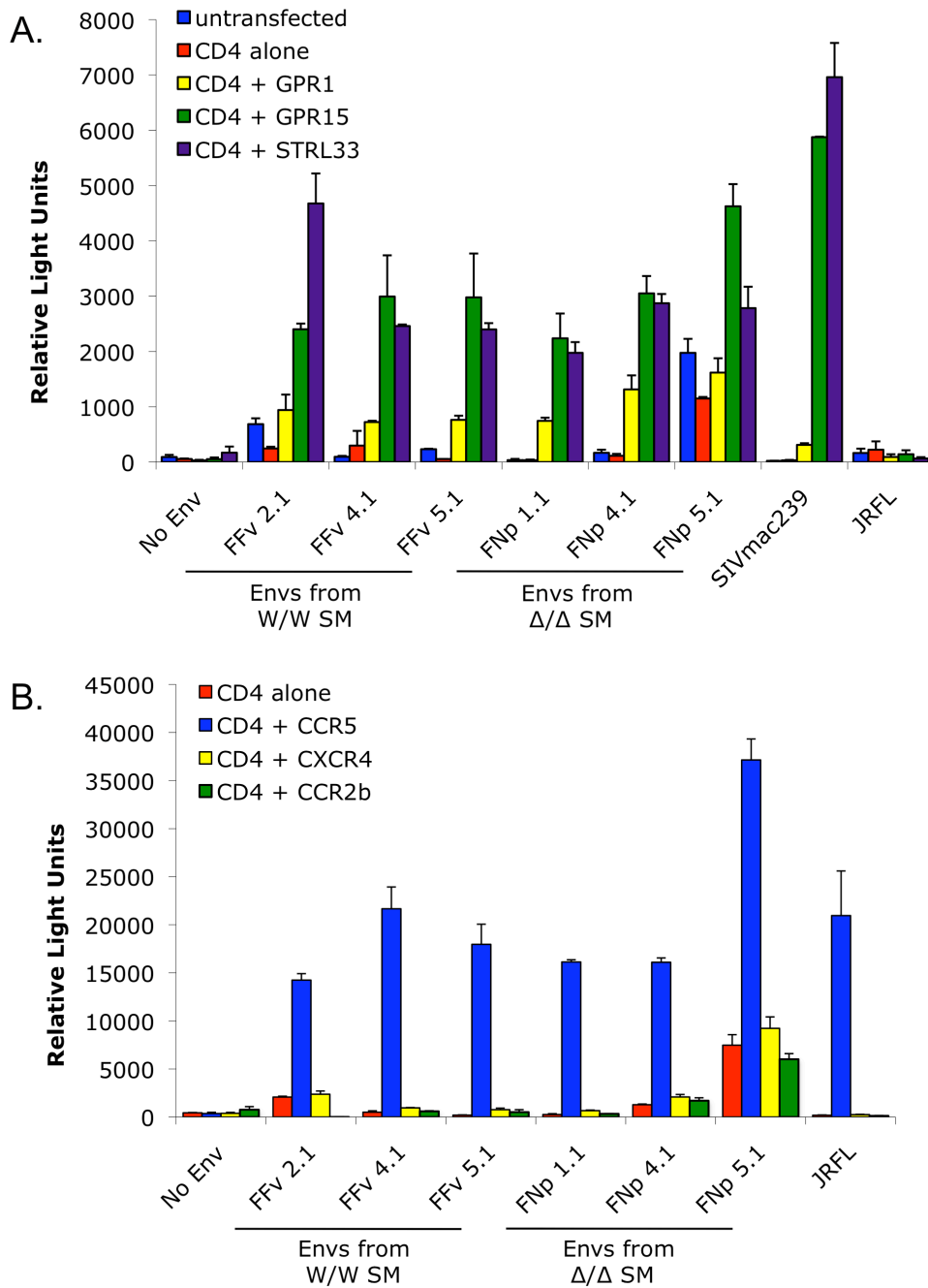
Figure 2-6. Alternative coreceptor utilization by SIVsmm Envs *in vitro*.
293T cells were transfected with CD4 alone or in combination with (A) the alternative
coreceptors GPR1, GPR15 and CXCR6, or (B) CXCR4, CCR2b or CCR5. Target cells were then
infected with luciferase-expressing pseudotype virions containing SIVsmm Envs from infected
CCR5 wild-type (FFv: W/W SM) and CCR5-null (FNp: Δ2/Δ2 SM) animals. Envs from
SIVmac239, HIV-1 JRFL and virions lacking Env served as controls. Infection was assayed by
RLU (mean ± SD) measured 3 days after infection.

71

SIVsmm infects sooty mangabey CCR5-null primary PBMC *in vitro* and infection of wild-type PBMC is not blocked by the CCR5 antagonist maraviroc

We next investigated the role of non-CCR5 pathways in SIVsmm infection of primary SM cells, utilizing both the specific CCR5 antagonist maraviroc and CCR5-null PBMC derived from CCR5Δ2 homozygous animals. Maraviroc blocks chemokine signaling and HIV-1 Env entry through human and rhesus macaque CCR5 (37, 62), but blocking of SM CCR5 coreceptor function has not been reported. Therefore, we first tested the effect of maraviroc on SIVsmm entry through smCCR5 in transfected cells. As shown in **Figure 2-7A**, maraviroc blocked SIVsmm pseudotype infection of target cells expressing CD4 and smCCR5, reducing luciferase expression to the level seen with target cells expressing CD4 alone (data not shown), indicating complete blocking of smCCR5-mediated entry by maraviroc. In contrast, maraviroc did not inhibit SIVsmm entry mediated by GPR15 (**Figure 2-7A**), nor did it affect entry by VSV-G pseudotypes (data not shown), confirming that blocking is not a nonspecific effect.

Next we asked if maraviroc would affect productive infection of SM primary PBMC by infectious isolates of SIVsmm. PBMC from an uninfected CCR5 wild-type (FAk) and a CCR5-null (FAz) animals were activated with PHA for three days, and then infected in the presence and absence of maraviroc with two SIVsmm primary isolates (M923 and M951), which were both derived from CCR5 wild-type infected animals. Viral replication was measured as SIV gag p27 levels in supernatants collected periodically post-infection (**Figure 2-7B**).

We first noted that both SIVsmm isolates were able to productively infect primary PBMC *in vitro* from the CCR5-null SM (FAz). In these cells, there was no difference in

replication associated with CCR5 blocking by maraviroc, as expected given the lack of functional CCR5 encoded by the mutant genes. This result indicates that SIVsmm infection of CCR5-null PBMC can occur independently of CCR5. We then tested CCR5 wild-type PBMC (FAk), and found that SIVsmm established productive infection in both the absence and presence of maraviroc. Furthermore, there was no difference in the level of infection achieved when CCR5 was blocked, based on p27 antigen production. This finding indicates that SIVsmm efficiently enters even CCR5-expressing SM primary PBMC through pathways independent of CCR5.

CCR5 genotypes in sooty mangabeys with SIV evolution to X4 coreceptor use

Unlike HIV-1 in humans, CXCR4 use by SIV in SM is rare. However, a few exceptions have been noted in which CXCR4 use emerged following experimental infection, which was associated with profound CD4+ T cell loss although not clinical AIDS (47). Therefore, to ask if restricted CCR5+ target cell availability due to genetic absence of the coreceptor might be linked to CXCR4 emergence, we genotyped two infected CD4-low SM previously described in which CXCR4-using SIVsmm variants emerged (47). Neither animal possessed a CCR5-null genotype: one was CCR5 homozygous wild type and the other was heterozygous for the CCR5Δ2 allele. Thus, the fact that acquisition of CXCR4 use by SIVsmm can occur but is not associated with animals that genetically lack CCR5 is consistent with the notion that alternative pathway-supported entry *in vivo* is robust and lack of CCR5 does not serve as a driving force in the rare cases with emergence of CXCR4 use.
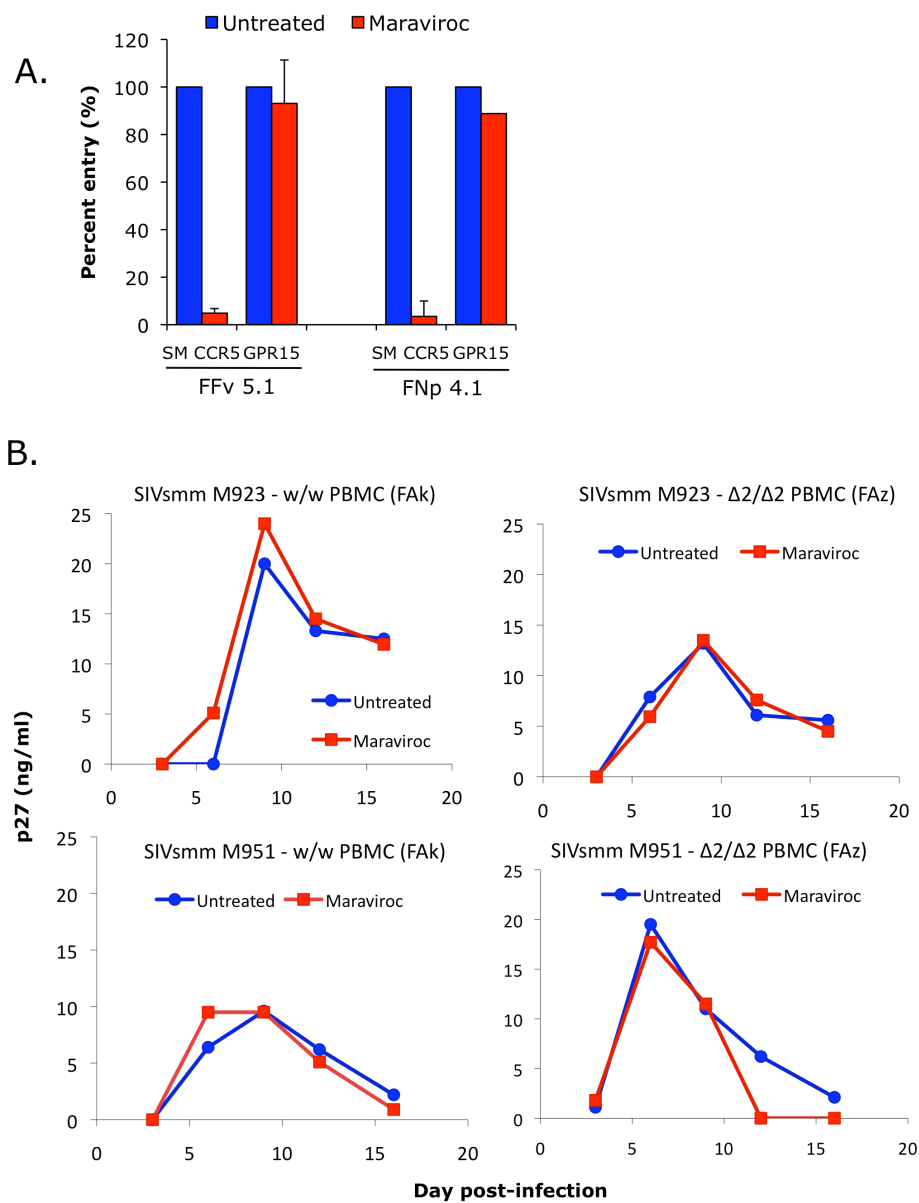
Figure 2-7. Effect of CCR5 blocking on SIVsmm use of CCR5 and entry into primary SM PBMC.
(A) 293T cells were transfected with CD4 in combination with wild-type smCCR5 or GPR15. Two days post-transfection, target cells were pretreated for one hour with or without the CCR5 antagonist, maraviroc (15uM), and then infected with pseudotype virions carrying SIVsmm Envs from a CCR5 wild-type animal (FFv) and a CCR5-null animal (FNp). Three days later, infection was measured based on RLU (mean ± SD) in cell lysates. (B) Growth curves from infection of primary SM PBMC (FAk: W/W SM; FAz: Δ2/Δ2 SM). Cells were stimulated for 3 days with PHA, then pretreated for one hour with or without maraviroc (15 uM), followed by infection with two different SIVsmm primary isolates (M923 and M951) in the continued presence or absence of maraviroc. SIV Gag p27 antigen levels in viral supernatants were measured by ELISA.

74

Discussion

We unexpectedly found that 8% of sooty mangabeys in a large US captive population lack functional CCR5 due to the high prevalence (29%) of mutations in the CCR5 gene that abrogate cell surface expression and SIV coreceptor function. Despite their CCR5-null status, homozygous mutant animals are susceptible to natural as well as experimental SIVsmm infection and display viral loads only modestly lower than CCR5 wild-type animals. *In vitro*, SIVsmm enters primary SM lymphocytes independently of CCR5, and Envs from both wild type and CCR5-null infected animals use several alternative coreceptors in addition to CCR5, but do not use CXCR4. These data indicate that both CCR5 and alternative coreceptor pathways mediate cell entry and robust viral replication *in vivo*. The recognition that both CCR5-dependent and independent pathways are used in the SM natural host has significant implications for understanding viral tropism *in vivo* and CD4+ T cell subset targeting that may regulate the outcome of natural host infection, and raises important questions about entry coreceptor use in pathogenic non-human primate models of AIDS. This finding also explains the previously obscure reason for widespread and efficient use of alternative coreceptors among the SIVmac/smm family of viruses. Finally, it provides a third example of convergent evolution resulting in disruption of CCR5 function among primates, with consequences for virus-host interactions in SM that differ from both humans homozygous for CCR5Δ32 and red capped mangabeys homozygous for CCR5Δ24.

From the original descriptions of alternative coreceptor use by SIVmac/smm viruses, and subsequently by other SIV strains, the reason for conserved use of these pathways has remained elusive (1, 18, 21). It has been repeatedly shown that various SIV

75

isolates can infect primary human Δ32 homozygous PBMC independent of both CCR5

and CXCR4, indicating that alternative coreceptors are expressed in a manner that

supports infection in primary lymphocytes *ex vivo*, at least in cells of human origin (13,

15, 23, 39, 49, 76). Our data show for the first time that the alternative coreceptors are

used by SIVsmm in primary simian T cells *ex vivo* and, more importantly, *in vivo* in the

natural host from which the SIVmac/smm family derived. Several important questions

are raised by this finding: (1) are alternative pathways also operative in SM with wild-

type CCR5 expression, or are they only relevant if CCR5 is absent; (2) what essential

role or selective advantage do they provide SIVsmm in SM infection *in vivo* that has led

to conservation of alternative coreceptor entry pathway use; (3) does alternative

coreceptor use define a novel population of CCR5-negative target cells that contributes to

the ability of host and virus to coexist without disease, and; (4) what role do alternative

pathways play in infection of macaques, the nonhuman primate model used to study

AIDS.

Prior to this confirmation that alternative pathways are used *in vivo*, it seemed

plausible that alternative coreceptor use by SIVsmm/mac was an *in vitro* epiphenomenon

of little biological significance. Recognizing that they are operative *in vivo*, it seems

more likely that conservation of their use among SIV isolates reflects some role that, if

not completely essential, offers a selective advantage for the virus. Comparison of viral

load data among the genotype groups showed robust replication in the absence of CCR5,

but a step-wise increase associated with the presence of one or two functional CCR5

alleles, and *ex vivo* blocking studies with maraviroc confirmed that both wild-type and

CCR5-null primary PBMC possess efficient CCR5-independent entry pathways. On one

hand, if the relevant coreceptor(s) are expressed only on cells that also express CCR5, the use of multiple pathways *in vivo* might be an example of functional redundancy acquired by SIV, reminiscent in part of the functional redundancy of the chemokine/chemokine receptor system. On the other hand, if CCR5 and alternative pathways are expressed on distinct or only partially overlapping CD4+ T cell subsets, the 0.5 log10 VL difference between Δ/Δ and W/W animals may also be consistent with separate components of plasma viremia supported by CCR5 and non-CCR5 pathways (and an intermediate gene-dosage effect in the presence of one CCR5 allele). It is notable that the degree of depletion in SM gut CD4+ T cells exceeds the proportion that express detectable CCR5 (28), and it will be important to determine if this is due to infection and targeting of CCR5-negative cells in wild-type animals mediated by other coreceptors.

As to why alternative coreceptor use is conserved among SIVsmm and related strains, it seems unlikely to result from the 8% prevalence of CCR5-null animals in SM, and more likely reflects a unique role that provides an advantage over CCR5 alone in transmission, establishment of reservoirs or other aspects of infection. Sooty mangabeys overall express very low levels of CCR5 on CD4+ T cells, which has been proposed as an evolutionary adaptive response to "protect" critical target cells and minimize pathogenesis (52, 53). If so, acquisition of alternative coreceptor use by the virus may have reflected a "counter-measure" to maximize replication capacity, although doing so in a manner that still retains the nonpathogenic nature of infection. Thus, the use of alternative entry pathways *in vivo* might enable infection of a novel CCR5-negative target cell population that is more expendable than CCR5+ cells, allowing the virus to replicate efficiently without causing disease in the face of extremely restricted CCR5 expression.

It will therefore be important to define the distribution of cells infected in animals with and without functional CCR5.

An important question raised by these SM findings is whether alternative coreceptors are utilized in pathogenic infection of macaques, which is widely used to model human AIDS. While CCR5 clearly plays a principal role as evidenced by substantial albeit variable viral suppression by CCR5 antagonists (74, 75), levels of CD4+ T cell infection in rhesus macaques can also substantially exceed the proportion of cells that express detectable CCR5 (45). While it is possible that infection of apparently CCR5-negative targets reflects entry mediated by CCR5 at levels below the threshold detectable by FACS, our findings revive the question of whether it may be mediated by additional entry pathways. Few studies have attempted to address alternative coreceptor use *in vivo*. One report showed that mutations that abrogated GPR15 use by SIVmac had little effect on replication or pathogenesis in rhesus macaques (57). Similarly, when pigtail macaques (*Macaca nemestrina*) were infected with SIVmne (also derived from SIVsmm), serial isolates exhibited decreasing ability to use alternative entry pathways when assayed *in vitro* (23). On the other hand, in cynomolgus macaques (*Macaca fascicularis*) infected with SIVsmm, animals with progressive disease showed retention or broadening of alternative coreceptor use while those without disease progression showed narrowing of alternative coreceptor use (39). Thus, it remains to be determined whether alternative pathways support entry in particular subsets of CD4+ T cells in the macaque model *in vivo*. Of note, emergence of CXCR4 use is common in HIV-1 infection of humans but exceedingly infrequent in macaques infected with SIVmac. HIV-1 rarely uses alternative coreceptors efficiently and evolution to CXCR4 use by

HIV-1 is believed to result, in part, from loss of CCR5+ target cells in late stage disease. Thus, the availability of efficient alternative entry pathways may be one reason that SIV rarely evolves to use CXCR4.

The SIVsmm Envs examined here use GPR15 and CXCR6 for entry quite efficiently *in vitro*, and use GPR1 somewhat less efficiently. This result is concordant with coreceptor use patterns of multiple other SIV isolates [22,23,30]. In human blood cells, CXCR6 is highly expressed on CD4+ and CD8+ memory but not naïve T cells, and on gamma-delta T and to a lesser extent NK cells (71), although others have reported expression by CD4+ naïve T cells as well (66). Interestingly, CXCR6 expression is regulated by T cell activation in a pattern that is tightly linked with CCR5 expression in response to some stimuli, but markedly different in response to others (71). GPR15 is also expressed on lymphoid and myeloid cells but with less information about specific distribution patterns (18, 19, 21). Of note, both CXCR6 and GPR15 are also highly expressed in intestinal tissues (18, 42), raising the question of whether alternative coreceptor use may be involved in mucosal events that play a central role in infection. Thus, CXCR6 and GPR15 are particularly likely candidates for mediating SM infection independent of CCR5, and further studies are required to determine which one or ones are responsible for entry into primary SM PBMC *ex vivo* and infection *in vivo*.

One of the most important priorities in HIV/AIDS research at present is understanding why infected natural hosts remain healthy while rhesus macaques infected with SIV, humans infected with HIV-1 or HIV-2, and chimpanzees infected with SIVcpz develop AIDS. Many features are shared by non-pathogenic natural host and pathogenic non-natural host infection including sustained high level viremia, vigorous immune

activation during acute infection, and extensive depletion of gut mucosal CD4+ T cells. In pathogenic infections it is believed that in addition to infection and loss of short-lived T effector and T effector memory cells, damage to long-lived CD4+ Tcm populations that impairs the capacity to maintain immune cell homeostasis is a critical factor in progressive immunodeficiency (29, 48, 56). CCR5 levels are profoundly lower on SM CD4+ T cells compared with RM and humans, which may restrict the target cells available for infection *in vivo* (52, 53). More recently it has been found that CD4+ Tcm in SM have particularly impaired CCR5 expression upon activation, and this corresponds with markedly lower levels of cell-associated infection in Tcm compared with Tem cells in SM, whereas both populations are similarly infected in RM (50). Thus, in addition to the role of CCR5, it will be important to define the distribution and use of other coreceptors by SIVsmm in its natural host as well as in pathogenic rhesus macaque infection. Another prominent difference between pathogenic infection and nonpathogenic natural host infection is the presence of sustained high level generalized immune activation in rhesus and humans, whereas acute infection in natural hosts is associated with transient immune activation that rapidly resolves (7, 20, 22, 27, 40, 68). Sustained immune activation during chronic infection may be an additional factor driving T cell turnover and depletion. A principal mechanism driving chronic immune activation is believed to be translocation of microbial products due to disruption of gut mucosal barrier integrity that occurs early in infection (9). However, gut mucosal lymphocyte infection and CD4+ T cell depletion occurs in natural host as well as pathogenic host infection (28, 54). One potentially critical difference is the loss in human and rhesus macaque infection, but preservation in natural hosts, of mucosal CD4 Th17 cells, which

play a critical role in gut mucosal immune defense (8, 12, 22, 58). Why gut Th17 CD4+

T cells are preserved in infected natural hosts but depleted in other hosts remains to be

determined, but the critical role of entry coreceptors in determining tropism and cell

subset infection *in vivo* suggest that both CCR5 and alternative coreceptor pathways must

be defined in order to understand the targeting versus protection of critical CD4+ cell

subsets.

In addition to the YNPRC and TNPRC SM colonies, we also found the Δ2 allele

in SM in the Tai forest of Cote d'Ivoire, confirming its presence not just in captive but

also in wild-living West African animals, albeit at a lower frequency. Sooty mangabeys

at YNPRC were derived from multiple sources and their history is not well documented

(5), so it is difficult to know for sure from what geographic regions in West Africa these

animals descended. Of note, the Δ2 allele was not described in earlier studies that

reported the Δ24 allele in SM from West Africa and the YNPRC colony (14, 51).

However, CCR5 alleles in those studies were screened by PCR amplicon size, which

could discriminate a 24 bp size difference but is unlikely to distinguish a 2 bp difference

between the wild type and Δ2 alleles.

The identification here of SM CCR5Δ2 brings to three the number of primates

known to have a high prevalence of defective CCR5 alleles. Interestingly, each of the

three examples of populations with homozygous mutant CCR5 individuals shows distinct

patterns of host/virus interactions. For HIV-1 in humans, which have the lowest

prevalence of defective CCR5 genes (~1% among Caucasians), CCR5 use is a stringent

requirement for establishment of new infections, and its absence in the host provides

almost complete protection from infection even though late-stage variants can use

CXCR4 and, occasionally, other coreceptors. In RCM, which have the highest prevalence of CCR5-null individuals (≥70%), SIVrcm has adapted to use CCR2b as a coreceptor and lost the ability to use CCR5 (14). SIVsmm infection in SM, which have an intermediate prevalence of CCR5-null individuals (8% in this population), demonstrate an intermediate relationship, in which alternative coreceptors efficiently mediate both natural and experimental infection in the absence of CCR5, but CCR5 use by the virus is retained, perhaps because both CCR5 and alternative pathways together maximize replication in the majority of animals, while the non-CCR5 pathways are required in the CCR5-null animals.

It is somewhat unexpected that SIVsmm does not use CCR2b, which is the route of entry taken by SIVrcm in the absence of CCR5. SM and RCM are closely related and sometimes considered sub-species within the same species (14, 26). The presence of the same CCR5Δ24 allele in RCM and SM has been interpreted as indicating an origin prior to separation of these populations, although it is uncertain if the remarkably high frequency CCR5Δ24 in the RCM population reflects selective pressure exerted by an environmental or infectious cause, or founder effect (14). Another question raised by our results is whether the SM CCR5Δ2 and the SM/RCM CCR5Δ24 emerged independently, resulting in deletions in the same region due to multiple nucleotide repeats enabling recombination, or given the overlapping sites whether Δ2 emerged first and additional events led to Δ24. Since Δ2 is not expressed, it is unlikely that further deletions would lead to any additional selective advantage, and two separate recombination deletion events in the same region of the same gene seem more probable.

82

What selective pressures might have led to three independent primate CCR5 deletion alleles is uncertain. CCR5Δ32 has been present in the human population for at least 3000 years (34), far longer than HIV-1, and despite considerable speculation on infectious or other pressures, both what factors fueled its emergence and when it occurred remain enigmatic (30). In contrast, SIV has been endemic in the SM and RCM populations for much longer, although whether its entry predated separation of the populations is uncertain (26). The CCR5 mutation is currently not essential for protection from SIV-induced pathogenesis, but it is plausible that each of the mutations result from ancestral evolutionary pressure by pathogenic SIV infection. For SM, genetic abrogation of CCR5 expression may have been an additional, complementary response to control pathogenesis along with phenotypic CCR5 downregulation (50, 52). If so, in both SM and RCM the virus then acquired mechanisms to circumvent the restriction, by expanding coreceptor use for SIVsmm or switching for SIVrcm, yet the hosts then acquired other additional mechanisms to avoid pathogenesis. Alternatively, it may be that all three CCR5 mutations, human, SM and RCM, reflect evolutionary adaptation to some other as-yet unidentified infectious or other environmental factor acting similarly on all three types of primates. Nevertheless, whatever its origin and frequency, the SM CCR5Δ2 mutation here expands the pathways known to support infection in this important natural host model, the identity, distribution and utilization of which must be taken into account in understanding SM infection *in vivo,* and the similarities or differences from RM infection that determine outcome from infection.
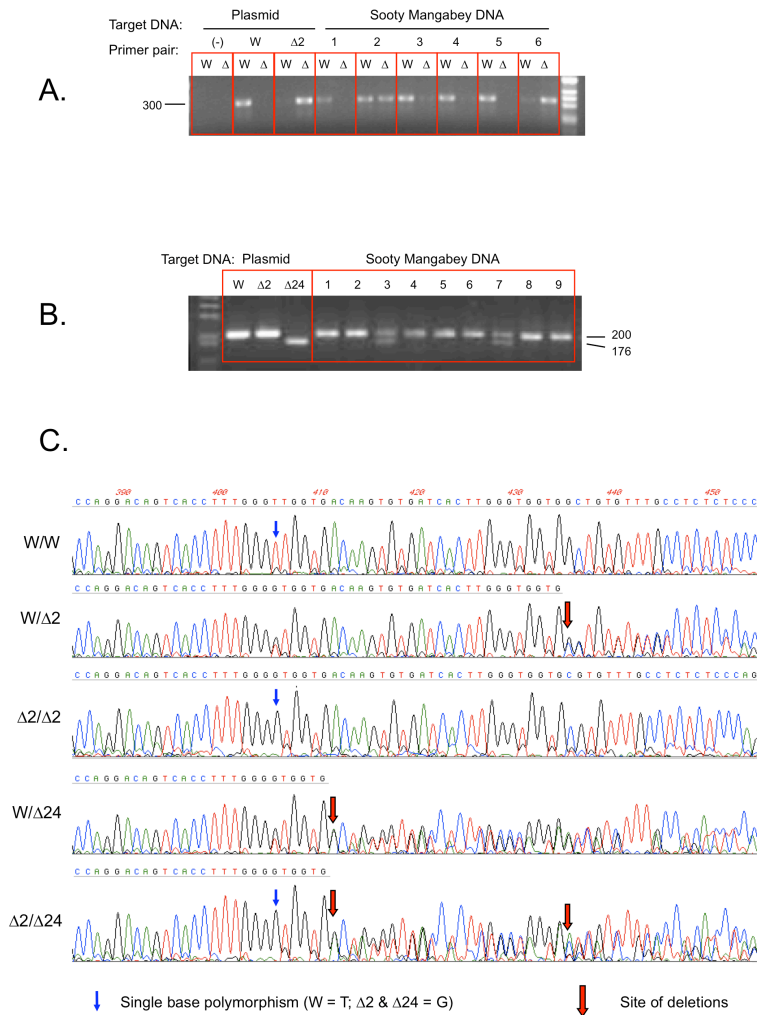
83

Figure 2-S1. Analysis of sooty mangabey CCR5 genotypes.
(A) Genomic DNA was analyzed by PCR in two separate reactions that contained primers specific for the wild-type or Δ2 CCR5 alleles. (B) Genomic DNA was amplified with CCR5-specific primers that generate an amplicon of 227-229 bp for the Δ2 and wild-type alleles, or 205 bp for the Δ24 allele. Note that animals 1-6 in panel A do not correspond to numbers in panel B. (C) Direct sequence validation of genotypes is shown in representative chromatographs of animals from each of the 5 genotypes identified in this analysis: W/W, W/Δ2; Δ2/Δ2; W/Δ24 and Δ2/Δ24. Red arrow indicates the site of Δ2 or Δ24 frameshifts and blue arrow indicates a single nucleotide polymorphism (T in wild-type; G in Δ2 and Δ24 alleles) that results in a coding change (Leu in wild-type; Val in Δ2 and Δ24 alleles).
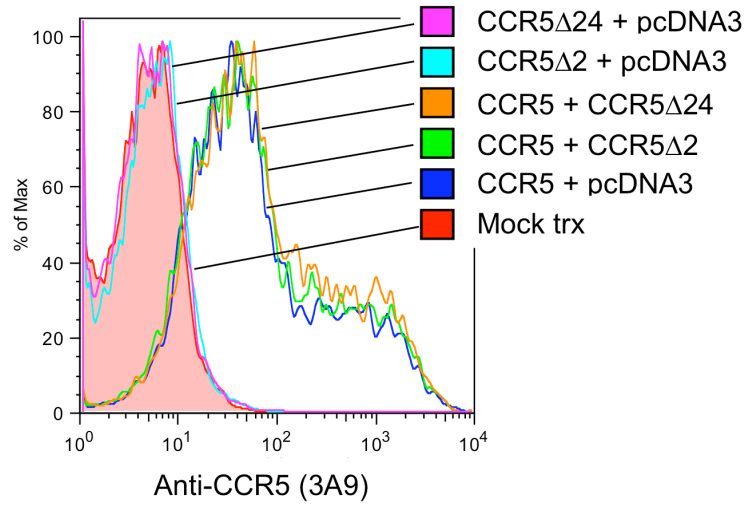
Figure 2-S2. Lack of dominant negative effect of mutant CCR5 alleles.
293T cells were transfected with wild-type smCCR5 plasmid (1 ug) along with plasmids encoding CCR5Δ2, CCR5Δ24 or pcDNA3 (1 ug of each plasmid). CCR5 expression was determined by staining with mAb 3A9 and FACS analysis.
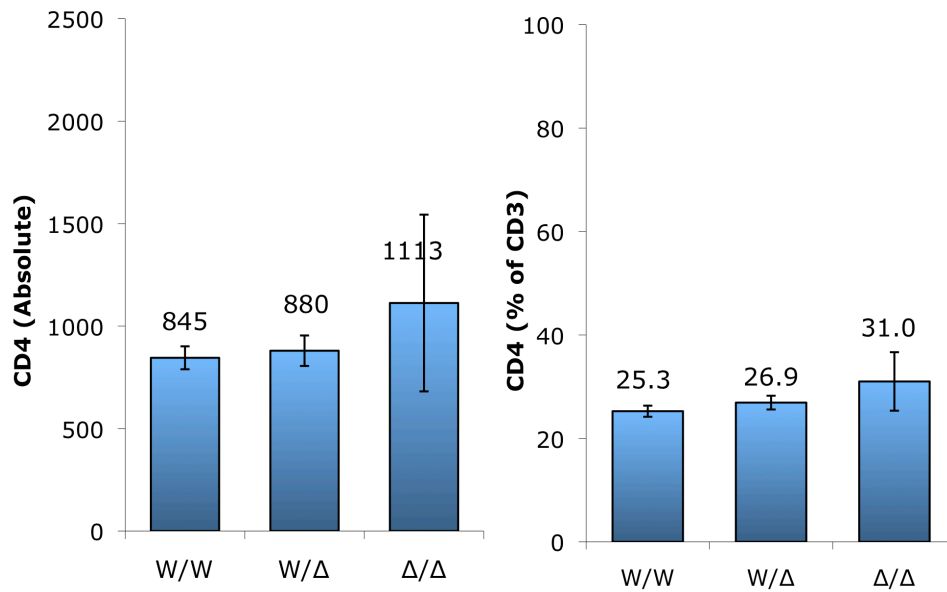
Figure 2-S3. Blood CD4+ T cell levels in infected sooty mangabeys between genotype groups.
(A) CD4+ T cell counts (cells/ul; mean ± SEM; left) and (B) CD4+ T cell as a percentage of CD3+ cells (mean ± SEM; right) from infected animals in the wild-type (n=60), heterozygote (n=49) and homozygous mutant (n=7) genotype groups. CD4+ T cells are not significantly different between groups (p=ns for absolute cell counts by Kruskal-Wallis test; p=ns for %CD4+ cells by ANOVA).
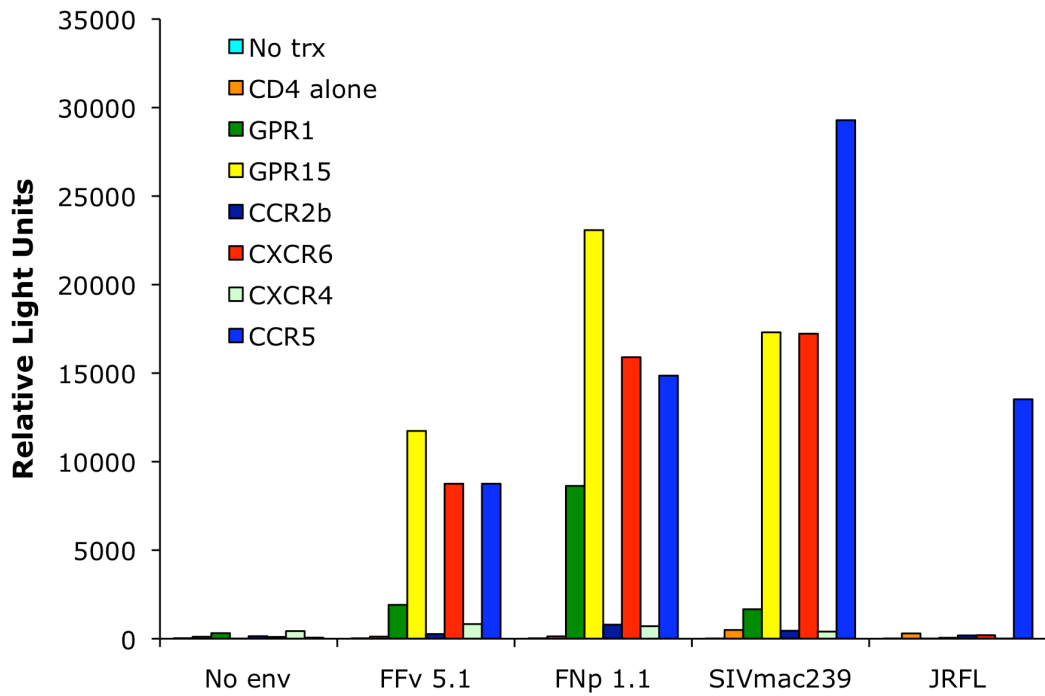
Figure 2-S4. Relative use of alternative coreceptors compared with CCR5.
Representative experiment in which CCR5 and alternative coreceptors were tested in parallel with
a subset of SIVsmm Env pseudotype viruses.

Acknowledgement

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CHAPTER III

CXCR6 AND GPR15 EXPRESSION:
PRELIMINARY EXAMINATION ON HUMAN PBMC SUBSETS

Abstract

Understanding the virus/host relationship in natural hosts will enable better understanding of pathogenic HIV infection of humans. SIV infection of sooty mangabey (SM) natural hosts does not cause disease, despite robust virus replication. SIV uses CCR5 for entry into target cells, but SM and other natural hosts express very low CCR5 levels on CD4+ T cells. I have shown that SIVsmm can utilize non-CCR5 entry pathways in the SM natural host, *in vivo*. Based on *in vitro* infection assays, we hypothesized that CXCR6 and GPR15 serve as alternative SIV entry coreceptors *in vivo*. Ultimately, we wish to determine whether alternative coreceptor expression patterns in SM effects target cell tropism in these animals. Here, I sought to examine the expression patterns of CXCR6 and GPR15, in addition to CCR5 on primary human cells using multi-color flow cytometry. I show that on human CD4 T cells, CXCR6 and GPR15 are: i) predominantly expressed on memory cell subsets, ii) detected to some extent on effector cells, and iii) present at extremely low levels on naïve cells. On lymphocytes, the percentage of cells expressing CCR5 and CXCR6 increased upon CD3/CD28-stimuation, whereas the percentage of GPR15-expressing cells did not change. A large percentage of resting and stimulated CD4+ memory cells expressed CCR5 alone, while a similar percentage of CD4+ memory cells co-expressed CCR5 and CXCR6 upon stimulation. Notably, co-expression of CXCR6 and GPR15 was extremely rare. This work serves as a framework for future studies comparing cell subset specific expression patterns in SM and non-natural hosts.

Introduction

Several chemokine receptors and chemokine receptor-like orphan receptors serve as entry coreceptors, along with CD4, for HIV/SIV. *In vivo*, CCR5 and CXCR4 are the primary entry coreceptors used by HIV while CCR5 was long thought to serve as the main coreceptor for SIV (1-4, 6, 7, 16). Numerous studies report that SIV has a broad coreceptor range *in vitro*, and has been shown to utilize CXCR6 (Bonzo/STRL33), GPR15 (BOB), GPR1, ChemR23 and APJ for entry (8, 9, 19-21, 25). However, SIV use of alternative coreceptors was considered an *in vitro* phenomenon, with the notion that CCR5 served as the exclusive entry coreceptor, *in vivo*. My identification of SIVsmm-infected CCR5-null SM indicated otherwise. This observation indicates that SIVsmm can utilize alternative coreceptors for transmission and replication in CCR5-null SM, *in vivo* (22), and that the expression and distribution of alternative coreceptors may define novel cell subsets that are more expendable allowing for high virus replication without causing disease.

This finding raises many questions: i) which alternative coreceptors mediate SIV entry *in vivo*, ii) what primary cell subsets express alternative coreceptors, iii) is alternative coreceptor use by SIV seen in other natural host species besides SM, and iv) does alternative coreceptor expression differ between natural hosts, that have presumably evolved to avoid pathogenesis, and non-natural hosts, which have not? Based on *in vitro* entry assays by myself and others, I hypothesize that CXCR6 and GPR15 are likely entry coreceptors used by SIVsmm (8, 22). Many of these studies used human-derived alternative coreceptors, however, a recent study from our lab confirmed that SM-derived

97

CXCR6 and GPR15 support efficient SIV entry in a transfection-based assay system suggesting that these alternative coreceptors may support SIVsmm entry *in vivo* (10).

Since a SM-specific anti-CXCR6 antibody is not available, we set out to first define CXCR6 and GPR15 expression profiles on human primary cells. CXCR6 is the receptor for CXCL16 (17), while GPR15 is an orphan receptor for which the natural ligand is not yet known (13). Although, the expression profile of CXCR6 on human primary cells has been reported, the findings are conflicting. One group reported that CXCR6 expression is mainly detected on naïve CD4+ T cells as well as natural killer (NK) cells and B lymphocytes (25), while another group reported CXCR6 expression is restricted to memory subsets of CD4+ and CD8+ T cells (26). We attempted to clarify CXCR6 expression profiles on resting and stimulated human PBMC subsets. The other coreceptor of interest is GPR15; although not much expression data is available, GPR15 mRNA has been detected in human alveolar macrophages and CD4 lymphocytes as well as in activated RM PBMC (5, 11). Additionally, a recent study revealed low level GPR15 expression on resting and stimulated human PBMC (14).

The current study will allow us to compare CXCR6 and GPR15 expression patterns, in addition to CCR5, on human CD4+ T cell subsets. This work serves as a framework for future studies comparing CXCR6 and GPR15 expression patterns in SM, which we hypothesize evolved to restrict expression to dispensable cell types. In addition to implications for SIV, understanding alternative coreceptor expression patterns on human cells is important because i) while rare, HIV alternative coreceptor use has been described, and ii) with CCR5 knock-out efforts, alternative coreceptor use might become an issue.

98

Materials and Methods

Isolation and stimulation of primary human cells

Fresh human PBMC were obtained from normal donors from the University of

Pennsylvania Immunology core. Human PBMC from a single donor were divided into

two equal groups. Ten million cells were rested overnight at 37°C, and the remaining

cells were stimulated for three days in the presence of plate-bound CD3/CD28 antibodies.

Three days post-antibody stimulation, recombinant IL-2 was added to cells for four

additional days in the continued presence of CD3/CD28 antibodies.

Staining procedure

Prior to antibody stimulation, cells were stained with Live/Dead Dye according to the

manufacturer's instructions. On appropriate days, PBMC were stained with antibodies

for the following: (i) lineage markers - CD3 (APC-Cy7), CD4 (FITC), CD8 (PE-Texas

Red) and CD14, (ii) memory markers - CD27 (PE-Cy7) and CD45RO (PE-Cy5), and (iii)

coreceptors - CCR5 (PE), CXCR6 (APC) and GPR15 (PerCP-Cy5.5). Cells were stained

at 4ºC in the dark for 30 minutes. After incubation, cells were washed with 1ml FACS

buffer (PBS + 1% FBS + 0.1% sodium azide) three times. Cells were fixed with 0.4ml

2% paraformaldehyde in FACS buffer.

Analysis

Approximately, 2 x 10^5 cells were acquired on an LSR-II, and data was analyzed using

FloJo software. As shown in **Figure 3-1**, cells were gated based on characteristic light

scatter properties followed by the selection of live cells (**Figure 3-1; A,B**). Cells were

then separated based on CD3 and CD14 expression (**C**), and CD3-positive cells were divided based on expression of CD4 and CD8 (**D**). CD3+CD4+ cells were then divided into naïve and memory cell subsets based on CD45RO and CD27 co-expression (**E**). Lastly, coreceptor expression levels were measured on each CD4 T cell subset (naïve, CD27+ memory, CD27- memory and effector cells).

Quantitative PCR – Coreceptor mRNA levels

Primary PBMC from SM were left unstimulated or cultured in the presence of Concanavalin A and IL-2 for up to 5 days. CD4+ cells were sorted from PBMC at 0 and 120 hours post-stimulation using negative selection. Total RNA was isolated from purified CD4) cells using the RNAeasy extraction kit (Qiagen) according to the manufacturer's instructions. Quantification of mRNA levels for all coreceptors tested was performed by RT-PCR using the SYBR-Green assay. Levels of CCR5, CXCR6, GPR15, GPR1 and APJ mRNA were normalized to the expression of the housekeeping mRNA, GAPDH.

<u>**Results**</u>

CXCR6 and GPR15 are detected on CD4+ and double negative (CD4-/CD8-) T cells; CXCR6 are detected on CD8+ T cells

I aimed to define the expression profile of CXCR6 and GPR15 on human primary cells; these receptors have been shown to support SIV entry *in vitro*. I first determined the expression levels of CXCR6 and GPR15 on various T cell populations. For the sake of comparison and since CCR5 expression levels are well established on human cells, we also measured CCR5 expression on various T cell populations. On resting lymphocytes, the average percentage of cells expressing CXCR6 was 1.3% on CD4+ and CD8+ T cells, and 5.5% on DN T cells. On average, GPR15 expression was detected on 2.4% CD4+ T cells and 12.2% DN T cells; GPR15 expression was extremely low to undetectable on resting CD8+ T cells. The average percentage of resting cells expressing CCR5 was 8.6% on CD4+ T cells, 22.3% on CD8+ T cells and 63.2% on DN T cells. Flow plots from a representative donor are shown in **Figure 3-2**. In all, these data demonstrate that CXCR6 and GPR15 expression is detected on a small percentage of resting lymphocytes.

I next examined CXCR6 and GPR15 expression on CD3/CD28-stimulated cells from matching donors. On stimulated lymphocytes, the average percentage of cells expressing CXCR6 was 8.5% on CD4 T cells, 4.2% on CD8 T cells and 13.1% on DN T cells. On average, GPR15 expression was detected on 5.1% CD4 T cells and 12.2% DN T cells; GPR15 expression was also extremely low on stimulated CD8 T cells. The average percentage of stimulated lymphocytes expressing CCR5 was 18.8% on CD4 T cells, 13.3% on CD8 T cells and 33.9% on DN T cells. These results show that

101

CD3/CD28-stimulation increased the percentage of cells expressing CXCR6 and GPR15, as well as CCR5 (**Figure 3-2**). Percentages of CXCR6, GPR15 and CCR5 expression on resting and stimulated cells from both donors are listed in **Table 3-1**.
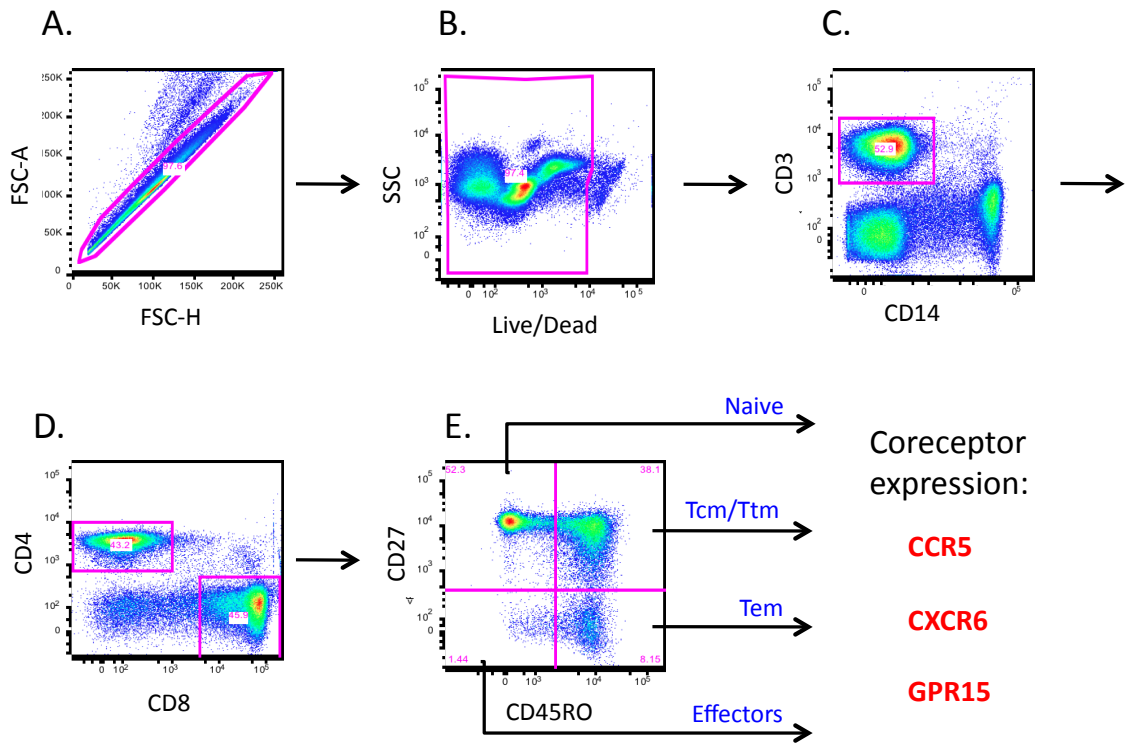
Figure 3-1. Gating strategy for analysis of coreceptor expression levels on total CD4 T cells and CD4 T cell subsets.
Representative FACS plots showing the successive gating strategy used on unstimulated (rested) human PBMC stained for lineage markers (CD3, CD4, CD8 and CD14), memory markers (CD45RO and CD27), and coreceptors of interest (CCR5, CXCR6 and GPR15). Live cells were separated using a Live/Dead stain. Gated populations (cells within pink gated region) from one FACS plot are the total cells shown in the subsequent FACS plot. This gating strategy was used to determine coreceptor expression on total CD4 T cells (plots A – D) and CD4 T cell subsets (plots A – E). The percentage of cells in a particular gated area or quadrant are located at the top right-hand corner of the plot.
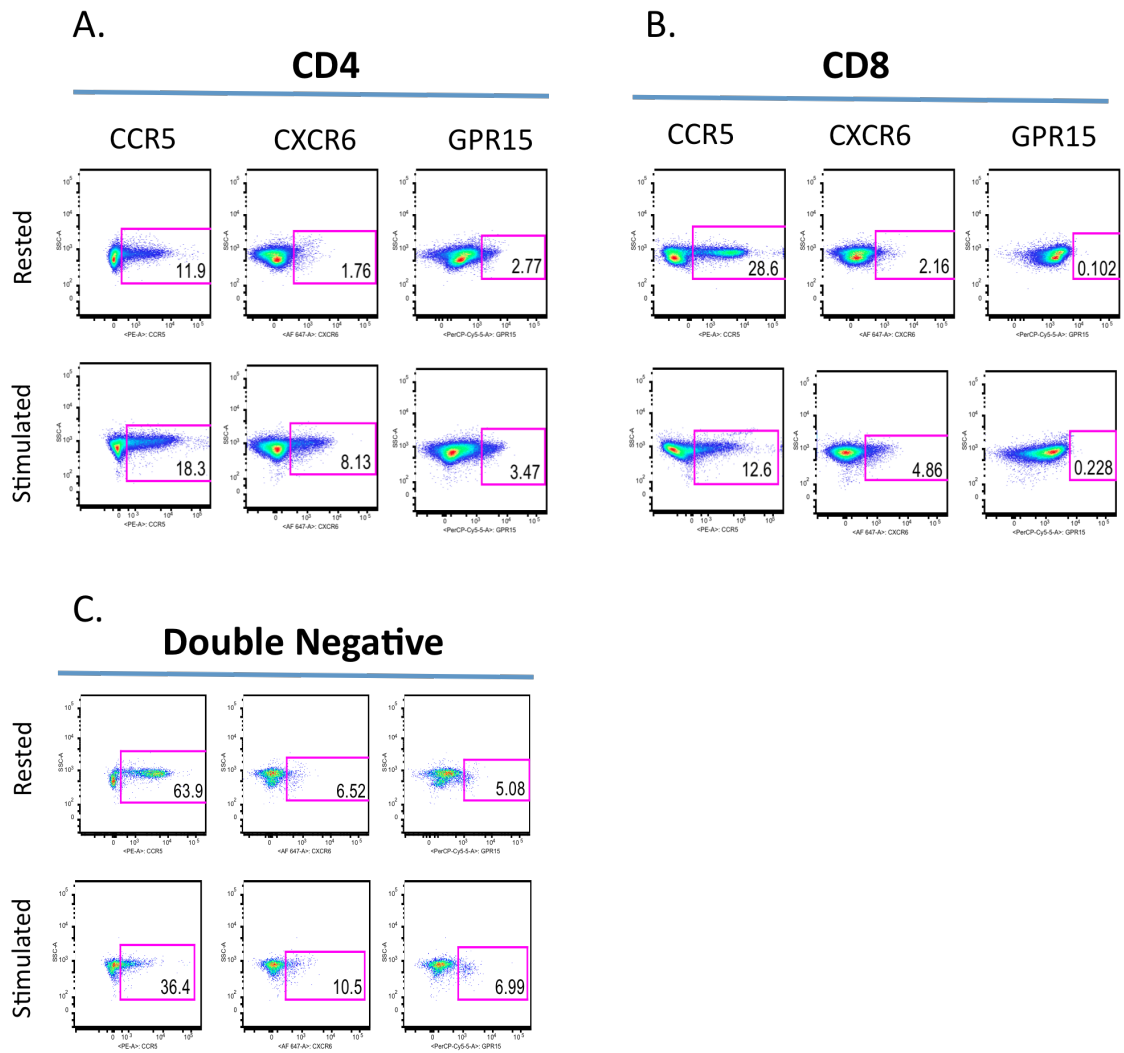
Figure 3-2. CCR5, CXCR6 and GPR15 expression levels on rested and stimulated human CD4, CD8 and double negative (CD4-CD8-) T cells.
Resting (overnight incubation at 37°C) and stimulated (one week incubation: three days in the presence of CD3/CD28 antibodies followed by four days in the presence of IL-2) human PBMC were stained with lineage markers (CD3, CD4, CD8 and CD14), memory markers (CD45RO and CD27), and coreceptors of interest (CCR5, CXCR6 and GPR15). The percentage of cells expressing each coreceptor of interest on total A) CD4, B) CD8 and C) double negative (CD4-/CD8-) T cells is displayed in each FACS plot. A combination of florescence minus one (FMO), isotype-matched antibodies and staining with secondary antibody alone served as negative controls (data not shown). Representative data set from one of two donors.

CCR5				
	Stimulation	**CD4**	**CD8**	**DN**
ND383	None	5.2	15.8	62.4
	CD3/CD28	19.2	13.8	31.4
ND366	None	11.9	28.8	63.9
	CD3/CD28	18.3	12.8	36.4

CXCR6				
	Stimulation	**CD4**	**CD8**	**DN**
ND383	None	0.8	0.5	4.6
	CD3/CD28	8.8	3.6	15.7
ND366	None	1.8	2.2	6.5
	CD3/CD28	8.1	4.9	10.5

GPR15				
	Stimulation	**CD4**	**CD8**	**DN**
ND383	None	2.0	0.3	8.4
	CD3/CD28	6.7	0.9	17.5
ND366	None	2.8	0.1	5.1
	CD3/CD28	3.5	0.2	7.0

Table 3-1. Percentage of CCR5, CXCR6 and GPR15 expression on resting and stimulated human PBMC cell subsets

CXCR6 and GPR15 are predominantly expressed on CD4+ memory cell subsets; CXCR6 and GPR15 expression is extremely low on CD4+ naïve T cells

Since cells expressing CD4 are potential targets for HIV/SIV, we wanted to determine the expression levels of CXCR6 and GPR15, in addition to CCR5, on CD4 T cell subsets. CXCR6, GPR15 and CCR5 expression is extremely low (~1.0%) on naïve CD4 T cells (CD45RO-/CD27+) on both resting and CD3/CD28-stimulated cells (**Figures 3-3 & 3-4**). Similar to CCR5 expression, CXCR6 and GPR15 were both detected primarily on CD45RO+/CD27+ memory cells (predominantly central memory cells; Tcm), CD45RO+/CD27- memory cells (primarily effector memory cells; Tem) and effector (CD45RO-/CD27-) cell subsets, although CXCR6 and GPR15 expression was relatively lower than CCR5 expression on all subsets examined.

Both CXCR6 and CCR5 expression levels are higher on CD45RO+/CD27- memory cells (primarily Tem) than CD45RO+/CD27+ memory cells (predominantly Tcm), and expression of these coreceptors is upregulated upon CD3/CD28 antibody stimulation. The percentage of CXCR6-expressing cells increased from an average of 1.3% to 10.0% on resting versus stimulated CD45RO+/CD27+ memory cells (predominantly Tcm), respectively, while CCR5-expressing cells increased from an average of 12.9% to 24.7%, respectively. The average percentage of CD45RO+/CD27+ cells expressing GPR15 modestly increased from 3.9% on resting cells to 6.5% on stimulated cells. On CD45RO+/CD27- memory cells (mostly Tem), CD3/CD28-stimulation increased CXCR6 and CCR5 expression. On average, the percentage of CXCR6-expressing cells increased from 3.1% on resting cells to 35.8% on stimulated cells, while the average percentage of CCR5-expressing cells increased from 34% to 60%

on resting and stimulated cells, respectively. The average percentage of GPR15-

expressing cells increased from 5.2% on resting cells to 11.6% on stimulated cells.

However, increased GPR15 expression upon stimulation was not observed in both donors

examined. One donor did not show in increase in GPR15 expression on resting versus

stimulated CD45RO+/CD27- cells (6.3% to 5.7%, respectively), while the other donor

did (4.3% to 17.4%, respectively). Overall, these results suggest that CXCR6 and GPR15

are predominantly expressed on memory cell subsets (CD45RO+/CD27+ and

CD45RO+/CD27-) and are expressed at extremely low levels on naïve CD4 T cells. The

percentage of cell expressing CXCR6, GPR15 and CCR5 from various cell subsets are

displayed in **Figures 3-3** and **3-4**. In addition to CD3/CD28 antibody stimulation, cells

were also stimulated with phytohemagglutinin (PHA), which yielded similar results in

that most coreceptor expression was detected on memory cell subsets (**Table 3-2**).
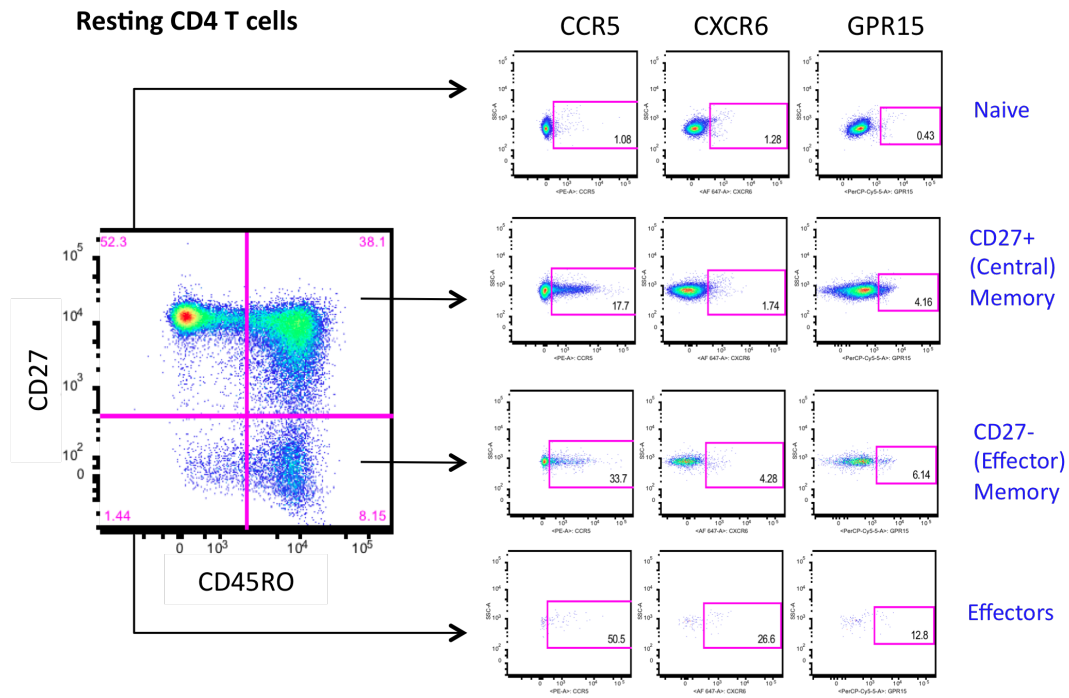
Figure 3-3. CCR5, CXCR6 and GPR15 expression levels on rested CD4 T cell subsets.
Resting human PBMC were stained as previously described in Figure 3-1. Total CD4 T cells
were divided into naïve, memory and effector cell subsets based on combinations of CD45RO
and CD27 co-expression (plot on far left). Arrows point from each quadrant to corresponding
FACS plot of coreceptor expression for that particular subsets. For each subset, the percentage of
cells expressing a particular coreceptor, CCR5 (left), CXCR6 (middle) and GPR15 (right), is
displayed in the FACS plot. Representative data set from one of two donors.

Figure 3-4. CCR5, CXCR6 and GPR15 expression levels on CD3/CD28-stimulated CD4 T cell subsets.
CD3/CD28-stimulated human PBMC were stained as previously described in Figure 3-1. Total CD4 T cells were divided into naïve, memory and effector cell subsets based on combinations of CD45RO and CD27 co-expression (plot on far left). Arrows point from each quadrant to corresponding FACS plot of coreceptor expression for that particular subsets. For each subset, the percentage of cells expressing a particular coreceptor, CCR5 (left), CXCR6 (middle) and GPR15 (right), is displayed in the FACS plot. Representative data set from one of two donors.
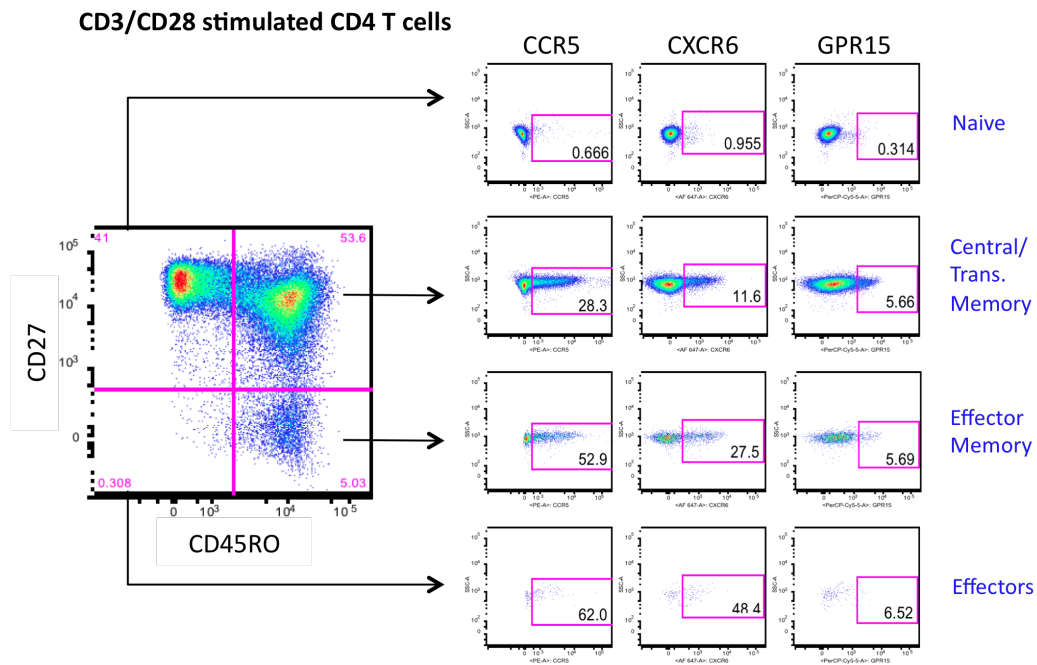
CCR5					
	Stimulation	Total	Naïve	Central mem (CD27+)	Effector mem (CD27-)
ND147	None	**13.8**	2.0	23.7	58.6
	PHA	**22.9**	6.1	29.9	49.1
ND383	None	**5.2**	0.8	8.0	34.3
	CD3/CD28	**19.2**	0.9	21.0	67.0
ND366	None	**11.9**	1.1	17.7	33.6
	PHA	**7.3**	2.3	7.0	39.0
	CD3/CD28	**18.3**	0.7	28.3	52.9

CXCR6					
	Stimulation	Total	Naïve	Central mem (CD27+)	Effector mem (CD27-)
ND147	None	**0.5**	0.8	0.5	1.1
	PHA	**1.1**	1.1	0.9	3.6
ND383	None	**0.8**	0.5	0.9	1.9
	CD3/CD28	**8.8**	1.9	8.3	*44.0*
ND366	None	**1.8**	1.3	1.7	4.3
	PHA	**3.3**	2.7	3.0	19.9
	CD3/CD28	**8.1**	1.0	11.8	27.5

GPR15					
	Stimulation	Total	Naïve	Central mem (CD27+)	Effector mem (CD27-)
ND147	None	**2.8**	1.5	6.5	2.6
	PHA	**2.8**	1.4	3.3	8.6
ND383	None	**2.0**	0.6	3.5	4.3
	CD3/CD28	**6.7**	1.3	7.4	17.4
ND366	None	**2.8**	0.4	4.2	6.1
	PHA	**1.1**	0.4	1.1	5.1
	CD3/CD28	**3.5**	0.3	5.7	5.7

Table 3-2. Percentage of CCR5, CXCR6 and GPR15 expression on resting and stimulated (PHA and CD3/CD28) CD4 T cell subsets.

CXCR6/CCR5 co-expression increases dramatically upon CD3/CD28-stimulation; CXCR6/GPR15 co-expression is rare on resting and stimulated CD4+ T cells

Although HIV use of alternative coreceptors is rare *in vivo*, we wanted to determine if CXCR6 and GPR15 are co-expressed on human cells expressing CCR5. Furthermore, it will be interesting to ultimately compare and contrast co-expression profiles of CXCR6, GPR15 and CCR5 between natural and non-natural hosts. We hypothesize that alternative coreceptor expression profiles on natural host primary cells may reveal novel target cell subsets that lack CCR5 yet express CXCR6 and/or GPR15, which may support viral entry and replication, *in vivo*. Here we are studying human cells, which have not evolved under such pressure so CXCR6 and GPR15 may be expressed predominantly on CCR5-expressing cells.

We examined the co-expression of CXCR6, GPR15 and CCR5 on CD4+ memory subsets (CD45RO+/CD27+ and CD45RO+/CD27-) because memory subsets expressed most robust levels of each coreceptor alone (and effector cells were rare). We first determined the percentage of cells expressing CXCR6, GPR15 and CCR5 alone on resting and CD3/CD28-stimulated cells. On resting CD45RO+/CD27- memory cells, roughly 30% of cells expressed CCR5 alone, ~1% cells expressed only CXCR6 and 2% of cells expressed GPR15 alone. Notably, the percentage of CD3/CD28-stimulated cells expressing CCR5 or GPR15 were similar to expression levels seen on resting cells while CXCR6 expression increased to roughly 3% on stimulated CD45RO+/CD27- memory cells (**Figure 3-5A**).

The percentage of cells expressing at least one coreceptor substantially increased upon CD3/CD28-stimulation. This increase in coreceptor expression can be appreciated

by a decrease in the total percentage of coreceptor-negative CD45RO+/CD27- memory cells (primarily effector memory cells) upon stimulation. When examining coreceptor co-expression on this subset, the most dramatic effect was in cells expressing both CXCR6 and CCR5. CXCR6/CCR5+ cells increased substantially upon stimulation from 2.5% on resting cells to ~30% on stimulated cells. Less than 5% of resting and stimulated CD45RO+/CD27- memory cells were positive for both GPR15 and CCR5, with a modest increase in co-expression of these two coreceptors on stimulated cells. Surprisingly, CXCR6 and GPR15 co-expression was not detected on resting CD45RO+/CD27- memory cells, and only ~1% of stimulated cells were positive for these receptors. Additionally, on average, 5% of stimulated CD45RO+/CD27- memory cells express all three coreceptors, which is considerably higher than the percentage of triple positive resting cells (**Figure 3-5A**). Thus, CD3/CD28 stimulation leads to a large increase in CD45RO+/CD27- (Tem) cells co-expressing CXCR6 and CCR5.

Examination of coreceptor expression profiles on CD45RO+/CD27+ memory cells, reflecting predominantly Tcm cells, revealed similar co-expression trends as seen on CD45RO+/CD27- memory cells (primarily Tem cells). Approximately, 85% of resting CD45RO+/CD27+ memory cells lacked CXCR6, GPR15 and CCR5, while the largest proportion of coreceptor-expressing cells were singly positive for CCR5 and GPR15 (**Figure 3-5B**). Stimulation led to upregulation of coreceptor expression on CD45RO+/CD27+ memory cells, however the overall percentage of CXCR6, GPR15 or CCR5-expressing CD45RO+/CD27+ memory cells was considerably lower than the percentage of CD45RO+/CD27- memory cells expressing these coreceptors. As seen in CD45RO+/CD27- memory cells, a large proportion of stimulated CD45RO+/CD27+

memory cells expressed CCR5 alone or CXCR6 in combination with CCR5. Of note,

CXCR6 and GPR15 co-expression was not detected on resting or stimulated

CD45RO+/CD27+ memory cells, and less than 1% of these cells expressed all three
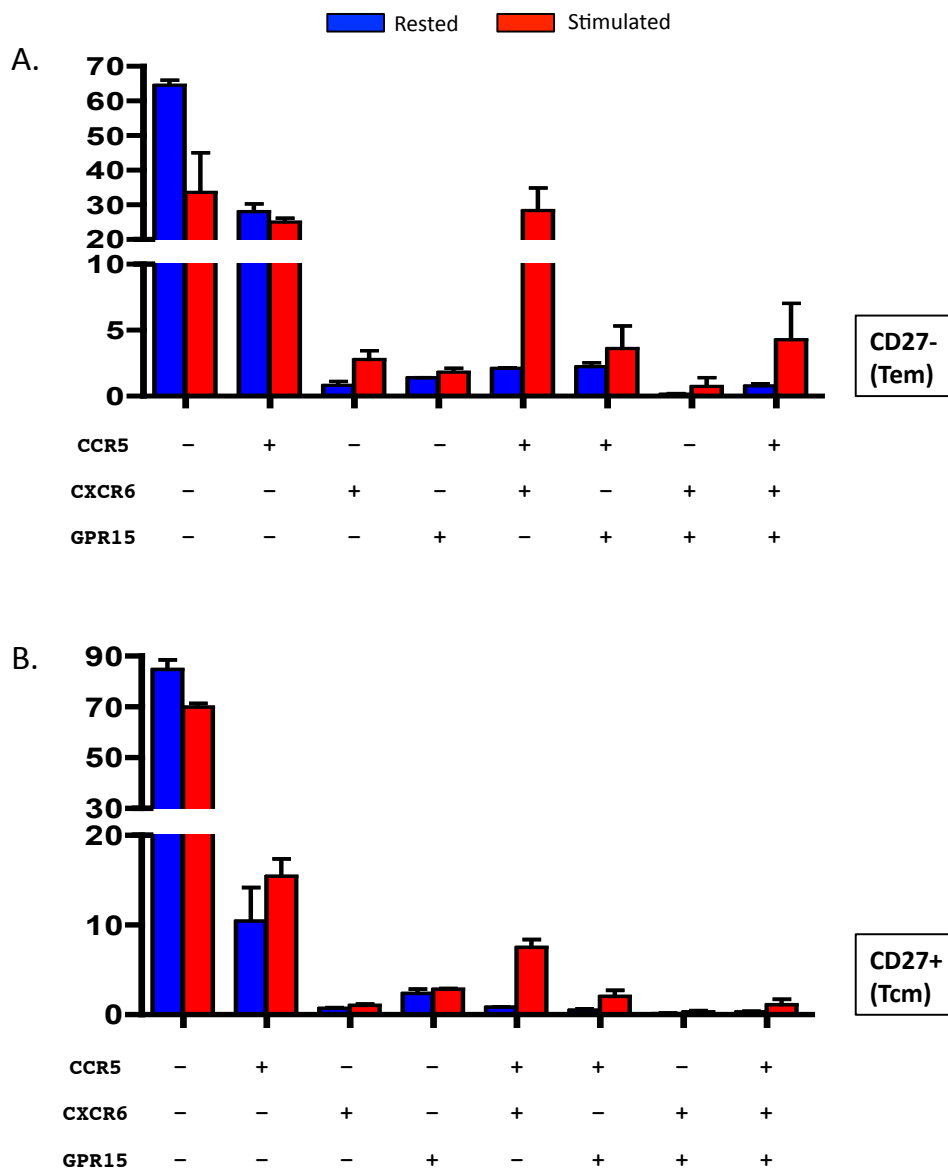
coreceptors (**Figure 3-5B**).

Figure 3-5. Co-expression of CCR5, CXCR6 and GPR15 on resting and stimulated CD4 T cell subsets.
Resting (blue columns) and stimulated (CD3/CD28; red columns) human PBMC were stained and analyzed, gating on CD3+, CD4+ CD45RO+ cells as previously described in Figure 3-1. Co-expression of CCR5, CXCR6 and GPR15 on A) CD27- (primarily Tem) and B) CD27+ (primarily Tcm) subsets was determined using Boolean gating. For each cell subset, the mean percentage of coreceptor expression for all single and multiple combinations are shown (mean + SEM; n=2).

114

CXCR6 and GPR15 mRNA levels are relatively higher than CCR5 mRNA levels in resting and stimulated SM CD4 cells

My work (Chapter 2) showed CCR5-independent SIVsmm infection *in vivo* and in primary SM cells *ex vivo*, and that human-derived CXCR6 and GPR15 support robust SIVsmm entry *in vitro*. A follow-up study from our lab showed that SM-derived CXCR6 and GPR15 support SIV entry *in vitro* (10). We hypothesize that these alternative coreceptors are likely used *in vivo*. Antibodies to SM-CXCR6 are not currently available, therefore we sought to determine the mRNA levels of these alternative coreceptors on SM CD4+ cells using quantitative PCR. As shown in **Figure 3-6**, CXCR6 and GPR15 mRNA levels are relatively high compared to CCR5, GPR1 and APJ mRNA levels in unstimulated SM CD4+ cells. Of note, GPR1 and APJ are additional alternative coreceptors that have been shown to mediate SIV entry *in vitro*. Furthermore, a decrease in CCR5, GPR1 and APJ mRNA levels occurred in SM CD4+ cells stimulated with concanavalin A, while the average mRNA levels of CXCR6 and GPR15 remained the same. However, there is considerable variation in CXCR6 and GPR15 mRNA levels between individual animals tested (**Figure 3-6**). Although mRNA levels do not directly predict protein expression levels, these results in SM primary cells are consistent with our hypothesis that CXCR6 and GPR15 may act as entry coreceptors *in vivo* in addition to CCR5.
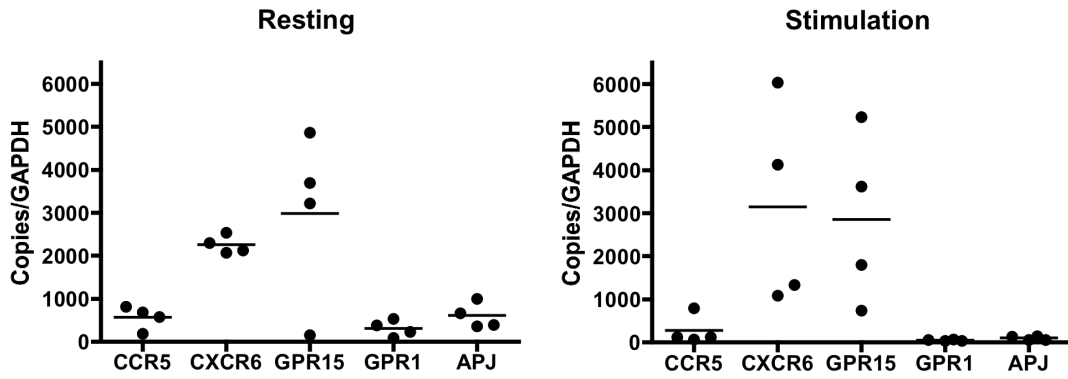
Figure 3-6. Coreceptor mRNA levels in resting and stimulated sooty mangabey CD4+ cells. Purified SM CD4+ cells were left untreated (resting) or stimulated with concanavalin A for five days at 37°C. Coreceptor-specific primers were used to amplify cDNA derived from each cell source to determine mRNA levels using quantitative PCR. Plasmid DNA containing each coreceptor of interest was used to create a standard curve. GAPDH was used as an internal control and coreceptor copy numbers were plotted relative to GAPDH mRNA levels. (Data from N. Francella, unpublished)

Discussion

Chemokine receptors other than CCR5 and CXCR4 can support SIV, and to a lesser extent HIV, entry *in vitro*. SIV use of alternative coreceptors such as CCR2, CCR4, CCR8, CX3CR1, APJ, GPR1, GPR15 and CXCR6 has been described extensively (5, 8, 19, 23, 24), and most SIV strains can propagate in human T cell lines that do not express CCR5 as well as in CCR5-null (Δ32 homozygous) primary human lymphocytes (2, 12). Since CXCR4 use by SIV is extremely rare, these data suggest that non-CCR5 pathways can mediate SIV infection *ex vivo*. HIV primary isolates have been shown to use alternative coreceptors for entry, although in most cases HIV use is far less robust than SIV use (25). Much of the evidence on alternative coreceptor use came from *in vitro* infection assays, and little is known about alternative coreceptor use or expression patterns *in vivo*.

Our lab recently published the first piece of conclusive evidence that non-CCR5 pathways support robust SIV transmission and replication *in vivo*. I identified a novel mutant SM-CCR5 allele (Δ2), and genotyped a large cohort of SM to determine the prevalence of mutant SM-CCR5 alleles (Δ2 and Δ24), which led to the identification of a group of CCR5-null animals. Surprisingly, half of the CCR5-null SM were naturally infected with SIVsmm. This observation revealed that alternative coreceptors support SIV transmission and replication *in vivo*, indicating that these receptors must be expressed on SM CD4+ cells. I then showed that CXCR6 and GPR15 (of human origin) serve as efficient SIVsmm entry pathways *in vitro* (22). A follow-up study from our lab showed that SM-derived CXCR6 and GPR15 supported SIV entry as efficiently as CCR5 in a pseudotype infection assay (10). Based on these findings, we sought to study the

117

expression profiles of CXCR6 and GPR15 on human and SM primary cells. I first set out to examine the expression profile of CXCR6, GPR15 and CCR5 on various human cell populations since there is no anti-CXCR6 antibody currently available that cross-reacts with the SM molecule.

In human primary cells, I found CXCR6 and CCR5 expression detected on CD4+, CD8+ and DN T cells, whereas GPR15 expression was detected on CD4+ and DN T cells, but not on CD8+ T cells. Since CD4+ T cells are the target cells of HIV/SIV, we sought to investigate the expression patterns of CXCR6 and GPR15, with that of CCR5, on resting and stimulated CD4+ T cell subsets. Functional CD4+ T cell subsets (naïve/memory/effectors) can be distinguished based on the expression of a number of surface antigens such as CD45RA, CD45RO, CD27, CD62L and CCR7. In our study, specific combinations of CD45RO and CD27 expression were used to define naïve, memory and effector cell subsets. Of note, CD27+ memory cells (CD45RO+) are comprised primarily of central memory cells while CD27- memory cells are predominantly made up of effector memory cells.

Chemokine receptor expression patterns are important for determining viral tropism. It is well established that CCR5 is restricted to memory and effector subsets on CD4+ T cells, which is consistent with depletion of CD4+ memory cells in mucosal tissues during acute SIV infection (18). It is likely that memory CD4+ T cells are preferentially targeted by HIV because they express high CCR5 levels and exist in a relatively activated state. Here we found that like CCR5, CXCR6 and GPR15 are predominantly expressed on memory (CD27+ and CD27-) CD4+ T cell subsets, and are expressed at extremely low levels on naïve CD4+ T cells. Prior studies, reporting

118

CXCR6 expression patterns were discordant; one group described CXCR6 expression on naïve CD4+ T cells, B cells and a subset of natural killer (NK) cells, while another group showed CXCR6 expression is predominantly restricted to memory CD4+ and CD8+ T cells (25, 26). Our finding that CXCR6 expression is highly restricted to memory CD4+ T cells is consistent with the latter study.

Meanwhile, the regulation of CXCR6 has also been examined (15, 26). One study showed that the expression pattern and regulation of CXCR6 paralleled that of CCR5, and inversely correlated with that of CXCR4 (26). Our results corroborate these findings in regard to CXCR6/CCR5 co-expression. Like CCR5, CXCR6 expression is highly restricted to memory CD4+ T cells and is up-regulated upon CD3/CD28 stimulation. Interestingly, when examining co-expression of CXCR6, GPR15 and CCR5 on CD27+ and CD27- memory CD4+ T cells, the percentage of cells expressing both CXCR6 and CCR5 increased significantly upon stimulation. Notably, a small percentage of cells expressed CXCR6 alone while the majority of CXCR6+ cells were also CCR5+; CXCR6 and GPR15 co-expression was extremely rare.

The CD3/CD28 antibody stimulation method (plate-bound) used in this study resulted in the up-regulation of CXCR6, GPR15 and CCR5. An examination of the percentage of cells in each CD4 T cell subset revealed that while the percentage of CD27+ memory cells (mostly Tcm) increased upon stimulation (38.1% of resting cells; 53.6% of stimulated cells), a substantial proportion of cells sustained the naïve phenotype (41%) after stimulation. Although our findings show a considerable increase in CXCR6 and CCR5 expression using the CD3/CD28 plate-bound method, it is likely that up-regulation of coreceptor expression may have increased more dramatically if the cells

were more uniformly stimulated, such as through the use of CD3/CD28 beads. Notably,

PHA-administration provided a more homogenous stimulation resulting in 7.2% naïve

CD4 T cells compared to 41% naïve CD4 T cells after CD3/CD28 (plate-bound)

stimulation. However, PHA stimulation did not induce a substantial increase in

coreceptor expression (**Tables 3-1 and 3-2**). This finding indicates that coreceptor

regulation differs under different stimulation conditions.

Although alternative coreceptor use by HIV is rare, a recent study identified a

transmitted/founder virus that efficiently infected cell lines over-expressing GPR15,

FPRL and APJ (14). Based on our GPR15 expression patterns on human primary cells,

one could speculate that this particular HIV isolate would exhibit similar viral tropism as

an R5-tropic virus since CCR5 and GPR15 were predominantly expressed on human

memory CD4+ T cells.

Nevertheless, expression profiles on primary human cells may not be indicative of

expression on primary NHP cells, which emphasizes the importance of defining CXCR6

and GPR15 expression profiles, relative to that of CCR5, on primary SM cells. Using

quantitative PCR, we detected CXCR6 and GPR15 mRNA levels in SM CD4+ cells.

Notably, CXCR6 and GPR15 mRNA levels were relatively higher than CCR5 mRNA

levels in these cells. Despite this promising result, we do not know how mRNA levels

correspond to protein expression levels. However, detection of CXCR6 and GPR15 on

SM CD4+ cells is consistent with our hypothesis that alternative entry pathways mediate

SIV infection in CCR5-null SM.

These results provide a background for further studies in NHP. Once a SM anti-

CXCR6 antibody is available, it will be important to explore CXCR6 and GPR15

expression profiles on primary SM cells. Ultimately, it would be interesting to determine

if alternative coreceptor expression profiles differ between natural and non-natural hosts,

which may reveal novel target cell subsets that contribute to the non-pathogenic nature of

SIV in infected natural hosts.

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CHAPTER IV

DISCUSSION & CONCLUSIONS

Presently, one of the most important priorities in HIV/AIDS research is understanding why infected natural hosts remain healthy while SIV-infected RM, HIV-infected humans and SIVcpz-infected chimpanzees develop AIDS. Although enormous progress has been made in HIV/AIDS research over the past few decades, HIV infection of humans remains a significant global issue. The use of NHP models of AIDS has greatly enhanced our current knowledge of HIV pathogenesis, however, a comprehensive understanding of the mechanisms underlying non-pathogenicity seen in SIV-infected natural hosts is not completely understood.

Intriguingly, natural and non-natural hosts exhibit divergent responses to SIV infection. When SM and RM are inoculated with unpassaged SIVsmm, infection results in high viremia in both species. SIV-infected RM exhibited severe CD4 T cell depletion, persistent generalized immune activation, chronic proliferation of lymphocytes, and ultimately died from an AIDS-like disease, while SIV-infected SM displayed modest lymphocyte proliferation and remained disease-free (25). This study clearly demonstrated that the host dependent factors, rather than properties of the virus, play a central role in immunodeficiency in SIV-infected RM, and highlights the importance of fully investigating the underlying mechanisms responsible for non-pathogenicity in SIV-infected natural hosts. Many studies indicate key features of natural host SIV infection that are associated with lack of disease progression. Such features include: preferential sparing of critical cell subsets (i.e. central memory cells) from infection (1, 19), lack of chronic immune activation (3, 10, 17), maintenance of mucosal immunity (8), preservation of gut Th17 cells (4), and preservation of T cell homeostasis (18).

For this dissertation, I identified a novel, non-functional SM-CCR5 mutant allele (CCR5Δ2), and determined the prevalence of this allele in a large SM colony. Remarkably, animals homozygous for mutant CCR5 alleles were susceptible to SIV infection *in vivo*, indicating that non-CCR5 pathways support SIV transmission and replication in these CCR5-deficient animals. Moreover, this finding challenges the long-standing notion that CCR5 is the exclusive coreceptor used by SIV *in vivo*, and raises many questions regarding the role alternative entry pathways play in SIV infection of natural hosts.

In natural host SM, alternative coreceptor use in conjunction with restricted CCR5 expression may redirect infection to more expendable cell types, thus contributing to the maintenance of immune cell homeostasis (**Figure 4-1**). While SIVmac utilizes alternative coreceptors *in vitro*, CCR5 expression in RM is high and the role alternative entry pathways play, *in vivo,* is less clear (**Figure 4-2**). In addition to implications for SIV, alternative coreceptor expression and use may play a role in HIV infection of humans since i) while rare, HIV alternative coreceptor use has been described, and ii) with CCR5 knock-out efforts, acquisition of alternative coreceptor use by HIV may become an issue.

Regulation of cellular receptors required for HIV/SIV entry and viral susceptibility in CCR5-null hosts

SIV infection of multiple African NHP species is endemic and evidence suggests that natural hosts have been infected with SIV since ancient times, which has likely led to evolutionary adaptations in both virus and host. A potential host adaptation to SIV

infection is the regulation of receptors and/or coreceptors required for SIV entry in a manner that protects critical cells from infection. In AGM, selective downregulation of CD4 was observed on primary lymphocytes transitioning from a naïve to memory phenotype. Surprisingly, CD4-deficient memory cells maintained T cell functions, while the loss of the surface-bound CD4 molecule protected these cells from SIVagm infection *in vivo* (1).

Many natural host species (i.e. SM, AGM, sun-tailed monkeys and mandrills) have developed mechanisms to differentially regulate CCR5 on specific cell subsets. Natural hosts express extremely low levels of CCR5 on CD4+ T cells found in the peripheral blood and in mucosal tissues, however CD8+ T cells from these hosts, express considerably higher levels of CCR5 (21). Differential regulation of CCR5 expression is also exhibited on SM CD4+ central memory T cells, which express significantly lower levels of CCR5 than SM CD4+ effector memory T cells (19). Currently, the mechanisms underlying CCR5 regulation remain unknown. However, this observation raises the question, is low CCR5 expression on natural host CD4+ T cells an evolutionary adaptation in direct response to SIV infection?

In contrast to intrinsic regulation of CCR5, CCR5 genetic polymorphisms that abrogate surface expression have been identified in different host populations. In humans, a 32 base-pair deletion in ECL2 has been described. Approximately, 1% of Caucasians are homozygous for CCR5Δ32 mutant alleles, and these individuals are highly resistant to HIV infection. Notably, investigation of CCR5Δ32 homozygous individuals confirmed that CCR5 was the main coreceptor utilized by M-tropic strains of HIV-1 and that these strains were critical for person-to-person transmission (16, 24).

Another CCR5 mutant allele, CCR5Δ24, was determined to be extremely common in the RCM population, where ≥70% of these animals were homozygous for the CCR5Δ24 mutant allele (5). Despite a high prevalence of non-functional CCR5 alleles in the RCM population, CCR5-null animals are *not* protected from SIV infection. SIVrcm has adapted to use CCR2b as an entry coreceptor and lost the ability to use CCR5. Notably, wild-type RCM-CCR5 is functional and supports entry for HIV-1, HIV-2 and various strains of SIV (5).

In SM, I recently identified a novel CCR5 polymorphism, SM-CCR5Δ2, and determined a 26% allelic frequency for this mutant allele in a large SM colony housed at the Yerkes primate center (23). I found that 8% of the SM housed at Yerkes were homozygous for CCR5-defective alleles including CCR5Δ2 and CCR5Δ24 (20), which had an allelic frequency of 3% in this colony. Surprisingly, 50% of the CCR5-null SM were naturally infected with SIVsmm, thus the CCR5-null phenotype in SM does *not* protect against SIV infection. In contrast to SIVrcm, SIVsmm Envs cloned from a CCR5-null animal efficiently used CCR5 for entry as well as various alternative coreceptors, but not CXCR4 or CCR2b (7, 23). Interestingly, SIVrcm and SIVsmm have evolved to utilize different entry pathways in the absence of CCR5.

Remarkably, each of the three examples of populations with CCR5-null individuals (SM, RCM and humans) demonstrates distinct patterns of host/virus interactions. Intriguingly, CCR5-null humans (non-natural host) are protected from HIV infection *in vivo*, whereas CCR5-null SM and RCM (natural hosts) remain susceptible to SIV infection. The fact that natural hosts exhibit multiple mechanisms to reduce or completely abrogate CCR5 expression suggests that low or restricted CCR5 levels may

129

be advantageous to these hosts, possibly due to protecting critical cell subsets and a reduction in the homing of CD4+ T cells to sites of inflammation. Additionally, it will be interesting to explore if utilization of non-CCR5 entry pathways by SIV contribute to the non-pathogenic nature of SIV in natural hosts.

Convergent evolution of CCR5 mutant alleles

Many polymorphisms in CCR5 have been identified and investigated (2, 14, 15), and while a few mutant alleles were functionally defective, only three primate CCR5 deletion alleles have resulted in CCR5-null phenotypes to date: Δ32 in humans, Δ24 in RCM/SM and Δ2 in SM. Identification of the SM-CCR5Δ2 allele is the third example of convergent evolution resulting in disruption of CCR5 expression and function in primates.

Two questions regarding these mutant alleles remain unanswered: i) when did they arise, and ii) what selective pressure(s) led to the retention of CCR5Δ32, CCR5Δ24 and CCR5Δ2 in their respective species? It is estimated that the CCR5Δ24 mutant allele arose in an ancestral mangabey species over 10,000 years ago, prior to the formation of natural barriers, which likely caused a subspecies divide forming the SM and RCM populations (5). According to this estimation, the CCR5Δ24 mutant allele has been circulating in mangabey populations substantially longer than the CCR5Δ32 mutant allele, which is currently estimated to have entered the human population roughly 3,000 years ago (9, 11). Presently, it is unknown when the CCR5Δ2 allele entered the SM population; however, an estimate of the chronology of this mutation might be gained from evaluating the closely related RCM. The presence of CCR5Δ2 in the RCM

130

population would suggest that, like CCR5Δ24, CCR5Δ2 was present roughly 10,000 years ago in an ancestral mangabey species prior to the sub-speciation of RCM and SM. However, the absence of CCR5Δ2 in the RCM population would suggest that the CCR5Δ2 mutant allele arose after the RCM/SM subspecies divide.

Currently, the selective event that led to the emergence of CCR5Δ24 in the RCM/SM population is unknown, however the emergence of CCR5Δ32 in the human population has been attributed to infectious pathogens. The high frequency of the CCR5Δ32 allele in Northern European populations has been ascribed to the bubonic plague ("Black Death") pandemic in the 14th century (26), however subsequent predictions from a population genetic model suggest that the smallpox *Variola major* virus is a more likely candidate for selection pressure leading to CCR5Δ32 emergence (9). Evidence clearly indicates that CCR5Δ32 is not the result of selective pressure by HIV since this virus recently entered the human population. However it is plausible that endemic SIV infections in RCM and SM populations may have selected for the emergence of CCR5Δ24 and/or CCR5Δ2 in these hosts.

Another question raised by our finding is whether the SM-CCR5Δ2 and the SM/RCM CCR5Δ24 mutant alleles emerged independently resulting in deletions in the same region of CCR5. Both the Δ2 and Δ24 deletions occurred in a region of TM4 where multiple nucleotide repeats exist, which may have enabled recombination. However, this potential recombination site (-GTGGTG-) is conserved in other primate species (humans, chimpanzees and macaques) in which the Δ24 deletion (or any other deletion at this site) was not observed (5). Extensive CCR5-genotyping of other mangabey subspecies may identify deletions (Δ2, Δ24 or others) in this particular region of CCR5. On one hand, if

other deletions were observed, it would support the notion that CCR5Δ2 and CCR5Δ24 alleles arose independently in a region of CCR5 that has a high occurrence of recombination, likely leading to nucleotide deletions. On the other hand, the CCR5Δ2 and CCR5Δ24 mutant alleles contain two identical point mutations (436T>G and 538C>T), which result in amino acids identical to those in human CCR5 at those respective sites. If CCR5Δ2 and CCR5Δ24 arose from independent recombination events, it is peculiar that the resulting mutant alleles would contain more than one identical point mutation, in addition to deletions in the same region.

Alternative coreceptors: SIV use and viral tropism

It appears that natural hosts have developed multiple mechanisms to counter the potential pathogenic effects of SIV such as attenuated chronic immune activation and regulation of CCR5 expression through genetic polymorphisms or selective downregulation on specific T cell subsets (19, 21). SIV use of alternative coreceptors *in vitro*, has been extensively described over the past decade, however the potential biological role of alternative coreceptor use by SIV *in vivo* has not been investigated. Discovery of the SM-CCR5Δ2 mutant allele has led to the identification of SIV-infected CCR5-null SM emphasizing the biological relevance of non-CCR5 entry pathways in these hosts. Additionally, SM-PBMC, either genetically deficient in CCR5 or treated with a CCR5-specific inhibitor, supported SIV replication indicating non-CCR5 entry pathways are available on SM primary cells (23). Furthermore, SM-derived alternative coreceptors, CXCR6 and GPR15, efficiently supported entry by SIV Envs from CCR5-expressing and CCR5-null SM, *in vitro* (7). Taken together, these findings indicate that

alternative coreceptors play a major role in SIVsmm infection of CCR5-null SM, and explain the widespread and efficient use of alternative coreceptors *in vitro* among the SIVsmm/SIVmac family of viruses.

While alternative entry pathways solely support SIV transmission and replication in CCR5-null hosts, it is unknown to what extent alternative coreceptors contribute to SIV infection of CCR5-expressing SM. Remarkably, a modest yet significant difference in viral loads was observed (~3-fold) between CCR5-expressing and CCR5-null animals, with an intermediate gene dosage effect seen in the presence of one CCR5 allele. Based on this finding, one could speculate that CCR5 and alternative pathways are expressed on distinct or partially overlapping CD4+ T cell subsets, resulting in increased viral replication in animals possessing both CCR5 and non-CCR5 entry pathways. One way to address this question is by infecting SM-PBMC with replication-competent SIVsmm containing a GFP reporter gene, and subsequently staining infected cells for CD4 and coreceptors (CCR5, CXCR6 and GPR15). Analysis of coreceptor expression on SIV+ (GFP+) CD4 T cells will inform whether or not SIV infects cells expressing alternative coreceptors, but not CCR5.

The recognition that both CCR5 and non-CCR5 pathways are used by SIV in SM natural hosts has significant implications for understanding viral tropism *in vivo*. Since CD4/coreceptor interactions determine viral tropism and cell targeting, the findings of this thesis suggest that SIV infection in SM may involve target cells defined by the distribution and use of alternative coreceptors CXCR6 and GPR15, in addition to CCR5. Restricted expression of CCR5 on critical target cells (such as CD4+ central memory T cells) in SM has been previously described and thought to contribute to immune cell

homeostasis in these natural hosts (19). Combining the sparing of critical cell subsets through restricted CCR5 expression and alternative coreceptor-dependent targeting may allow virus replication in more expendable cells without the loss of immune cell homeostasis. We hypothesize that SM natural hosts may have evolved to restrict expression of CXCR6 and GPR15, in addition to CCR5, to dispensable cell subsets, thus allowing for high viral replication without causing disease. An understanding of the distribution of alternative coreceptors on primary SM cell subsets will elucidate SIVsmm tropism in these hosts. Additionally, strains of SIV (SIVagm and SIVrcm) from other natural hosts have been shown to use CXCR6 and/or GPR15 for entry *in vitro* (5, 6). It will be interesting to explore whether entry through alternative coreceptors is a conserved mechanism seen in multiple natural host species.

Another interesting question is do alternative coreceptors play a role in pathogenic HIV/SIV infections? In line with the hypothesis that alternative coreceptor use may contribute to non-pathogenicity seen in SIV-infected natural hosts, lack of alternative coreceptor use in pathogenic infections could result from host and/or viral parameters such as: i) species-specific polymorphisms that render non-natural host alternative coreceptors non-functional for viral entry, ii) differences in alternative coreceptor expression profiles between natural and non-natural hosts, which may affect viral tropism, iii) evolutionary adaptations in the virus, which may prevent the use of alternative coreceptors, and/or iv) high CCR5 expression in non-natural hosts that renders use of alternative coreceptors superfluous. Along these lines, studies indicate that the SIVmac virus family use CXCR6, GPR15 and a number of other alternative coreceptors

in vitro, however there is evidence to suggest that SIVmac does not efficiently use RM-derived CXCR6 (22).

In addition to implications for SIV, alternative coreceptor expression and use may play a role in HIV infection of humans since therapeutic strategies aimed at knocking out CCR5 genes, in an effort to engineer a CCR5-null phenotype, are currently underway. Some concerns with this approach is that the absence of CCR5 expression may drive the virus to utilize other coreceptors such as CXCR4, which is associated with accelerated disease progression (13). In an attempt to circumvent this issue, both CCR5 and CXCR4 can be targeted for gene disruption, however a CCR5 and CXCR4-null immune environment may further drive HIV to acquire the ability to use alternative coreceptors. Although rare, HIV use of alternative coreceptors does occur *in vivo*. Recently, an HIV transmitted/founder virus was identified that efficiently entered cells using GPR15, but had impaired CCR5 and CXCR4 use (12). Based on my preliminary findings in chapter 3, CXCR6 and GPR15 are predominantly expressed on human CD4+ memory cells, thus acquisition of alternative coreceptor use may result in infecting similar target cells as an R5-tropic virus.

Taken together, these data suggest that SM natural hosts may have evolved to restrict expression of CXCR6 and GPR15, in addition to CCR5, to dispensable cell subsets, thus allowing for high viral replication without causing disease. Future studies comparing CXCR6 and GPR15 expression profiles in natural and non-natural hosts will be of importance in determining the role of alternative coreceptors in natural hosts *in vivo*.

Potential role for alternative coreceptors *in vivo*
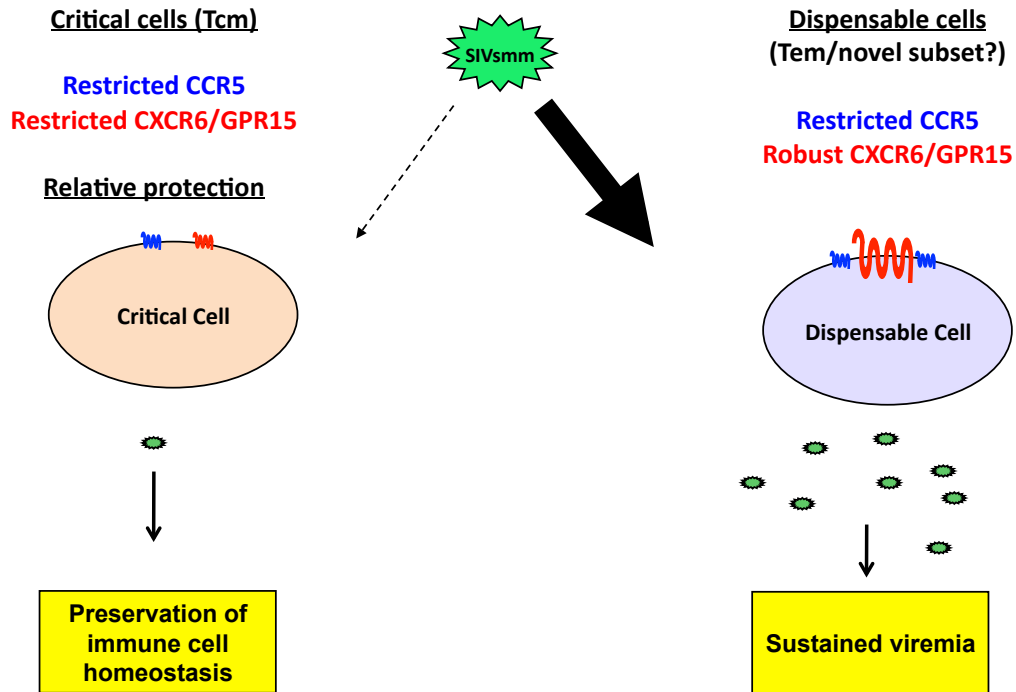## Natural Host (SM)

Figure 4-1. Model of potential coreceptor expression patterns on CD4+ T cells of natural hosts. Model demonstrating how restriction of CCR5 and alternative coreceptors (CXCR6 and GPR15) to specific cell subsets may redirect viral tropism, thus sparing critical cell subsets required for immune cell homeostasis, while targeting dispensable cell subsets allowing robust replication. Restriction of coreceptor expression (CCR5, CXCR6 and GPR15) to specific cell subsets may contribute to the non-pathogenic nature of SIV infection in natural hosts.

Potential role for alternative coreceptors *in vivo*
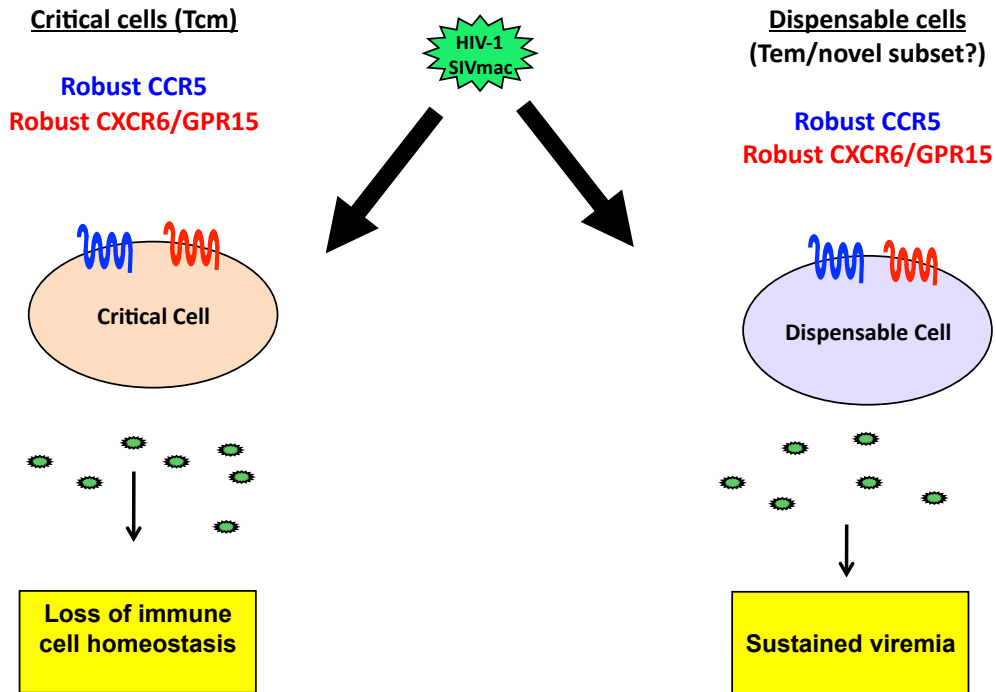## Non-Natural Host (Humans & RM)

Figure 4-2. Model of potential coreceptor expression patterns on CD4+ T cells of non-natural hosts. Model demonstrating that non-natural hosts express moderate to high levels of coreceptors on both critical and dispensable cell subsets. This type of expression likely promotes equal infection in both critical and dispensable cell subsets, ultimately leading to loss of immune cell homeostasis and immunodeficiency.

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