REGULATION OF CCR5 USE ON PRIMARY CD4+ LYMPHOCYTES BY R5X4 HIV-1

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ABSTRACT

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HIV-1 strains that use CCR5 predominate after transmission and during the asymptomatic period of disease. However, in up to half of infected people, variants that use CXCR4 emerge, coincident with accelerated disease progression. The earliest CXCR4 using strains to appear, called R5X4 viruses, usually retain CCR5 use. Prototype R5X4 HIV-1 isolates infect macrophages using CCR5 and CXCR4, but CD4+ lymphocyte infection by these viruses is mediated predominantly by CXCR4. Here, we sought to identify obstacles to CCR5 use on CD4+ lymphocytes by R5X4 HIV-1. Using a panel of R5X4 Envs we found that, although CXCR4 was the predominant coreceptor used to infect CD4+ lymphocytes, there was a spectrum of CCR5 use. Greater CCR5 use on lymphocytes correlated with relative resistance to inhibition by CCR5 mAbs and small molecule antagonists in CCR5+ indicator cells. Increasing CCR5 expression on primary lymphocytes through cytokine stimulation or lentiviral transduction increased the proportion of entry mediated by CCR5 for all R5X4 isolates except 89.6. Env dependence on CCR5 density was then evaluated using a cell line in which levels of CD4 and CCR5 could be independently regulated. At non-limiting CD4 levels, strains with greater lymphocyte CCR5 use were better able to exploit limiting levels of CCR5, whereas those that used lymphocyte CCR5 poorly were more sensitive to reductions in CCR5 levels. Evaluation of the V3 sequences of the R5X4 viruses using algorithms that

predict viral phenotype indicated an association between greater CCR5 use on lymphocytes and a predicted non-syncytium inducing phenotype. Introduction of an R306S mutation in the Env V3 domain of 89.6 enhanced its ability to use CCR5 at low levels and switched its preference to CCR5 for lymphocyte entry. Residue R306 in the 89.6 and C2-16 Envs was also associated with greater dependence on the amino terminus of CCR5 for infection. Thus, lymphocyte CCR5 is used by some R5X4 strains to a variable degree, low CCR5 expression coupled with inefficient Env-CCR5 interactions are the principal obstacles to lymphocyte CCR5 use by R5X4 HIV-1, and the spectrum of lymphocyte CCR5 use by R5X4 isolates is determined by variation in Env-CCR5 interactions.

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Chapter I - Introduction

Origin of the HIV-1 pandemic

Human Immunodeficiency Virus – type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), is a lentivirus in the family *Retroviridae*. HIV-1 can be grouped into three separate phylogenetic lineages that each entered the human population through cross species transmission of Simian Immunodeficiency Virus (SIV) from either chimpanzees (Group M and Group N) (Keele et al., 2006) or gorillas (Group O) (Van Heuverswyn et al., 2006) in west Africa. Recently a fourth lineage (Group P) (Plantier et al., 2009) was proposed, suggesting that cross-species transmission of SIV may still occur. HIV-1 from groups N and O are found almost exclusively in West Africa (Ayouba et al., 2000; Peeters et al., 1997); however, HIV-1 from group M spread beyond Africa leading to the global pandemic (Buonaguro, Tornesello, and Buonaguro, 2007). Since the early 20th century, the likely period during which HIV-1 Group M entered the human population (95), the evolution of this group from a common ancestor has resulted in the formation of distinct genetic subgroups, clades A-K, as well as numerous circulating recombinant forms of the virus (Buonaguro, Tornesello, and Buonaguro, 2007). The greatest diversity of HIV-1 Group M is found in Sub-Saharan Africa, while the pandemic in other parts of the globe is characterized by a high prevalence of one or a few specific clades or recombinant forms of the virus (Hemelaar et al., 2006). Over 25 million people have died of infection by HIV-1, and currently 33 million people globally, including 22 million in Sub-Saharan Africa, are infected by HIV-1 making it imperative that we understand the pathogenic mechanisms of this virus (Merson, 2006; UNAIDS-Epidemic-Update, 2009).

Overview of HIV-1 pathogenesis

HIV-1 is an insidious virus that, in the absence of antiretroviral therapy, slowly erodes the capacity of the immune system to respond to pathogens, which ultimately causes individuals to succumb to AIDS in an average of 5-7 years (Costagliola et al., 1989; Ward et al., 1989). The virus is spread by exposure to blood and other bodily fluids containing HIV-1, which occurs through blood product transfusion, IV drug use, perinatal exposure and, most commonly, during sexual contact (Curran et al., 1984; Harris et al., 1983; Scott et al., 1984).

HIV-1 infection and pathogenesis are studied using relevant human lymphoid cells and tissues *ex vivo* or SIV infection in non-human primates, and disease progression is monitored by specific measures that are adversely affected by the virus (Fig. 1.1). Following mucosal infection by SIV (Stahl-Hennig et al., 1999; Zhang et al., 1999) or HIV-1 infection of vaginal explant tissue (Collins et al., 2000; Gupta et al., 2002), virus is first found in small foci of infected resting and activated memory CD4+ lymphocytes and then later in macrophages and dendritic cells at the initial sites of infection. Within two weeks of SIV infection, virus disseminates from the site of local infection and amplification to local draining lymph nodes (Miller et al., 2005; Stahl-Hennig et al., 1999). From there, the virus travels through the lymphatic and vascular systems to other lymphoid tissue including the gut associated lymphoid tissue (GALT) (Miller et al., 2005; Stahl-Hennig et al., 1999). The majority of activated, memory CD4+ lymphocytes in the body are found in the GALT, and HIV-1 infection of CD4+ lymphocytes in this

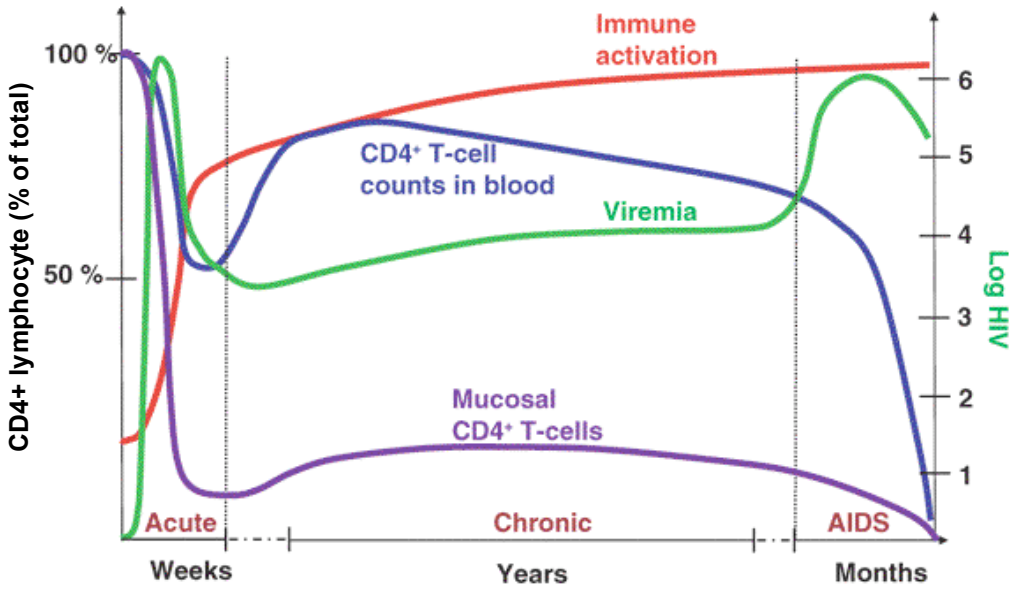

Figure 1.1. Markers of disease progression during HIV-1 infection.

Acute infection by HIV-1 is characterized by a rapid decline in CD4+ lymphocytes in multiple compartments that is coincident with a rapid increase in viral load. During this stage, the immune system is activated to respond to the virus. CD4+ lymphocytes in peripheral blood, but not in gut mucosal tissue, rebound as viral load decreases marking the transition to the chronic phase of infection. During the chronic phase, CD4+ lymphocytes in peripheral blood continue to decline and immune activation increases, which is thought to contribute to the immune exhaustion seen later in disease. Near the onset of AIDS, there is a loss of viral control that leads to a spike in viral load, depletion of the peripheral blood CD4+ lymphocytes and death. R5X4 strains typically appear near the onset of AIDS. Image obtained from *Grossman, Z. et al. Nature Medicine 2006*.

compartment leads to rapid and massive cell loss that is sustained throughout disease (Fig 1.1) (Brenchley et al., 2004; Schieferdecker et al., 1992; Schneider et al., 1994). HIV-1 replication in the GALT invokes a robust immune response and induces apoptosis of intestinal epithelium that damages the lining of the gut (Li et al., 2008; Sankaran et al., 2008; Schmitz et al., 2001). Microbial products are thought to enter peripheral blood as a consequence of the breakdown of the intestinal barrier and induce immune activation that increases as disease progresses and has been hypothesized to directly contribute to the immunodeficiency that occurs at the end of disease (Fig 1.1) (Brenchley et al., 2006).

Although HIV-1 infection after the acute phase is clinically asymptomatic, covert viral replication proceeds for years until the final stage of disease (Embretson et al., 1993; Pantaleo et al., 1993). Humoral and cellular HIV-1-specific immune responses arise weeks after transmission; however, in most cases, they fail to adequately control viral replication, instead providing selective pressure that contributes to viral evolution (Allen et al., 2005; Wei et al., 2003). As HIV-1 disease progresses, the number of CD4+ lymphocytes in blood slowly declines (Polk et al., 1987). The opportunistic infections that characterize AIDS typically begin to occur once CD4+ lymphocyte levels fall below a threshold (Eyster et al., 1989), and infected individuals ultimately die from an inability to clear virulent as well as opportunistic microorganisms (Fig. 1.1) (Quagliarello, 1982).

HIV-1 pathogenesis begins at the interface between the virus and cellular membranes. HIV-1 requires a receptor, CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984), and a seven-transmembrane domain chemokine coreceptor, either CCR5 (Choe et al., 1996; Deng et al., 1996) or CXCR4 (Berson et al., 1996; Feng et al., 1996), for infection of target cells. Viruses are typically classified based on the coreceptors they use

to infect CD4+ cells lines: R5 viruses use CCR5, X4 viruses use CXCR4, while R5X4 viruses are able to use either coreceptor (Robertson et al., 2000). Differences in coreceptor use between HIV-1 variants have important implications for transmission and pathogenesis. R5 HIV-1 predominates in the early and asymptomatic stages of infection (Schuitemaker et al., 1991); however, in up to 50% of subtype B-infected individuals, viruses that use CXCR4 emerge (Spijkerman et al., 1995). Memory lymphocytes are the preferential targets of R5 viruses, but the emergence of CXCR4 use leads to an expansion in target cell tropism to include naïve lymphocytes as well (Blaak et al., 2000; Ostrowski et al., 1999). The appearance of these variants and the expanded tropism that results is associated with an accelerated decline in the number of CD4+ lymphocytes in peripheral blood and rapid progression to AIDS and death (Koot et al., 1993), but presently, it is unclear whether the emergence of CXCR4-using viruses is a cause or consequence of immune decline. Interestingly, regardless of virus quasispecies in the donor or the route of transmission, nearly all HIV-1 infections are initiated by R5 viruses (van't Wout et al., 1994; Zhu et al., 1993). Although there is evidence suggesting mutational barriers and selective immune pressure oppose the emergence of CXCR4-using viruses (Harouse et al., 2003; Pastore, Ramos, and Mosier, 2004), it is unclear what factors regulate the appearance of these variants and why predominately R5 strains transmit. R5X4 viruses are the first CXCR4-using variants to appear (van Rij et al., 2000), and despite CCR5 use on indicator cells, these viruses rarely establish infections after transmission (Cornelissen et al., 1995; Pasquier et al., 1998). Consequently, studies focused on R5X4 HIV-1 may shed light on these two critical aspects of HIV-1 infection: the emergence of CXCR4 use and the bottleneck at transmission that selects for R5 viruses.

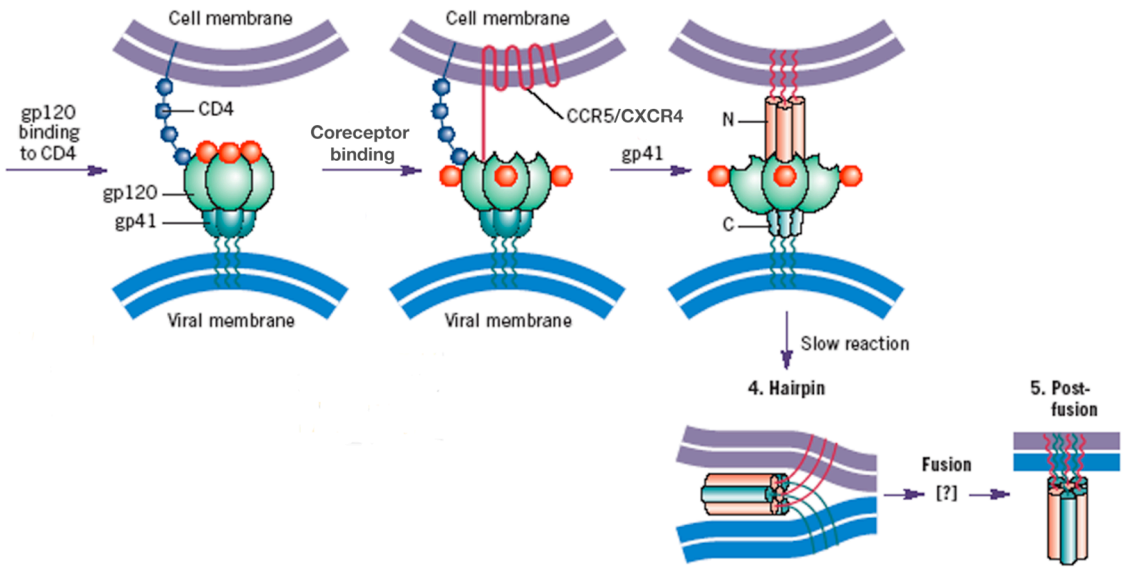

Figure 1.2. Steps in the gp120 mediated membrane fusion process.

The HIV-1 Env and the cellular receptor (CD4) and coreceptors (CCR5/CXCR4) are shown on the viral and host cell membranes, respectively. CD4 binding leads to conformational changes in gp120 that result in the formation of a coreceptor-binding site. Coreceptor binding causes additional structural changes in gp120 that lead to insertion of the fusion peptide of gp41 into the target cell membrane. Creation of the six-helix bundle in gp41 brings the virus and target cell membrane into close proximity ultimately resulting in membrane fusion and viral entry. This image was modified from *Nathanson and Overbaugh, Viral Pathogenesis and Immunity 2006*

Function and structure of the HIV-1 envelope protein

The HIV-1 lipid membrane is studded with the envelope (Env) glycoprotein, which is arranged on the surface of the virion as a trimer (Gelderblom et al., 1987). This protein is composed of gp120, the surface subunit, non-covalently attached to the transmembrane subunit gp41, which contains the fusion peptide (Earl, Doms, and Moss, 1990; Freed, Myers, and Risser, 1990). As outlined in Figure 1.2, HIV-1 entry is a multistep process that begins with binding between gp120 and CD4 (Dalgleish et al., 1984; Klatzmann et al., 1984). In addition to tethering the virus to target cells, CD4-gp120 binding also triggers conformational changes in Env that create a coreceptor binding site (Sattentau and Moore, 1991). A number of seven-transmembrane G-protein-coupled receptors serve as coreceptors for HIV-1 *in vitro* (Edinger et al., 1998a; Edinger et al., 1998b), but only CCR5 and CXCR4 are thought to play a significant role for infection *in vivo* (Scarlatti et al., 1997). Coreceptor binding is required to complete the conformational changes in Env that lead to membrane fusion (Mkrtchyan et al., 2005; Oravecz, Pall, and Norcross, 1996).

Virus-host membrane fusion is mediated by gp41. Gp41 has a an N-terminal fusion peptide that is inserted into the target cell membrane following receptor binding (Freed, Myers, and Risser, 1990; Gallaher, 1987) and two heptad repeats (HR1 and 2) that fold back on each other following insertion of the fusion peptide (Chan et al., 1997; Weissenhorn et al., 1997). Formation of this 6-helix bundle brings the HIV-1 and target cell membranes into close proximity (Melikyan et al., 2000). The exact mechanisms that

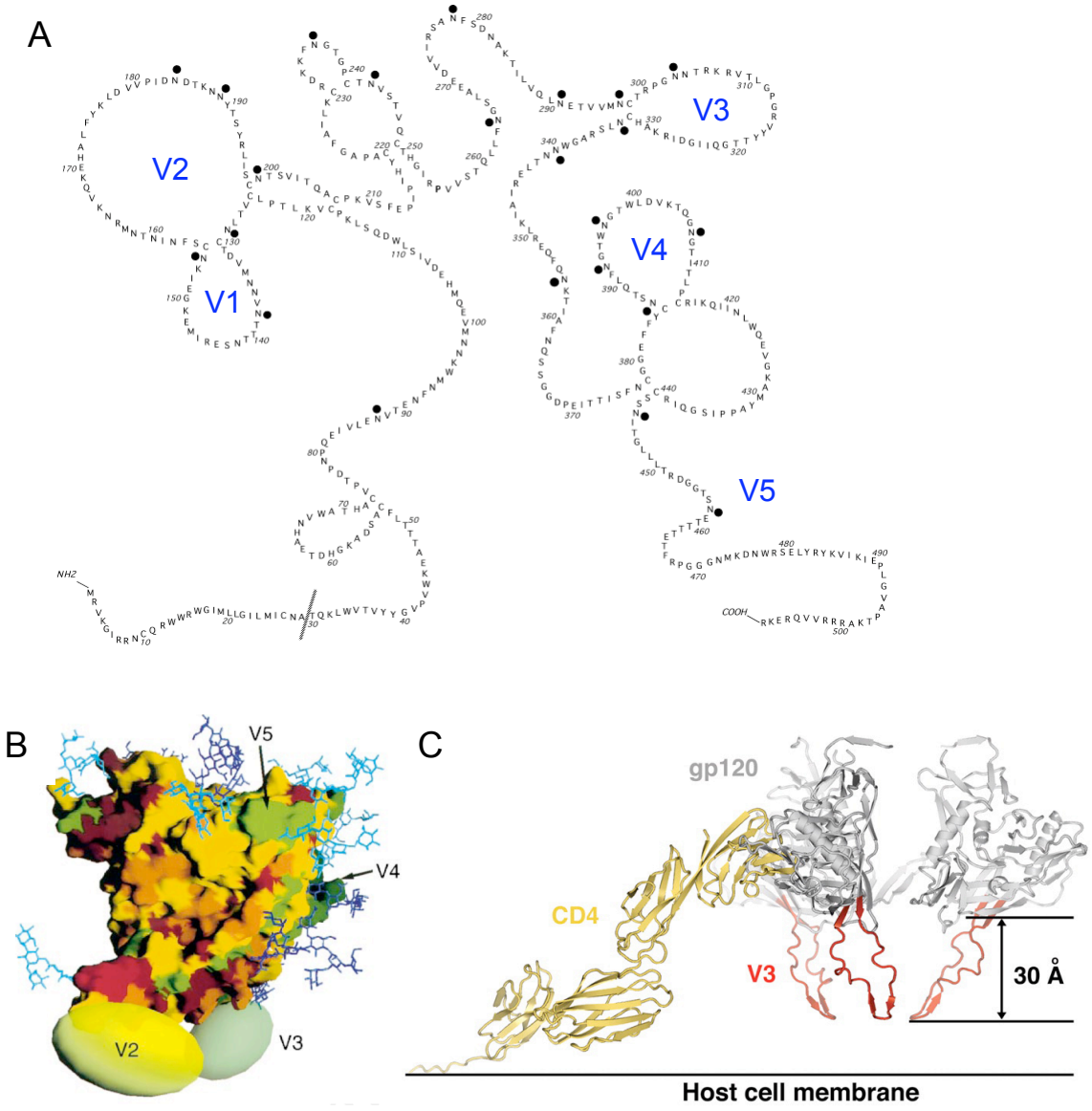

Figure 1.3. Env structure and variability.

The 2D amino acid structure of the R3A Env is pictured with the location of the variable loops denoted (A). Predicted N-linked glycosylation sites are indicated by black dots, and the N-terminal leader sequence is cleaved where indicated. The structure of gp120 is shown without the variable loops (B) or CD4-triggered with the V3 loop (C). (A) The molecular surface of monomeric gp120 core showing the inner domain (left side) and the heavily glycosylated outer domain (right side), which is modeled with complex (light blue) or high-mannose (dark blue) sugars. The location of the variable loops is indicated. Color coding signifies the sequence variability of the indicated residues: red, highly conserved between primate lentiviruses, orange, highly conserved between HIV-1 viruses, yellow, variable between HIV-1 viruses, green, significant variability between HIV-1 viruses. (B) CD4-triggered gp120 structure with V3 loop extended toward the cell surface. The CD4 structure is superimposed showing the orientation of the gp120 trimer towards the host cell membrane. R3A 2D sequence obtained from G. Leslie (U. of Pa.). Gp120 images from *Wyatt, R. et al. Nature 1998* (B), and *Huang, C. et al. Science 2005* (C)

join the viral and cellular lipid bilayers are unknown, but following membrane fusion, the capsid core is released into the cell. Upon viral entry, reverse transcription, nuclear import, integration of viral cDNA, viral gene expression, mRNA export, protein synthesis and virion assembly occur, ultimately leading to the formation of new HIV-1 viruses (Greene and Peterlin, 2002).

The conformational changes that have to occur in gp120 for entry to proceed are directly related to the complex structure of the protein. The surface subunit of Env can be divided based on patterns of genetic diversity between isolates into five conserved regions and five variable regions, V1-V5, which correspond to disulfide bonded loops (Fig. 1.3a) (Starcich et al., 1986). Additionally, gp120 is heavily glycosylated (Leonard et al., 1990). Structural studies of gp120 monomer without its variable loops reveal a core that is divided into an inner and outer domain (Fig. 1.3b) (Chen et al., 2005; Kwong et al., 1998). The more conserved inner domain forms the interacting surface within the trimer, while the glycosylated outer domain is exposed (Kwong et al., 1998). The CD4 binding site and portions of the coreceptor binding region, including the bridging sheet, are non-contiguous on the surface of gp120, and the coreceptor binding domains are also partially concealed by variable loops V1/V2 and V3 (Chen et al., 2005). CD4 binding causes conformational changes, primarily within the inner domain, that result in formation of the bridging sheet and extension of the V3 loop toward the cell surface, ultimately facilitating coreceptor binding (Fig. 1.3c) (Huang et al., 2005; Kwong et al., 1998).

Molecular determinants of coreceptor use

The V3 loop is the most critical determinant of coreceptor use by HIV-1. The V3 loop is typically around 35 amino acids long and its structure consists of a base, stem and tip (Leonard et al., 1990). The tip and stem of V3 are thought to interact with the extra cellular loops of the coreceptor, while the base interacts with the coreceptor N-terminus (Huang et al., 2007; Huang et al., 2005). Studies have shown that only a few mutations within V3 are necessary to alter coreceptor use (de Jong et al., 1992a; Shioda, Levy, and Cheng-Mayer, 1992). The presence of positively charged amino acids at positions 11 and 24 or 25 within the stem of V3 are associated with the ability to use CXCR4 (Cardozo et al., 2007; de Jong et al., 1992a; Fouchier et al., 1992). Moreover, deletion of three amino acids in the stem of V3, Δ9-12, eliminates CXCR4 use by prototype R5X4 viruses, while deletions within the base of V3 abolish CCR5 use by these strains (Nolan, Jordan, and Hoxie, 2008). Another characteristic frequently associated with CXCR4 use by HIV-1 is a high net positive charge within V3 (Fouchier et al., 1992). Post-translational modifications within V3 can also impact viral coreceptor use; the presence of N-linked glycosylation in the base of V3 has been shown to determine CCR5 versus CXCR4 use for some isolates (Ogert et al., 2001; Pollakis et al., 2001).

The bridging sheet and V1/V2 loops are in close proximity to the coreceptor-binding site, and changes within these regions can also have implications for coreceptor use. Mutations within the bridging sheet can increase sensitivity to CCR5 or CXCR4 antagonists, indicating that this domain contributes to the efficiency of interaction with coreceptor (Reeves et al., 2002). Mutations within V1/V2 also affect how efficiently Env interacts with the coreceptor. Removing either of two N-linked glycans within the V1/V2

domain of DH12, an R5X4 virus, decreased CCR5 and CXCR4-mediated infection (Ogert et al., 2001). Furthermore, replacing the V1/V2 of R5 Envs with the corresponding domains from Envs that use CXCR4 can permit use of this coreceptor (Cho et al., 1998; Pollakis et al., 2001), and the efficiency of CXCR4 use in this context is regulated, in part, by glycosylation within V1/V2 (Pollakis et al., 2001). As critical determinants of coreceptor use, the bridging sheet, V1/V2 and V3 also influence target cell tropism.

Target cell tropism and viral phenotype

HIV-1 entry is a critical aspect of viral pathogenesis and a primary determinant of which cell types are infected by the virus. CD4, CCR5 and CXCR4 are expressed on a number of immune cells including T helper lymphocytes, monocytes, macrophages and dendritic cells (Bleul et al., 1997; Terhorst et al., 1980; Wood, Warner, and Warnke, 1983; Zaitseva et al., 1997). CD4+ lymphocytes are the primary targets for HIV-1 infection and replication *in vivo* (Massari et al., 1990; Spear et al., 1990), while macrophages are though to be a source of viral replication in numerous tissues (Eilbott et al., 1989; Jeffrey et al., 1991). Although HIV-1 infects monocytes and dendritic cells *in vivo*, infection of these cells is infrequent (Cameron et al., 1992; Spear et al., 1990), and in the case of monocytes, is more common late in disease (McElrath, Pruett, and Cohn, 1989). Maturation-dependent intracellular blocks to HIV-1 replication in dendritic cells and monocytes may explain why a low percentage of these cells are infected *in vivo* (Bakri et al., 2001; Sonza et al., 1996).

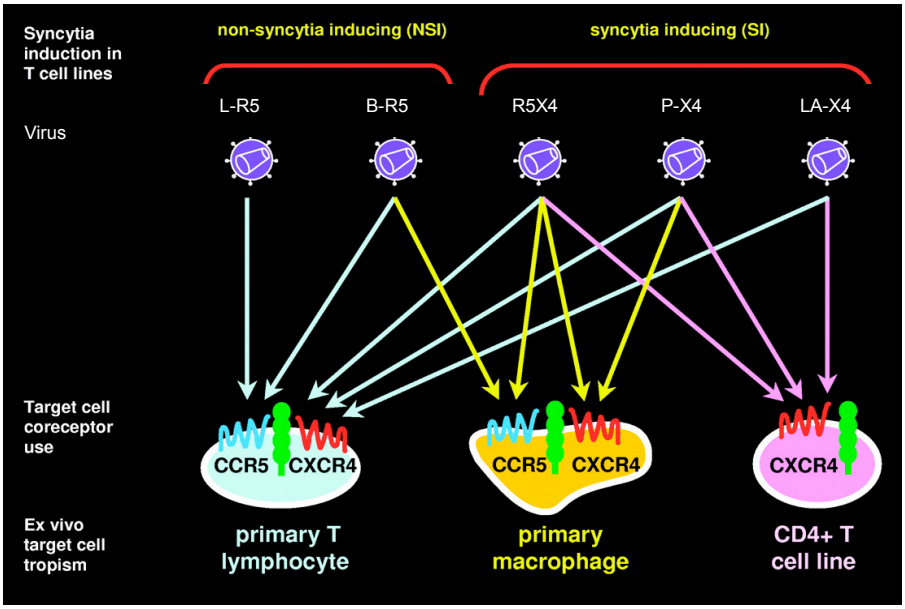

Figure 1.4. HIV-1 coreceptor use and target cell tropism.

Target cell tropism is indicated for the particular HIV-1 virus. L-R5, lymph node derived R5 isolates that poorly infect primary macrophages, B-R5, brain derived R5 viruses infect macrophages and lymphocytes; P-X4, primary isolate X4 viruses infect all three cell types; LA-X4, lab-adapted X4 viruses infect primary lymphocytes, and R5X4 viruses infect both primary cell types. Illustration modified from *Collman and Goodenow. Journal of Immunology 2006*

Early characterization of HIV-1 target cell tropism revealed that some viral isolates infected primary CD4+ lymphocytes and lymphocyte cell lines but not macrophages. These isolates were called syncytium-inducing (SI) or T cell (T) tropic isolates because of their ability to induce cell-cell fusion, or syncytia, in CD4+ lymphocyte cell lines (Fig. 1.4). SI isolates grew rapidly *in vitro* and were typically recovered at late stages of infection (Asjo et al., 1986; Tersmette et al., 1988). Another class of HIV-1 isolates was found at earlier stages of disease. These isolates infected CD4+ macrophages and primary lymphocytes, replicated slowly, did not infect CD4+ transformed cell lines (and thus did not induce syncytia in them) and were therefore called non-syncytium-inducing (NSI) or macrophage (M) tropic isolates (Fig. 1.4) (Asjo et al., 1986; Tersmette et al., 1988). Interestingly, M-tropic isolates did not infect T cell lines and most T-tropic isolates replicated poorly in macrophages (Schuitemaker et al., 1991; Schwartz et al., 1989). Following the identification of CCR5 and CXCR4 as coreceptors, it became apparent that differences in target cell tropism for M-tropic and T-tropic isolates were in large part related to target cell expression of CCR5 or CXCR4, respectively, and current classification schemes are now largely based on coreceptor use. R5 viruses use CCR5 for infection and X4 use CXCR4, while R5X4 viruses can infect cells expressing CCR5 or CXCR4 (Berger et al., 1998).

Coreceptor use and viral tropism

The combination of receptor and coreceptors expressed on a cell clearly influences HIV-1 tropism. However, viruses within a strain can vary considerably in the

use of CCR5 or CXCR4 when expressed on different cells, which also has an impact on cell tropism and pathogenesis. As mentioned, lab-adapted and primary X4 isolates efficiently infect primary lymphocytes using CXCR4 (Gartner et al., 1986; Tersmette et al., 1988); however, lab-adapted X4 isolates show a reduced ability to use CXCR4 on macrophages when compared to some X4 primary isolates (Fig. 1.4) (Verani et al., 1998; Yi et al., 1999). This difference in tropism results from the inability of lab-adapted X4 viruses to use the low levels of CXCR4 expressed on primary macrophages (Tokunaga et al., 2001), which may be a consequence of prolonged passage on CD4+ cell lines *in vitro*. There are other examples of physiologically relevant variation in receptor use within a strain. R5 Envs cloned from brain typically infect macrophages more effectively than R5 Envs recovered from peripheral blood and tissue despite similar infection levels on lymphocytes (Fig. 1.4) (Peters et al., 2004; Peters et al., 2006). Furthermore, differences in macrophage tropism between brain-derived R5 viruses have important implications for viral pathogenesis. Neurovirulent R5 HIV-1 from patients with dementia show an even greater capacity to infect macrophages than R5 viruses isolated from brains of patients with no signs of neurological impairment (Gorry et al., 2002a). In both cases, enhanced macrophage tropism is mediated either by increased CCR5 affinity (Gorry et al., 2002a) or by an ability to efficiently utilize low levels of CD4 or CCR5 for infection (Dunfee et al., 2006; Gorry et al., 2002a; Peters et al., 2004).

The relationship between coreceptor use and target cell tropism is quite complex and is unique for each HIV-1 strain. It is critical that we fully understand the variation in coreceptor use by HIV-1 strains and its affects on target cell tropism because of the important role these two factors play in HIV-1 transmission and pathogenesis.

Emergence of CXCR4 use: an overview

Longitudinal studies of HIV-1 reveal that R5 isolates predominate during the early and the asymptomatic period of disease (Schuitemaker et al., 1991). However, nearly half of subtype-B infected individuals develop CXCR4-using variants (Spijkerman et al., 1995) (as do a number of people infected with other subtypes, except for subtype C which acquires CXCR4 use infrequently (Abebe et al., 1999; Tscherning et al., 1998)) . The emergence of CXCR4-using viruses is associated with accelerated loss of CD4+ lymphocytes and rapid progression to AIDS (Koot et al., 1993). However, it remains unclear whether viruses capable of using CXCR4 arise in response to a weakened immune system, whether these viruses cause the accelerated immune decline associated with their emergence or if both phenomena occur. Unraveling this complex relationship as well as identifying the factors that regulate the emergence of CXCR4 use will help more clearly define the role of viral evolution in disease progression and may have implications for other stages of infection.

Emergence of CXCR4 use: intrinsic mutational barriers

In some cases, as little as two amino acid changes are sufficient to convert a virus from R5 to R5X4 (Kiselyeva et al., 2007; Pastore, Ramos, and Mosier, 2004), yet variants that use CXCR4 arise in only half of infected patients, suggesting barriers to their emergence. Studies of viral evolution *in vitro* and *in vivo* reveal complex mutational pathways from CCR5 to CXCR4 use that are beset with obstacles. In one study, nearly all R5X4 viruses derived *in vitro* replicated more poorly and were more

sensitive to CCR5 antagonists than the parental R5 viruses (Pastore, Ramos, and Mosier, 2004). Additionally, when the mutations that accrued were individually reinserted into the parental R5 virus, the mutations within the V3 loop that switched coreceptor use resulted in non-functional viruses. Mutations within the V3 loop only resulted in functional Envs with CXCR4 use when they were preceded by mutations in V1/V2 (Pastore et al., 2006). In a patient followed for years before and after coreceptor switch, there was a reduction in the efficiency of CCR5 use by R5 viruses and rapid divergence, not only within V3, but in the V4/V5 loops as well, prior to the emergence of R5X4 viruses (Coetzer et al., 2008). These studies suggest that mutations within other domains of Env are required prior to or concurrent with the changes in V3 for CXCR4 use to emerge, and this multistep process is a barrier to the acquisition of CXCR4 because it negatively impacts CCR5-mediated entry and frequently results in non-functional Envs.

Emergence of CXCR4 use: immune suppression of CXCR4-using viruses

The difficult mutational pathways that lead to CXCR4 use are an intrinsic obstacle to coreceptor switching. However, the immune response to the virus also appears to impede this process. It has been reported that, following the acquisition of CXCR4 use, X4 viruses are more sensitive to neutralizing antibodies than coexisting R5 viruses (Bunnik et al., 2007). Support for the notion that CXCR4-using viruses are preferentially targeted by the host immune response also comes from acute infection. A few anecdotal cases have suggested that after a brief period of replication, CXCR4-using HIV-1 variants can be selectively suppressed following coinfection with viruses that use

CCR5 (Cornelissen et al., 1995; Veenstra et al., 1995). A similar observation was made in a macaque coinfection study in which the R5 SHIV remained detectable while the X4 SHIV became undetectable in some animals coincident with the establishment of a SHIV-specific immune response. Depletion of CD8+ lymphocytes in these animals led to the reemergence of the X4 SHIV to levels similar to those of the R5 SHIV, and the X4 SHIV returned to undetectable levels after the CD8+ lymphocytes rebounded (Harouse et al., 2003). These findings suggest the immune response to HIV-1 suppresses X4 variants and that the loss of immune control may contribute to the emergence of CXCR4-using viruses. On the other hand, recent data suggests that most people who sexually acquire HIV become infected with a single variant, which is usually R5 (although whether X4 viruses are suppressed by local innate immune responses during mucosal replication is unknown) (Keele et al., 2008a).

While it remains unresolved, there is a substantial body of work suggesting that immune decline precedes the emergence of CXCR4 use because the antiviral immune response is more effective against these strains. The transmission and establishment of R5X4 viruses have been associated with poor humoral and cytotoxic cellular immune responses (Dalmau et al., 2009), although, it is not clear whether pronounced immune dysfunction enabled R5X4 infection or whether infection by R5X4 strains precipitated the immune destruction. In this regard, macaque models of coreceptor switch may be informative. The emergence of CXCR4-using SHIVs in macaques infected with CCR5-using viruses occurred in animals with high viral load, poor cellular immune response and undetectable antibody response to the virus (Ho et al., 2007; Ren et al., 2010), suggesting that immune decline precedes the appearance of variants that use CXCR4.

Emergence of CXCR4 use: CCR5+ target cell depletion

Another factor proposed to drive the acquisition of CXCR4 use is target cell depletion. R5 HIV-1 preferentially infects memory lymphocytes, while naïve lymphocytes, which lack CCR5 expression, are rarely targeted by these viruses (Blaak et al., 2000; Rabin et al., 1999). Acquisition of CXCR4 use results in a marked increase in infection of naïve lymphocytes (Blaak et al., 2000; Ostrowski et al., 1999). Preferential infection of CCR5+ memory lymphocytes coupled with expanded targeting of naïve lymphocytes following acquisition of CXCR4 use has lead to the hypothesis that depletion of CCR5+ targets cells creates pressure that selects for viruses with CXCR4 use. CCR5+ target cell depletion and lack of immune control are not mutually exclusive; these factors may act in concert to promote the acquisition of CXCR4 use. One interpretation of the results from the macaque model of coreceptor switch mentioned above is that poor immune control results in high levels of viral replication, increasing the chances of the emergence of mutant viruses that use CXCR4 while creating an environment that would allow greater depletion of CCR5+ target cells.

Conversely, an expansion in target cell tropism coupled with more rapid, cytopathic infection by R5X4 and X4 viruses suggests a viral component to the decline in cell numbers seen when these viruses are present. Infection by viruses that use CCR5 or CXCR4 are equally cytopathic for their target cells *in vitro* (Kwa et al., 2001) and in *ex vivo* lymphoid tissue (Grivel and Margolis, 1999). However, in blood, central and effector memory as well as naïve CD4+ lymphocytes express CXCR4, resulting in much greater lymphocyte depletion following infection with X4 and R5X4 viruses (Grivel and Margolis, 1999; Kwa et al., 2001). On the other hand, in some CD4+ lymphocytes

subsets, such as resting memory cells, CXCR4-using viruses are more cytopathic (Zhou et al., 2008), and these variants induce substantially more apoptosis in uninfected bystander cells in lymphoid tissue compared to R5 viruses (Jekle et al., 2003). Animal models also indicate that infection with X4 SHIV is more pathogenic than R5 SHIV infection (Harouse et al., 1999; Nishimura et al., 2004), resulting in rapid depletion of peripheral CD4+ lymphocytes, including naïve cells (Nishimura et al., 2005). Greater infection of naïve lymphocytes likely impairs lymphocyte differentiation and exacerbates immune decline. Although rare, in the instances in which CXCR4-using HIV-1 is transmitted and establishes infection, CD4+ lymphocyte numbers decline rapidly and progression to AIDS is much faster than the time frame following R5 infection (Yu et al., 1998).

HIV-1 coreceptor use and transmission

Heterosexual intercourse is the most common route of HIV-1 transmission, but it is nonetheless a very inefficient process. The probability of HIV-1 transmission from an infected individual is infrequent, ranging from 0.04-0.38% per sex act (Boily et al., 2009). However, HIV-1 transmission through other routes occurs at higher rates; the mother-to-child transmission rate, pre- and intrapartum combined, is approximately 20% (Kourtis et al., 2006). Efforts to understand HIV-1 transmission in different settings have revealed a bottleneck at transmission that is associated with genotypic and phenotypic selection of newly transmitted HIV-1 variants.

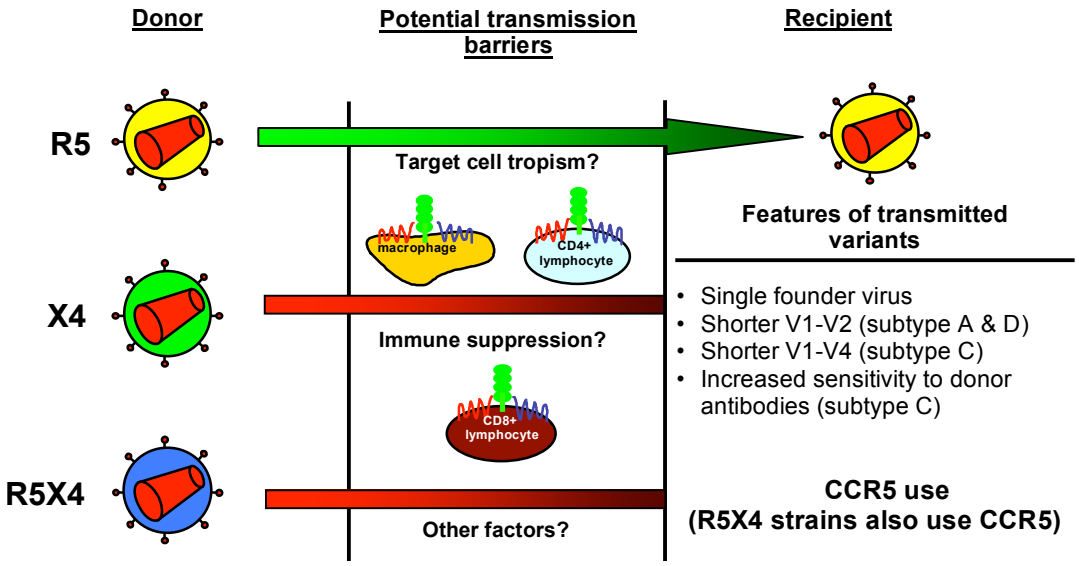

Figure 1.5. Selective transmission of R5 HIV-1 from donor to recipient.

Regardless of the composition of the viral quasispecies in the donor, only R5 viruses are transmitted to the recipient. It is unclear what factors impede the transmission of CXCR4 using viruses, or what selectively promotes the transmission of R5 strains. R5 target cell tropism and X4 immune suppression are two possible barriers to transmission of X4 strains, but there are likely others. It is clear that certain features are common in viruses from the recipient, such as the requirement for CCR5 use, which suggests selection targeted at the viral Env.

HIV-1 transmission is associated with a loss in genetic diversity from the donor to the recipient that suggests a selective barrier to transmission. HIV-1 infection is generally associated with high levels of genetic diversity (Hahn et al., 1986; Saag et al., 1988). However, after transmission, viral diversity in the recipient is much lower than in the donor, and this reduced diversity is most evident in the *env* gene (Verhofstede et al., 2003; Wolinsky et al., 1992; Zhu et al., 1993). In fact, in the majority of new sexually transmitted HIV-1 infections the env is completely homogeneous (Abrahams et al., 2009; Keele et al., 2008a). Furthermore, recent studies using PCR amplification and phylogenetic reconstruction of the successfully transmitted virus indicate a single viral variant is usually responsible for seeding infection in the recipient (~80%) with two or more variants responsible for the rest (Abrahams et al., 2009; Keele et al., 2008a).

A number of classic studies suggested that genetic homogenization in the recipient was associated with selection at mucosal transmission that favored M-tropic/NSI isolates with specific exclusion of T-tropic/SI variants. In these studies, viruses from the acute phase of infection were highly M-tropic where as T-tropic variants did not arise until later stages of disease (Schuitemaker et al., 1992; van't Wout et al., 1994; Zhu et al., 1993). The preponderance of M-tropic variants in a recipient during the earliest periods following transmission was seen even when the donor harbored T-tropic variants (van't Wout et al., 1994; Zhu et al., 1993), and a few anecdotal studies indicated that SI variants could be selectively suppressed following transmission (Cornelissen et al., 1995; Pratt et al., 1995). These early studies concluded that the ability to replicate in macrophages is essential for establishing infection in recipients and implied that macrophages are likely a major source of virus replication during acute infection.

However, more recent *in vitro* and *in vivo* studies strongly suggest CD4+ lymphocytes are the first cells infected after mucosal transmission (Gupta et al., 2002; Zhang et al., 1999), variants from periods shortly after transmission do not have enhanced macrophage tropism (Isaacman-Beck et al., 2009), and the single, founder virus responsible for establishing infection after transmission actually replicates significantly better in CD4+ lymphocytes than macrophages (Salazar-Gonzalez et al., 2009).

It remains unclear whether target cell tropism is a factor in the bottleneck between donor and recipient, but it is apparent that selection for R5 variants and exclusion of CXCR4-using viruses occurs during HIV-1 transmission in association with characteristic changes in Env. The V3 domain in Env is a major determinant of target cell tropism, and initial characterization of V3 within Envs from acute infection suggested selection at transmission that leads to a homogeneous population (Hwang et al., 1991; McNearney et al., 1992; Zhang et al., 1993). The discovery of CCR5 as the receptor for M-tropic viruses and the finding that V3 is the principal determinant of coreceptor use provided a genotypic basis for the phenotypic selection observed at transmission (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996). Furthermore, the identification of individuals that are protected from HIV-1 infection due to a 32 base pair deletion in the CCR5 gene, which prevents its expression on the cell surface, confirms CCR5 use is required for transmission (Liu et al., 1996; Samson et al., 1996). The requirement for CCR5 at transmission also suggests that V3, the major determinant of coreceptor use, is either a direct or an indirect target of selection during transmission. Additionally selection at transmission may also affect other regions of Env. Envs recovered from the earliest time point after transmission of subtype A or C

HIV-1 have shorter V1/V2 or V1-V4 length, respectively, and reduced N-linked glycosylation (Chohan et al., 2005; Derdeyn et al., 2004) when compared to matched donors. For subtype C, these changes are associated with greater sensitivity to neutralization by donor antibody (Derdeyn et al., 2004). However, these differences in Env appear to be subtype-dependent, since there are no changes in either Env length or the number of N-linked glycosylation sites following transmission of subtype B viruses (Chohan et al., 2005).

Selection for CCR5 use clearly occurs during transmission and is required for HIV-1 to establish infection in a recipient. Preferential immune suppression of CXCR4-using viruses may be one mechanism that accounts for this selection, although it remains unclear whether this is the principal means of exclusion or whether CXCR4-using strains are not transmitted for other reasons such as the inability to use CCR5 on a particular cell type.

Transmission, CCR5 use and tropism by R5X4 HIV-1

It is unclear why particular viral strains are the target of selection during transmission, which makes the study of R5X4 viruses intriguing and potentially informative. These viruses can infect indicator cells expressing either coreceptor, yet a dependence on CCR5 or CXCR4 for infection is associated with drastically different outcomes following HIV-1 transmission. R5 strains predominate following viral transmission whereas R5X4 viruses are typically not found during the early or asymptomatic period of infection (van't Wout et al., 1994). If variants that use CXCR4

are selected against, R5X4 viruses may be subject to the same repressive forces that are thought to oppose transmission of X4 viruses. Alternatively, if there is positive selection for CCR5 use, successful transmission of R5X4 viruses may not occur because these viruses uses CCR5 in a different manner than R5 viruses.

Numerous studies suggest that CCR5 use by R5X4 viruses differs from that of R5 viruses. CCR5 is a seven transmembrane protein with an N-terminus (NT) and three extracellular loops (ECLs). The NT of CCR5 is modified by tyrosine sulfation, and removal of sulfation within this domain by mutating tyrosine residues reduces infection by R5X4 viruses more substantially than infection by R5 viruses (Farzan et al., 1999; Rabut et al., 1998). Additionally, deleting amino acids from the NT of CCR5 has a larger impact on infection by R5X4 compared to R5 viruses (Rucker et al., 1996; Yi et al., 2003b). The ECLs of CCR5 are also critical domains for coreceptor function. Mutations within the ECLs cause a greater decrease in CCR5-mediated infection by R5X4 versus R5 viruses (Doranz et al., 1997; Genoud et al., 1999; Yi et al., 2003b). Inhibitors that target this region within CCR5 also have differing effects on R5X4 and R5 viruses. Antibodies with epitopes within the ECLs along with small molecule antagonists that disrupt this domain typically inhibit R5X4 infection more than infection by R5 viruses (Olson et al., 1999; Yi, Shaheen, and Collman, 2005). This suggests that, compared to R5 viruses, R5X4 viruses have less efficient interactions with CCR5. However, neither differences in the efficiency of Env-CCR5 interactions between R5X4 strains nor their ramifications for coreceptor use on primary cells have been addressed.

Coreceptor use by HIV-1 is typically determined on cell lines that express CD4 and CCR5 or CXCR4 (Björndal et al., 1997; Deng et al., 1997). While these cell lines

are invaluable tools for studying certain aspects of HIV-1 entry and infection, coreceptors used on these cell lines may not accurately reflect coreceptor use on primary cells (Collman, 1992; Tokunaga et al., 2001). Previous efforts by our lab to define the coreceptors used by a small number of prototype R5X4 HIV-1 on primary cells revealed these viruses can infect CD4+ macrophages using either CCR5 or CXCR4; however, in CD4+ lymphocytes, CXCR4 was the predominate coreceptor used by R5X4 HIV-1 while CCR5-mediated entry made only a marginal contribution to infection. In the same study, R5 viruses readily infected CD4+ lymphocytes suggesting CCR5 use by R5X4 viruses is severely impaired on these cells (Yi, Shaheen, and Collman, 2005). It is unclear how widespread the impairment to lymphocyte CCR5 use is for R5X4 viruses, and what factors regulate coreceptor use on primary lymphocytes. Given the selection for CCR5 use during transmission and the fact that CD4+ lymphocytes are likely the earliest cells infected in an HIV-1 recipient, understanding the factors that regulate CCR5 use on lymphocytes by R5X4 viruses may help elucidate why these viruses fail to establish infections following transmission.

Goals of this thesis

The goal of this thesis is to better understand the patterns of coreceptor use by R5X4 HIV-1 and to identify the factors that regulate CCR5 use on CD4+ lymphocytes by this virus strain. In chapter 3, I determine the extent of CCR5 use on lymphocytes by a panel of R5X4 HIV-1 viruses and examine the role of CCR5 expression on CCR5-mediated infection of CD4+ lymphocytes by these viruses. My findings reveal a limited

spectrum of lymphocyte CCR5 use by the R5X4 viruses in this panel that is determined, in part, by low CCR5 expression. Increasing CCR5 levels on CD4+ lymphocytes substantially increased CCR5-mediated entry by all but one virus, 89.6. In chapter 4, I determine the efficiency of the Env-CCR5 interaction based on sensitivity to CCR5 antagonist, CCR5 antibody blocking and infection of a cell line that expresses inducer-controlled levels of CD4 and CCR5. I observed a significant correlation between greater lymphocyte CCR5 use and both resistance to CCR5 blocking and reduced sensitivity to changes in CCR5 expression. My results suggest viruses with greater lymphocyte CCR5 use have more efficient interactions with CCR5, which indicates this interaction is the main determinant of CCR5 use on these cells. In chapter 5, I address the impact of mutations within V3 on the efficiency of Env interaction with CCR5 and coreceptor use by R5X4 HIV-1. My results show mutating an arginine at position 11 within the V3 of 89.6 to a serine increased the efficiency of interaction with CCR5 and changed coreceptor use on CD4+ lymphocytes from preferential CXCR4 use to predominant CCR5-mediated entry. Mutating this position resulted in 89.6 and C2-16 variants with altered use of the CCR5 N-terminus. This indicates that V3, and position 306 in particular, is a principal determinant of Env-CCR5 interactions and CCR5 use for some, but not all, R5X4 viruses. Thus, low CCR5 expression is a major obstacle to CCR5 use on lymphocytes by R5X4 viruses, and the limited lymphocyte CCR5 use by R5X4 viruses is regulated by the efficiency of the Env-CCR5 interaction. For most R5X4 viruses, the interaction is inefficient resulting in poor lymphocyte CCR5 use; however, mutations that increase the efficiency of this interaction can enhance CCR5-mediated infection of CD4+ lymphocytes.

Chapter II - Materials and Methods

Primary cells and cell lines

CD4+ lymphocytes were isolated by negative selection from whole blood (Stem Cell Technologies, Vancouver, BC, Canada). Purified lymphocytes were maintained at 10^6 cells/ml in RPMI (Invitrogen, Carlsbad, CA) with 10% FBS (Thermo Scientific, Waltham, MA) and stimulated for 3 days with 5 μg/ml of phytohemagglutinin (PHA; MP Biomedical, Solon, OH) or in 24 well plates coated with 5 μg/ml of anti-CD3 (OKT3; a gift of M. Betts, U. of Pa.) and anti-CD28 (clone 28.2; Beckman Coulter, Fullerton, CA) antibodies. Cells were then either infected and maintained thereafter in IL-2, or cultured for 10 days in IL-2 (300 U/ml; Proleukin, Novartis, Basel, Switzerland) to upregulate CCR5 prior to infection. 293 Affinofile cells that express CCR5 and CD4 under independent regulation were maintained in DMEM (Thermo Scientific) with 10%FBS, blasticidin, G418, hygromycin and zeocin (Invitrogen) (Johnston et al., 2009; Lassen et al., 2009). 293, U87/CD4, U87/CD4/CXCR4 and U87/CD4/CCR5 cells were obtained from the NIH AIDS Research and Reference Reagent Program (Björndal et al., 1997; Graham et al., 1977). 293 cells were maintained in DMEM with 10% FBS, and the U87 cell lines were maintained in selective media containing 1 μg/ml of puromycin (MP Biomedical, Solon, OH) or 300 μg/ml of G418 (Invitrogen, Carlsbad, CA) for selection of CD4 or CCR5 and CXCR4 respectively.

HIV-1 Env, CD4 and coreceptor expression vectors

HIV-1 R5X4 prototypes 89.6, DH12, and primary isolate NR Env clones were derived from patients with advanced disease and have been previously described

(Collman et al., 1992; Ray et al., 2007; Shibata et al., 1995). Env clone R3A was derived from an unusual acute seroconverter found to harbor R5X4 variants during acute infection, and it has been described previously (Meissner et al., 2004; Yu et al., 1998). R5X4 variants C2 and DR were obtained from two HIV-infected CCR5-null individuals homozygous for the Δ32 allele (Gray et al., 2006). The X4 isolate Tybe (from CSF) and R5 strains JRFL (from brain) and Bal (from lung) have also been described previously (Gartner et al., 1986; Koyanagi et al., 1987; Yi et al., 2003a). Prototype 89.6, R3A, JRFL and Tybe Envs along with NR Envs were subcloned into the expression vector pCAGGS (Niwa, Yamamura, and Miyazaki, 1991) using standard methods. The expression vectors used to subclone the remaining vectors have been previously described (Helseth et al., 1990; Kim et al., 2001). 89.6 and C2-16 envelope clones with mutations at position 11 within the V3 loop were generated using a Quick Change XL mutagenesis kit (Stratagene, La Jolla, CA). NR Env clones were a gift from R. Doms (U. of Pa.), R3A was obtained from J. Hoxie (U. of Pa.) and kindly provided by L. Su (UNC-Chapel Hill), and JRFL, DH12 and Bal Env clones were generously provided by M. Cho (Case Western Reserve).

CCR5 N-terminal deletion (Δ2-5, Δ2-9, and Δ2-13) mutant coreceptors were kindly provided by R. Doms and have been previously described (Blanpain et al., 1999). CCR5 N-terminal tyrosine (Y3A, Y10A, Y14A and Y15A) mutant coreceptors were kindly provided by G. Leslie and J. Hoxie (U. of Pa.), and were created using the Quick Change XL mutagenesis kit and sequence specific primers containing the appropriate nucleotide substitutions. Coreceptors with these mutations have been described elsewhere (Dragic et al., 2000; Genoud et al., 1999; Lee et al., 1999a; Siciliano et al., 1999)

Pseudotype virus production

HIV-1 Env luciferase pseudotype viruses were generated by cotransfecting a plasmid that expressed HIV-1 structural proteins (pCMVΔP1ΔenvpA), a plasmid that expressed the packaged luciferase reporter (pHIV-1 luc), and an HIV-1 or VSV-G Env plasmid. Plasmids were cotransfected into 293 cells using Fugene transfection reagent (Roche, Palo Alto, CA) at ratios of 1:3:1 as previously described (Sterjovski et al., 2007; Yang et al., 2004). GFP reporter viruses were created by cotransfection of structural gene plasmid pHp1, GFP reporter plasmid pHRET-GFP, the *tat* expression plasmid pCep4-tat, and a plasmid expressing an HIV-1 or VSV-G Env. Plasmids were cotransfected into 293 cells at a ratio of 10:10:1:10 as previously described (Chang et al., 1999). The CCR5 lentiviral expression vector pNL-CCR5 was created from pNL-CD4 by digestion with NotI and XhoI to remove CD4, and the CCR5 gene was amplified (sense primer 5'-TAG TGC TGT TAA CTT GCT CAA TGC-3' and antisense 5'-GAT CAA GGA TAT CTT GTC TTC-3') and subcloned into the NotI and XhoI sites. CCR5 transduction vectors were produced by cotransfecting pRev, pNL-CCR5 and pVSV-G into 293 cells at a ratio of 1:2:1.

HIV-1 Env pseudotype viruses and lentiviral expression vectors were harvested 48 hours after transfection, clarified by centrifugation at 250xg, then stored in 5% sucrose at -80°C until use. Plasmids pCMVΔP1ΔenvpA (Parolin et al., 1996) and pHIV-1-luc (Yang et al., 2004) were generously provided by J. Sodroski (Harvard University), and pNL-CD4 (Tokunaga et al., 2001) was kindly provided by B. Cullen (Duke University). Plasmid pHRET-GFP (Lin et al., 2002) was provided by P. Corbeau (Hôpital Saint Eloi)

and pHp1 and pCep4-tat (Chang et al., 1999) were a gift from J. Zucali (University of Florida) obtained through the NIH AIDS Research and Reference Reagent Program.

CCR5 over-expression in CD4+ lymphocytes

PHA-stimulated CD4+ lymphocytes were pelleted (250xg at 25°C for 5 minutes) and resuspended at $2x10^6$ cells per ml in media containing 8 μg/ml of Polybrene. Cells were then transduced using 200 μl of CCR5 expression vector pNL-CCR5, spin inoculated for 2 hours as described (O'Doherty, Swiggard, and Malim, 2000), then incubated in the presence of 10 U/ml of IL-2 at 37°C and 5% CO_2 for 72 hours. After this period, coreceptor expression was analyzed by FACS and cells were used for infection.

Infection of cell lines and CD4+ lymphocytes

U87 cells were plated in 96 well plates at $1.5x10^4$ cells per well one day prior to infection, infected by spin inoculation at 1200xg for 2 hours with HIV-1 pseudotype viruses (5 ng of p24 antigen per virus), and cultured at 37°C and 5% CO_2 for three days. For blocking studies, U87/CD4/CCR5 cells were pretreated for 1 hour and then infected in the presence of 10 μg/ml of CCR5 mAb or serial dilutions of the CCR5 small molecule antagonists. Luciferase activity was measured by lysing cells in PBS containing 0.1% Triton X-100, combining cell lysate 1:1 with luciferase assay substrate (Luciferase Assay System; Promega, Madison, WI) and measuring luciferase relative light units (RLUs)

using a Dynex technologies microtiter plate luminometer. CCR5 monoclonal antibodies 2D7, 45531 and CTC8 were obtained from the NIH AIDS Research and Reference Reagent Program, and mAb MC-1 (Blanpain et al., 2002) was a gift from M. Mack (University of Munich). The CCR5 blocker M657 (Finke, 2000) was a gift from M. Miller (Merck, W. Point, PA), and Maraviroc (Dorr et al., 2005) (Pfizer Inc., New York City, NY) was obtained through the NIH AIDS Research and Reference Reagent Program.

CCR5 wt and mutant coreceptor use was evaluated in 293 cells by plating cells in 6 well plates at $8x10^5$ cells/well 24 hours before transfection. Cells were transfected using Fugene transfection reagent with 1μg of a plasmid that expressed CD4 either alone or in combination with 1μg of plasmid expressing wt or mutant CCR5. Cells were washed twice with media the day after transfection, and 48 hours post transfection, cells were removed with 2mM EDTA in PBS and replated either at $1.5x10^4$ cells/well in 96 well plates for infection or at $8x10^5$ cells/well in a 6 well plate for FACS. The following day, cells were analyzed for CD4 and coreceptor expression as described below or incubated with AMD3100 (1 μg/ml; Sigma-Aldrich, St. Louis, MO) for one hour then infected with 5 ng of HIV pseudotypes by spinoculation as previously mentioned. Infection levels were determined after 72 hours by lysing cells and measuring luciferase activity in the cell lysates as detailed for U87 cells.

For infection of primary cells, PHA or CD3/CD28-stimulated CD4+ lymphocytes were added to 96 well plates at $2x10^5$ cells per well. Cells were pretreated for 1 hour with saturating concentrations of CCR5 antagonists M657 (5 μg/ml) or Maraviroc (2 μM), the CXCR4 antagonist AMD3100 (5 μg/ml) or a combination of CCR5 and

CXCR4 blockers. Following pretreatment, CD4+ lymphocytes were infected in the presence of the inhibitors using an equal volume or 10 ng of p24 antigen per virus for PHA or CD3/CD28-stimulated cells, respectively. CD4+ lymphocytes were infected by spin inoculation at 1200xg for 2 hours, and then cultured at 37°C and 5% CO_2 in the presence of IL-2 for 3 days. Infection was measured by luciferase activity in the cell lysate as previously described, or by FACS analysis of GFP expression.

Infection of 293 Affinofiles has been described in detail previously (Johnston et al., 2009; Lassen et al., 2009). To briefly summarize, cells were plated at $1.5x10^4$ cells per well in 96 well plates in media containing 2% dialyzed FBS. Cell were allowed to adhere for two days, then media was removed and replaced with media containing a fixed concentration of the CD4-inducing reagent Minocycline (0.625 ng/ml) and varying concentrations of the CCR5 inducer Ponasterone (0-2 μM). After an overnight incubation, cells were pre-treated for at least 1 hour with 1 μg/ml of AMD3100 to block CXCR4 in media containing 10% dialyzed FBS, then infected by spin inoculation using 1 ng of p24 antigen per luciferase pseudotype virus. Infected cells were cultured for 4 days at 37°C and 5% CO_2, after which luciferase activity was measured as described. FACS analysis of CCR5 and CD4 expression was carried out the day of infection on cells that were plated in 6 well plates and maintained in an identical manner.

FACS analysis of CD4, CCR5, CXCR4 and GFP expression

CD4+ lymphocytes were pelleted (250xg for 5 minutes), washed with FACS buffer (PBS containing 1% fetal bovine serum and 0.1% NaN_3), resuspended in 50 μl of

FACS buffer and stained with 1 μl of mAbs to CD4 (clone RPAT-4- Fluorescein isothiocyanate [FITC]-conjugated), CCR5 (clone 2D7-phycoerythrin [PE]-conjugated), and CXCR4 (clone 12G-PE-Cy5-conjugated) (all from BD Biosciences, San Jose, CA). Fluorescence minus one controls as well as cells stained with single antibodies were carried out in parallel. Cells were incubated at room temperature in the dark for 30 minutes then washed and resuspended in FACS buffer at 10^6 cells per ml. Cells were analyzed by a FACS Calibur flow cytometer (BD) using Cell Quest (BD) and FloJo software (Tree Star Inc, Ashland, OR).

To determine the expression of CD4 and wt or mutant CCR5 on transfected 293 cells, cells were detached by incubation for 10 minutes in PBS containing 2mM EDTA, pelleted, washed in FACS buffer then stained with CD4 FITC, CCR5 PE (clone 2D7) and CCR5 (clone 3A9-Allophycocyanin [APC]-conjugated) as mentioned. Cells stained with single antibodies were carried out in parallel, and fluorescence was measured and analyzed as described. For CD4 and CCR5 quantification on 293 Affinofiles, cells were detached as previously mentioned then stained with the CCR5 PE mAb or with CD4 PE mAb (clone S3.5-Invitrogen, Carlsbad, CA) as described. Fluorescence was measured and analyzed by FACS as described, and CD4 and CCR5 binding sites were quantified using FloJo Software by comparing the average mean fluorescence intensity of stained cells to a standard curve created using PE-labeled beads with four distinct, defined quantities of fluorophores (QuantiBrite Beads, BD) as described (Davis et al., 1998). For detection of GFP expression after CD4+ T cell infection by HIV-1 pseudotypes, cells were washed and resuspended in FACS buffer as described 3 days post-infection and

analyzed for GFP fluorescence using the FACS Calibur flow cytometer and FloJo software.

Statistical analysis and PSSM matrix scoring

Statistical analysis was performed using GraphPad Prism 4 software. The EC50 values for M657 and Maraviroc were determined using a sigmoidal dose response equation. Correlations were derived by linear regression analysis, and correlation coefficients and p-values were determined using a 2-tailed Pearson test.

The predicted viral phenotype of R5X4 Envs used in this study was determined using the position-specific scoring matrix (PSSM) program (http://indra.mullins.microbiol.washington.edu/webpssm/). The PSSM algorithm compares amino acids at each position within the V3 of submitted envelopes to the corresponding residues from a database of Envs experimentally analyzed for coreceptor use (R5/X4 matrix) or viral isolates tested for T cell line syncytium-inducing capacity (SI/NSI matrix) as previously described *(Jensen et al., 2003)*.

Chapter III - Identification of the cellular factors that regulate CCR5 use on CD4+ lymphocytes by R5X4 HIV-1

Introduction

Pathogenesis by R5X4 viruses is linked to CXCR4 use in several models. Infection and depletion of lymphocytes in human lymphoid tissue infected *ex vivo* by R5X4 HIV-1 is completely blocked by the CXCR4 antagonist AMD3100 (Glushakova et al., 1999; Malkevitch et al., 2001). Moreover, infection of rhesus macaques with SIV/HIV (SHIV) chimeras carrying R5X4 envelopes induces a disease course similar to infection with X4 HIV-1 in humans (Nishimura et al., 2005; Nishimura et al., 2004). In light of these findings, our lab attempted to define the coreceptors used on CD4+ lymphocytes by R5X4 HIV-1 *in vitro* using prototype viruses. Although infection was quite robust on untreated lymphocytes, we were surprised to see almost no CCR5-mediated infection of lymphocytes by prototype R5X4 viruses when CXCR4 was blocked. Yet, R5 viruses used lymphocyte CCR5 for entry under the same conditions, showing that R5X4 HIV-1 was severely impaired in the use of an otherwise functional coreceptor. Conversely, when CCR5 was blocked, infection was not affected, indicating that R5X4 HIV-1 entry into CD4+ lymphocytes occurred almost exclusively through CXCR4 (Yi et al., 1999; Yi, Shaheen, and Collman, 2005). In contrast to those data, other studies have suggested that lymphocyte CCR5 can be used by some R5X4 strains (Ghezzi et al., 2001; Gray et al., 2006).

Thus, while CCR5 appears to be the secondary entry pathway into CD4+ lymphocytes by R5X4 HIV-1, it is unclear whether R5X4 viruses vary in their CCR5 use and if entry mediated by this coreceptor can substantially contribute to T cell infection. CCR5 expression is one factor that might regulate coreceptor use on CD4+ lymphocytes

by R5X4 HIV-1. CCR5 is expressed at low density on a small percentage of CD4+ lymphocytes, and low levels of receptor expression in other cell types has been associated with cell type-specific restriction of infection. Macrophages are poorly infected by many lab-adapted X4 strains, but increasing CXCR4 expression increases macrophage susceptibility to infection by these viruses (Tokunaga et al., 2001). Furthermore, studies focused on coreceptor use by viruses from different tissues revealed that M-tropic R5 Envs were uncommon in blood, semen and lymph nodes. Interestingly, these Envs were able to mediate infection of CD4+ lymphocytes at levels similar to M-tropic R5 Envs recovered from brain. The lack of M-tropism by peripheral R5 Envs was associated with an inability to infect cells expressing low levels of CD4 and CCR5 (Peters et al., 2004; Peters et al., 2006).

In this chapter, I examine the diversity among R5X4 HIV-1 in the extent to which CCR5 can be used for infection of lymphocytes, and identify factors that regulate coreceptor use by these viruses on CD4+ lymphocytes. To do this, I assembled a diverse panel of R5X4 envelope clones, determined the extent to which CCR5 contributed to infection of CD4+ lymphocytes by pseudotyped luciferase reporter viruses, and examined the effect of increasing CCR5 expression on lymphocyte coreceptor use. My findings show a limited range of lymphocyte CCR5 use among the R5X4 viruses with some viruses exhibiting extremely poor use of this coreceptor. Regardless of CCR5 use, CXCR4 remained the predominant coreceptor used by all viral clones. I also found that increasing CCR5 expression on lymphocytes could markedly enhance entry mediated by this coreceptor for most (but not all) R5X4 viruses. Thus, low CCR5 expression on

CD4+ lymphocytes is responsible for limiting and, in some cases, restricting CCR5-mediated infection of T cells by R5X4 HIV-1 isolates.

Results

A spectrum of restricted CCR5-mediated entry into CD4+ lymphocytes exists among R5X4 viruses

In order to examine the diversity in primary cell coreceptor utilization among R5X4 HIV-1 and facilitate the identification of factors that influence primary cell coreceptor use, we assembled a panel of diverse R5X4 *env* clones derived from divergent sources. Luciferase reporter viruses were generated that carried primary isolate R5X4 HIV-1 envelope clones from three different infected individuals (DR, C2, NR), using several individual Envs from within each swarm (Gorry et al., 2002b; Gray et al., 2006; Ray et al., 2007). We also studied three widely used prototype R5X4 clones (89.6, DH12 and R3A) which are also primary isolate-derived (Collman et al., 1992; Meissner et al., 2004; Shibata et al., 1995), along with control R5 and X4 Envs Bal and Tybe, respectively (Hwang et al., 1991; Yi et al., 2003a). Of note, 89.6, DH12 and NR *envs* were derived from late-stage AIDS patients (Collman et al., 1992; Ray et al., 2007; Shibata et al., 1995), which is the most common situation in which these variants are seen. R3A was an unusual R5X4 isolate in that is was obtained at the time of seroconversion from an individual infected via intravenous drug use who exhibited rapid disease progression (Meissner et al., 2004; Yu et al., 1998), whereas C2 and DR were isolated from two CCR5-null individuals (Gray et al., 2006). Dual CCR5 and CXCR4

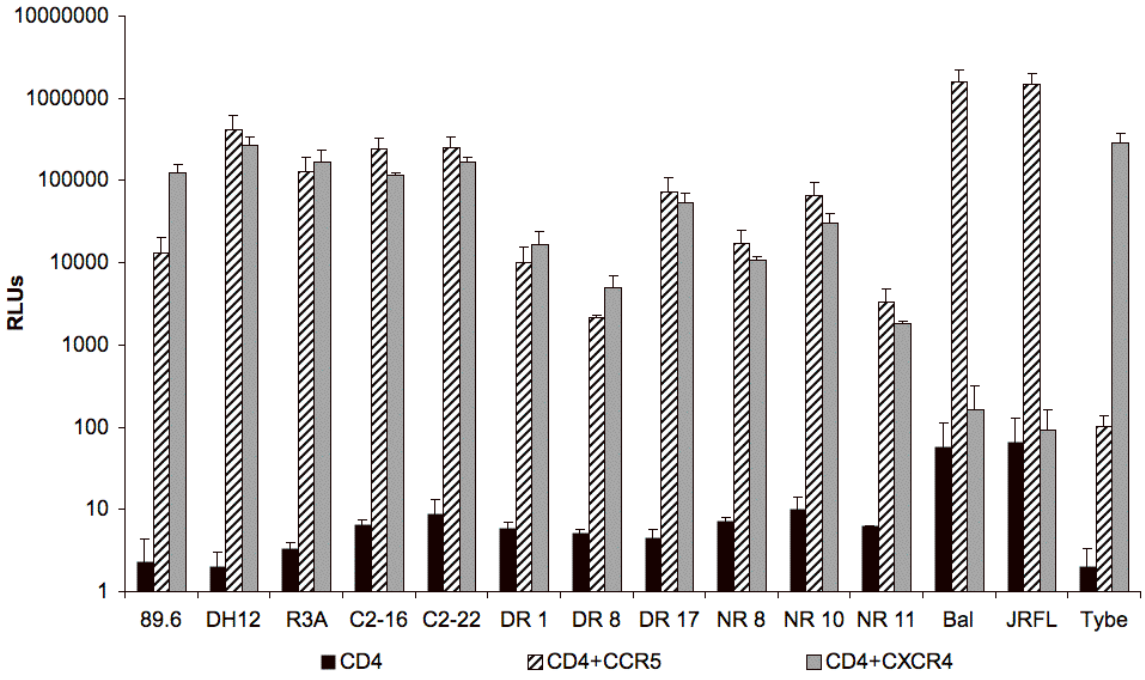

Figure 3.1. Coreceptor use on U87 indicator cells by HIV-1 pseudotype viruses.

U87 indicator cells expressing only CD4, CD4 and CCR5, or CD4 and CXCR4 were infected with HIV-1 luciferase reporter viruses pseudotyped with primary isolate envelopes (C2, DR, NR), prototype R5X4 envelopes (89.6, DH12 or R3A), R5 (Bal or JRFL) or X4 envelopes (Tybe) as controls. Three days later, infection levels were determined by measuring luciferase activity in cell lysates. The results are expressed in relative light units (RLUs) and represent means +/- standard error (SEM) for two experiments performed in triplicate.

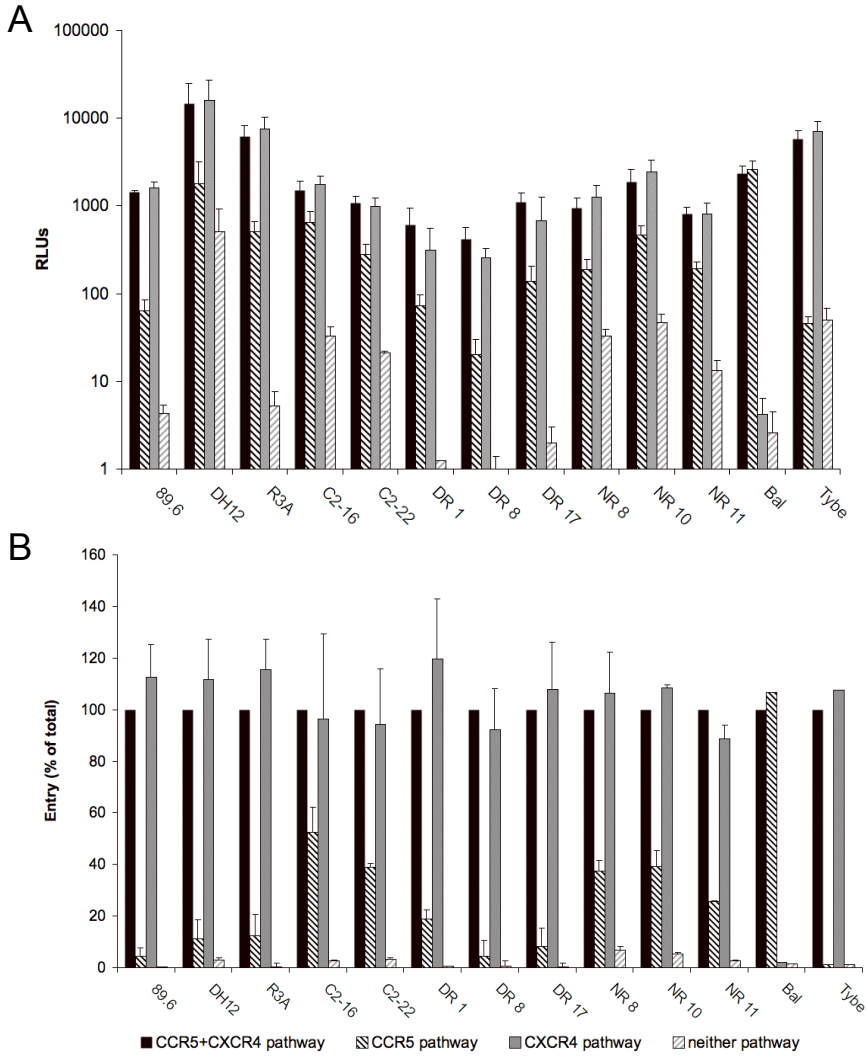

Figure 3.2. CXCR4 is the principal coreceptor on PHA/IL-2 stimulated CD4+ lymphocytes for R5X4 HIV-1 primary and prototype strains.

CD4+ T cells were stimulated with PHA for 3 days then treated for 1 hour before infection with or without the CXCR4 blocker AMD3100 (5µg/ml; "CCR5 pathway"), CCR5 blocker M657 (5µM; "CXCR4 pathway") or both ("neither pathway"). Cells were then infected with HIV-1 luciferase reporter viruses in the continued presence of coreceptor blockers and maintained with IL-2 (10 U/ml). Four days post infection, viral entry was determined by luciferase activity in cell lysates. Results shown for each virus are presented in RLUs (A) and as a percentage of the RLUs seen in the absence of coreceptor blockers (B). Data are means +/- SEM of three experiments using lymphocytes from different donors, each done in duplicate.

use for each virus was confirmed by infecting U87 cells that expressed CD4 and either CCR5 or CXCR4 (Fig. 3.1).

In order to determine coreceptor use by these viruses on primary lymphocytes, CD4+ T cells were infected in the absence or presence of CCR5 or CXCR4 antagonists M657 and AMD3100, respectively (Fig. 3.2). As expected, Bal was completely inhibited by the CCR5 antagonist M657 and Tybe by the CXCR4 antagonist AMD3100. When CD4+ lymphocytes were infected with R5X4 pseudotypes, a different pattern was evident. All R5X4 strains were markedly inhibited by CXCR4 blockade, whereas CCR5 blocking had no detectable effect. For all R5X4 strains, however, the addition of M657 to AMD3100 further reduced entry levels (Fig. 3.2a). These results indicate that all R5X4 viruses tested entered CD4+ lymphocytes through both CXCR4 and CCR5, but that infection through CCR5 was markedly less than that through CXCR4, and played little, if any, additional role when both coreceptors were available.

To quantitate the relative contribution of each coreceptor to infection of CD4+ lymphocytes, the luciferase levels in the presence of coreceptor antagonists were normalized to the total infection capacity for each virus in untreated cells (Fig. 3.2b). Blocking CCR5 alone had no inhibitory effect on infection for any of the viruses. However, CXCR4 blocking, when compared to dual coreceptor blocking, revealed CCR5-mediated infection that ranged from ≤10% for the three prototype strains and two of the DR primary isolate Envs, to 50% of untreated levels for the C2-16 primary isolate Env. Thus, CXCR4 is the predominant coreceptor used by R5X4 viruses to infect CD4+ lymphocytes. However, some entry can be mediated by CCR5, and that proportion varies between isolates indicating a spectrum of lymphocyte CCR5 use.

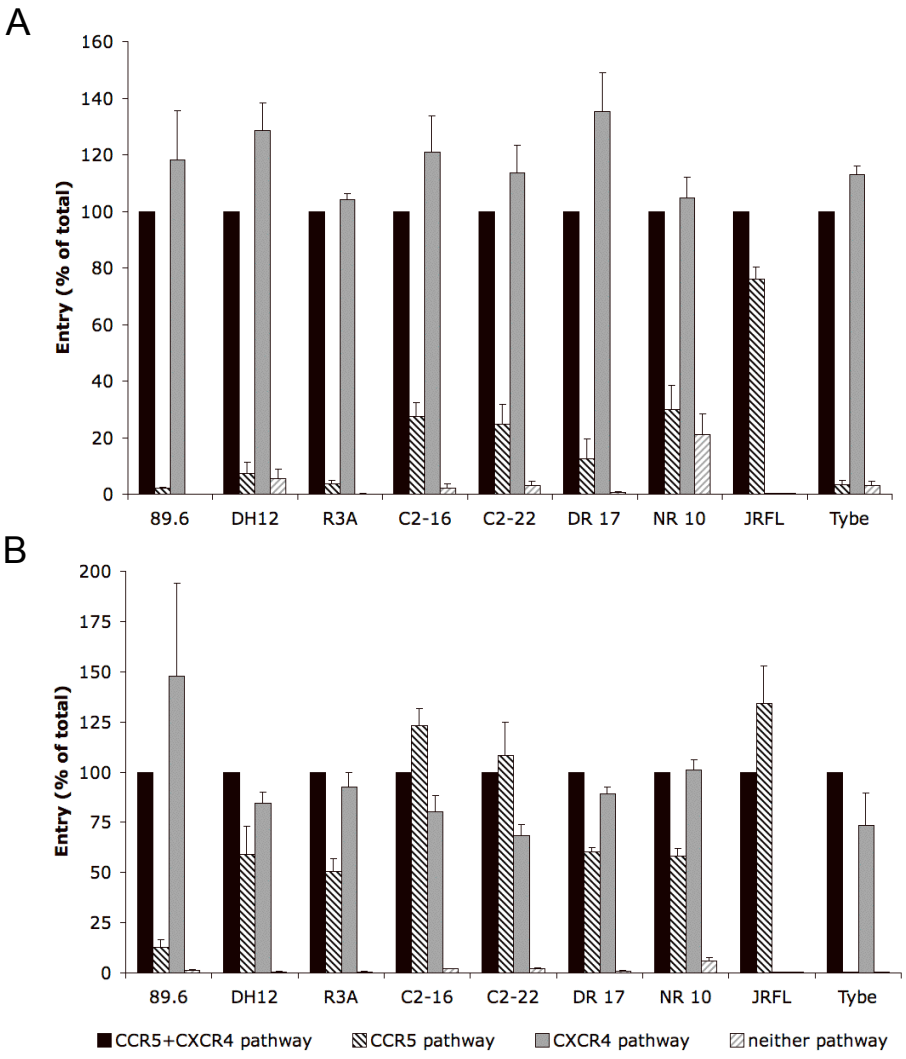

Figure 3.3. CCR5 upregulation on CD4+ lymphocytes increases the proportion of R5X4 infection mediated by CCR5.

CD4+ lymphocytes were CD3/CD28 costimulated for three days and then infected with HIV-1 luciferase pseudotype viruses (A) or cultured with IL-2 for 10 additional days to upregulate CCR5 expression prior to infection (B). Infections were carried out with or without AMD3100, M657 or both antagonists to block CXCR4 and/or CCR5. Four days later, infection was determined by luciferase activity in cell lysates. Entry was normalized as a percentage of the RLUs seen in the absence of coreceptor blockers for each virus. Data are means +/- SEM of three experiments using lymphocytes from different donors, each done in duplicate.

CCR5 upregulation on CD4+ lymphocytes increases the proportion of R5X4 HIV-1 entry mediated by CCR5

These data indicate that CCR5-mediated infection of CD4+ lymphocytes by many R5X4 HIV-1 is restricted (Fig. 3.2), yet all viruses in question can use this coreceptor to readily infect indicator cells such as U87 CD4/CCR5 cells (Fig. 3.1). CCR5 on indicator cells is typically over-expressed, however, while peripheral blood lymphocytes express CCR5 at low density and on a minority of cells (Lee et al., 1999b; Wu et al., 1997). We therefore sought to test whether low levels of CCR5 expression were an obstacle to efficient CCR5 use on CD4+ lymphocytes by R5X4 viruses, and how CCR5 expression levels regulated pathway-specific R5X4 lymphocyte entry. To address the role of CCR5 expression, CD4+ lymphocytes were infected with HIV-1 luciferase pseudotype viruses immediately after 3 days of CD3/CD28 costimulation, or after 10 additional days of culture with 300 U/ml of IL-2, which has been shown to upregulate lymphocyte CCR5 (Creson et al., 1999; Yang et al., 2001). Infections were performed in the presence or absence of CCR5 and CXCR4 antagonists to determine proportional use of each entry pathway. Coreceptor expression as determined by surface staining indicated that few CD4+ lymphocytes were CCR5+, whereas 20-40% had detectable CCR5 after prolonged culture. In addition to the increase in percentage of cells within the CCR5+ gate, the mean fluorescence intensity (MFI) of cells within the CCR5 gate increased as well (approximately 2-fold). In contrast, the percentage of cells expressing CXCR4 decreased to a modest degree but remained higher after culture with IL-2 (50-70%).

As shown in Fig. 3.3a, CCR5 use by R5X4 HIV-1 was restricted in day 3 CD3/CD28-stimulated CD4+ lymphocytes, with CXCR4 being the predominant pathway used for entry. However, there was a modest spectrum of CCR5 use similar to that seen in PHA-stimulated lymphocytes (Fig. 3.2). In contrast, CCR5 upregulation following 10 days of IL-2 stimulation dramatically increased the proportion of infection mediated by CCR5 for most R5X4 viruses tested, with the exception of 89.6, which remained nearly completely CXCR4 dependent (Fig. 3.3b).

Nevertheless, for those viruses that did increase the proportion of entry mediated by CCR5, CXCR4 remained the predominant coreceptor used for infection of day 13 CD4+ lymphocytes by most R5X4 strains. Exceptions to this coreceptor usage pattern were seen in the two C2 Envs (C2-16 and C2-22) for which CCR5 consistently mediated an equal or greater proportion of entry than did CXCR4 on CCR5-upregulated CD4+ T cells (Fig. 3.3b). VSV-G pseudotypes, used as a control, showed no change in luciferase expression from day 3 to day 13 cells (data not shown).

The percentage of CD4+ lymphocytes infected through each coreceptor is modulated following CCR5 upregulation

In addition to total infection levels within the cultures, we also aimed to define the contribution of each pathway to infection of individual CD4+ T lymphocytes following CCR5 upregulation. CD4+ lymphocytes were cultured for 3 or 13 days as described above to establish standard or high CCR5 expression conditions, respectively, and then infected with HIV-1 Env pseudotype virions carrying a GFP reporter gene. Infection-

mediated GFP expression therefore enabled per-cell analysis of infection based on GFP expression as determined by FACS. As with luciferase infection, addition of both CCR5 and CXCR4 antagonists completely blocked GFP expression (data not shown).

As shown in Fig. 3.4a, CCR5-mediated entry into day 3 CD4+ lymphocytes resulted in a low percentage of GFP-positive infected cells. This value ranged from barely detectable (DH12) to ~5% (C2-16 and the R5 Env JRFL). While this result is concordant with the low percentage of cells positive for CCR5 expression by FACS, it was interesting to note that the fraction of GFP+ cells for C2-16 and JRFL often exceeded the proportion that were CCR5+ by FACS. Entry by these viruses was clearly mediated by CCR5, however, since infection was blocked by M657 or Maraviroc (data not shown). This finding indicates that susceptibility to infection by efficient CCR5-using Envs can be a more sensitive indicator of CCR5 expression than immunostaining, and thus, these isolates can use CCR5 at levels that are below the threshold of detection by FACS. Furthermore, the fact that some strains can exploit CCR5 to infect what appear to be CCR5-negative CD4+ T cells by FACS indicates that, following upregulation, the apparent increases in percentage of CCR5+ cells also reflects increased CCR5 expression levels by cells that are initially CCR5+ but at a level below the threshold detectable by flow cytometry.

When CCR5 was upregulated on CD4+ lymphocytes by prolonged culture in IL-2 (Fig. 3.4a, right), the percentage of GFP+ cells infected through CCR5 increased dramatically for the R5 isolate JRFL. Similarly, the percentage of GFP+ cells infected through CCR5 also increased for R5X4 viruses following CCR5 upregulation, although these percentages remained less than that for JRFL. Infection with VSV-G pseudotypes,

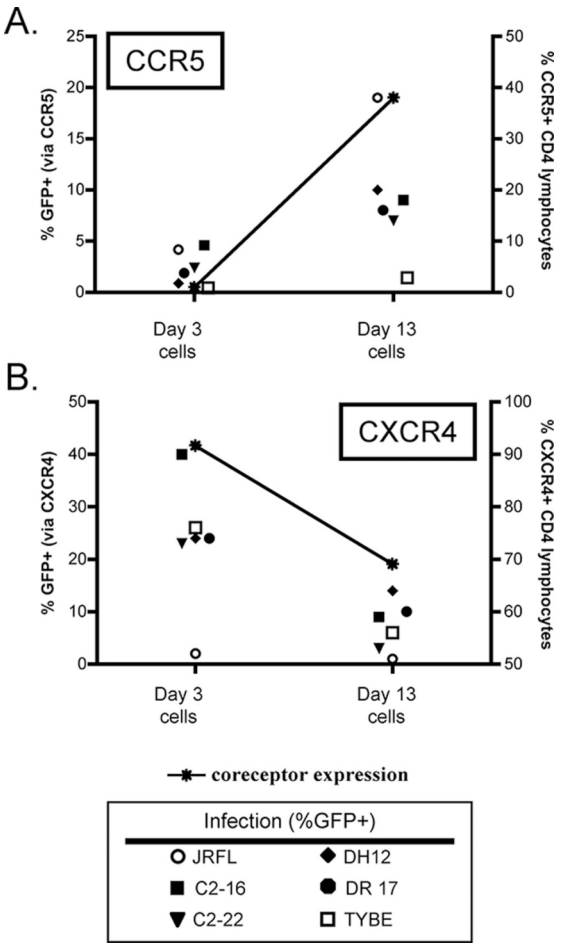

Figure 3.4. Stimulation-dependent changes in coreceptor expression affect the percentage of CD4+ lymphocytes infected via each pathway.

CD4+ lymphocytes were infected with HIV-1 GFP pseudotype viruses after 3 days of CD3/CD28 costimulation or following an additional 10 days of culture with IL-2 to upregulate CCR5. Cells were incubated with coreceptor antagonists for 1 hour prior to and throughout the infection to define the pathway of entry. CCR5 and CXCR4 expression were analyzed by FACS on the day of infection, and four days later, infected cells were determined by FACS analysis for GFP expression. Data indicate the percentage of GFP+ cells infected through CCR5 (A) or CXCR4 (B) at day 3 and day 13 after isolation, and are representative of three independent experiments using cells from different donors. The percentage of cells expressing CCR5 (A) and CXCR4 (B) at the time of infection is indicated by an asterisk (*).

used as a control for possible effects independent of coreceptor-mediated entry, showed no change in infection (54±6% GFP+ on day 3 cells and 44±8% on day 13 cells; data not shown). Thus, both R5X4 and R5 Envs are highly dependent on CCR5 expression levels, although R5 JRFL is more efficient at exploiting limiting levels than R5X4 Envs (Fig. 3.4a).

In contrast to CCR5, CXCR4 levels typically decreased somewhat from day 3 to day 13, and this was associated with a modest decrease in the percentage of cells infected via CXCR4 for the R5X4 viruses tested, as well as the X4 strain Tybe (Fig. 3.4b).

Coreceptor-dependent R5X4 entry following lentiviral vector CCR5 over-expression

Although 10 days in culture with IL-2 upregulated CCR5 levels and modulated coreceptor utilization, we considered the possibility that the extended period *in vitro* could also effect other factors that impact infection, despite the lack of changes in VSV-G pseudotype infection. For that reason, we used a second approach to upregulate CCR5. CD4+ lymphocytes were transduced after 3 days of PHA stimulation with a lentiviral vector expressing CCR5 or a mutated version of the coreceptor that is not expressed on the cell surface. CCR5 levels on transduced cells were measured by FACS, and cells were infected with prototype R5X4 Env luciferase viruses in the presence or absence of AMD3100. Transduction with the CCR5 vector increased the proportion of CCR5+ cells to 18-70% of CD4+ lymphocytes compared with 1-19% of cells transduced with a control vector (Fig. 3.5), and the MFI of CCR5+ increased as well (fold increase of 2.9±0.9; data not shown). CCR5 over-expression led to a marked increase in entry

mediated by CCR5 for R5X4 pseudotypes R3A and DH12 compared to control vector-transduced cells (Fig. 3.5). In contrast, entry mediated by CCR5 increased only marginally for strain 89.6. CCR5 transduction had no impact on the MFI of CD4 or CXCR4 expression and did not impact infection by the X4 virus Tybe (data not shown). Results from the lentiviral vector CCR5 over-expression studies are similar to those following stimulation-induced upregulation (Fig. 3.3), supporting the notion that entry as measured here reflects changes in coreceptor expression and not other features of the cell condition that might modulate infection.

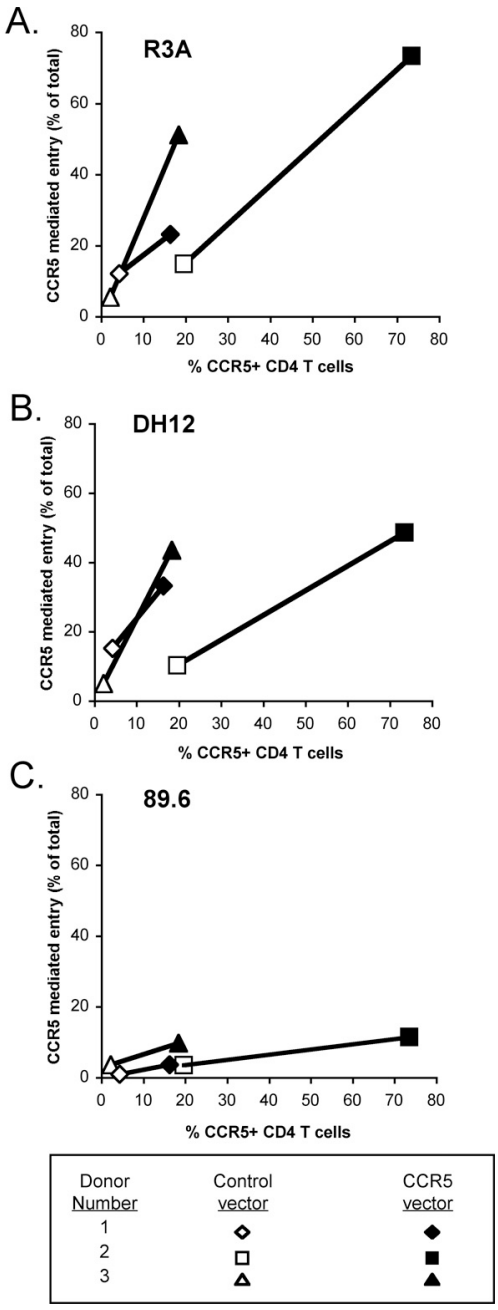

Figure 3.5. Lentiviral over-expression of CCR5 increases the proportion of R5X4 entry mediated by CCR5 on CD4+ lymphocytes.

CD4+ lymphocytes were stimulated with PHA for 3 days, transduced with either control or CCR5-expressing lentiviruses, and maintained in the presence of IL-2. Two days post-transduction, cells were pretreated for 1 hour with CXCR4 blocker AMD3100 and infected with HIV-1 luciferase pseudotypes in the continued presence of blocker. Infection was determined by luciferase activity in cell lysates four days later. The results are shown as the proportion of CD4+ lymphocyte infection mediated through CCR5 for R3A (A), DH12 (B) and 89.6 (C) on the Y-axis, plotted against the percentage of CD4+ lymphocytes that express CCR5 after transduction with a control or CCR5 expression vectors. Data are from three independent experiments using cells from different donors.

Conclusions

Coreceptor use and target cell tropism are important determinants of HIV-1 transmission and pathogenesis. In previous studies of primary cell tropism and coreceptor use by prototype R5X4 HIV-1 strains, we found that these viruses could use CCR5 and CXCR4 to infect macrophages, but in CD4+ lymphocytes, CXCR4 was the predominant entry pathway while CCR5 was used poorly, if at all. Here I sought to determine, employing a variety of dual-tropic isolates from disparate sources, whether R5X4 strains vary in their ability to use CCR5 on lymphocytes, and what factors were responsible for determining use of this pathway.

My findings reveal limited CCR5-mediated infection of CD4+ T cells by the R5X4 strains tested, which did not substantially contribute to overall infection levels when CXCR4 was available. However, a spectrum existed among the R5X4 variants in the extent to which lymphocyte CCR5 could be used if CXCR4 was not available. Notably, while one R5X4 virus could reach CCR5-mediated infection levels approaching half that of untreated cells, R5X4 strains with marginal CCR5 use ($\leq$ 10% of total entry) were more common. CCR5 is expressed at low levels on CD4+ lymphocytes. My results show that increasing CCR5 expression increased CCR5 use on CD4+ T cells by nearly all viruses, including R5X4 strains that were among the most restricted in the use of this coreceptor. However, 89.6 remained unable to use lymphocyte CCR5 even after increasing CCR5 expression on these cells. Thus, we conclude that CCR5 expression on CD4+ lymphocytes is an important regulator of the use of this coreceptor by many R5X4 viruses, but that other obstacles to lymphocyte CCR5 use may exist.

Even for R5X4 viruses with relatively greater lymphocyte CCR5 use, blocking this pathway had no impact on CD4+ T cell infection, as entry through CXCR4 alone was equivalent to infection when both coreceptors were available. CXCR4 is expressed on the majority of peripheral blood CD4+ lymphocytes, whereas CCR5 is expressed on a smaller subset of cells (Lee et al., 1999b; Ostrowski et al., 1998; van Rij et al., 2000; Wu et al., 1997). Most cells that are CCR5+ also express CXCR4; so blocking CCR5 may have little net effect on infection because entry can still occur through CXCR4. Conversely, since many CXCR4+ lymphocytes do not express CCR5, entry in the presence of CXCR4 blockers should be representative of the subset of cells that express CCR5. As demonstrated by my findings following CCR5 upregulation, increasing the subset of cells that express this coreceptor contributes to the increased proportion of infection mediated by CCR5.

If CCR5 use was determined solely by the percentage of lymphocytes that express this coreceptor, then CCR5-mediated infection should be equivalent for all R5X4 variants (and should be equivalent to infection by R5 variants), yet that was not observed. CCR5 use on lymphocytes varied among the R5X4 viruses, and this variation appeared to be related to the efficiency of interaction with CCR5. The percentage of lymphocytes infected using CCR5 by C2-16, the R5X4 strain with the greatest CCR5 use, were higher than the percentage of CCR5+ cells measured by FACS suggesting this virus can detect CCR5 better than some CCR5 specific antibodies. Furthermore, the two R5X4 strains with the greatest CCR5 use at endogenous levels were also the only viruses that showed a shift in preferential coreceptor use to CCR5 after upregulation of this coreceptor. This implies the R5X4 envs differ in the efficiency of interaction with CCR5 and the increased

CCR5 density following CCR5 over-expression was a critical factor in the increased CCR5-mediated infection of lymphocytes by R5X4 viruses. It is not clear from this study if CCR5 density or the percentage of CCR5+ cells plays a larger role in regulating use of this coreceptor on lymphocytes by R5X4 viruses.

The pattern of strain-dependent differences in the ability to use CCR5 on lymphocytes also strongly suggests a role for Env in regulating use of this coreceptor on lymphocytes by R5X4 viruses. Although CCR5 use by most of the viruses with extremely poor use of this coreceptor could be improved by increasing CCR5 expression, this was not the case for 89.6. Regardless of the method or magnitude of CCR5 over-expression, the virus remained extremely dependent on CXCR4 for infection. The nature of restricted CCR5 use by this virus is unclear, but results following CCR5 upregulation indicate CCR5 levels alone are not responsible for impaired lymphocyte CCR5 use by the 89.6 Env.

In summary, this study confirms with a panel of primary and prototype R5X4 HIV-1 variants that these strains are highly skewed towards CXCR4 use for entry into CD4+ T cells. However, there does exist a range among the isolates in the relative ability to use CCR5 on primary lymphocytes. CCR5 use by nearly all R5X4 viruses could be improved by CCR5 over-expression in lymphocytes, and this was sufficient to eliminate the barrier to CCR5 use for most, albeit not all, of the R5X4 isolates. These results indicate low lymphocyte CCR5 expression is a factor in the restriction to R5X4 entry through this pathway, although additional obstacles to primary cell coreceptor use exist. Better understanding of the viral and cellular factors that control coreceptor use by HIV-1 strains is critical to understanding the impact of viral evolution on target cell tropism.

Chapter IV - Efficiency of the Env-CCR5 interaction determines the spectrum of CCR5 use on CD4+ lymphocytes by R5X4 HIV-1

Introduction

HIV-1 Envs can differ in the ability to fuse viral and cellular membranes, and this variation in entry efficiency can lead to differences in infectivity and pathogenicity between viral strains. Longitudinal studies of HIV-1 infected patients that harbor only R5 viruses show that isolates from advanced stages of disease replicate to higher titers and are more cytopathic than isolates recovered during the asymptomatic phase of infection. Subsequent experiments using chimeric viruses indicated that greater infectivity of late R5 isolates was Env-dependent (Olivieri et al., 2007; Repits et al., 2005). Animal models of HIV-1 infection also suggest links between entry efficiency and pathogenesis. *In vivo* passage of SHIV-1 89.6 in rhesus macaques resulted in a variant that aggressively depleted CD4+ lymphocytes and caused much more rapid disease progression than infection with the parental virus (Karlsson et al., 1997). The difference in pathogenicity between these variants was associated with higher levels of replication on rhesus PBMCs and greater ability to induce syncytia by the passage virus. In these studies, the phenotypic differences between the parental and passaged SHIV-1 were dependent on the viral Env (Karlsson et al., 1998).

Env-coreceptor interactions and receptor density both influence entry efficiency. Changes in entry efficiency that result from differences in coreceptor binding or coreceptor expression level can impact sensitivity to entry inhibitors. Indeed, mutations that increase coreceptor binding and elevate CCR5 expression are both associated with reduced sensitivity to entry inhibitors and faster rates of Env-dependent membrane fusion (Heredia et al., 2007; Reeves et al., 2002). One study reported R5 Envs with lower

CCR5 affinity require higher CCR5 levels for infection (Gorry et al., 2002a). This observation implies that these two factors have an interdependent affect on viral entry, and it suggests that increased coreceptor expression may enhance entry for viruses with inefficient Env-coreceptor interactions.

R5X4 viruses are more sensitive to CCR5 antagonists than R5 viruses (Yi, Shaheen, and Collman, 2005), and in chapter 3, I presented results that showed restricted lymphocyte CCR5 use by R5X4 variants could be rescued by increasing CCR5 expression. These results imply that R5X4 viruses have inefficient interactions with CCR5, and these interactions contribute to impaired lymphocyte CCR5 use by some R5X4 viruses. I also observed a spectrum of CCR5 use by R5X4 strains on CD4+ T cells at endogenous CCR5 levels. Differences in the Env-CCR5 interaction between R5X4 strains may also account for the range of lymphocyte CCR5 use seen among the viruses in this group.

Therefore, in this chapter I examine the efficiency of the R5X4 HIV-1 Env interaction with CCR5, and determine whether differences in this interaction influence lymphocyte CCR5 use by these viruses. To do this, I measured the sensitivity of each R5X4 virus to entry inhibition by antibodies, CCR5 antagonists and the fusion inhibitor T20. Overall, my results show that R5X4 strains differed in their sensitivity to these agents. Furthermore, I discovered a significant correlation between greater CCR5 use on lymphocytes and increased resistance to inhibition by antibodies or antagonists; however, there was no relationship between lymphocyte CCR5 use by R5X4 viruses and sensitivity to T20. I also assessed the impact of CCR5 density on infection by various R5X4 strains using Affinofile cells that allow inducible regulation of CCR5 expression. I found that

R5X4 strains with greater lymphocyte CCR5 use were less affected by declining CCR5 levels. I also observed a significant positive correlation between the ability to exploit low levels of CCR5 on the Affinofile cells and CCR5 use on lymphocytes. My results indicate that the Env-CCR5 interactions regulate CCR5-mediated infection of lymphocytes by R5X4 HIV-1, and strain-dependent differences in this interaction are responsible for the spectrum of lymphocyte CCR5 use by the R5X4 virus panel.

Results

Resistance to inhibition by ECL2-specific CCR5 mAbs correlates with the capacity of R5X4 HIV-1 to use CCR5 for lymphocyte infection

Previous studies comparing CCR5 use by R5 and R5X4 Envs have suggested that R5X4 Envs: i) appear to have a lower affinity for CCR5, ii) are generally more sensitive to inhibition by CCR5 antagonists, and iii) are more affected by changes in the structure of CCR5 (Cormier et al., 2000; Doranz et al., 1997; Lu et al., 1997; Yi, Shaheen, and Collman, 2005; Yi et al., 2003b). Since R5 strains by definition use CCR5 efficiently for lymphocyte entry, our finding that R5X4 strains vary in their capacity to infect primary lymphocytes through CCR5 raised the possibility that these strains might also vary in their interaction with CCR5.

To examine the relationship between Env-coreceptor interactions and lymphocyte CCR5 use, we took advantage of CCR5-specific mAbs with well-characterized target epitopes (Fig. 4.1) (Blanpain et al., 2002; Lee et al., 1999a). U87/CD4/CCR5 cells were infected in the presence or absence of mAbs that target epitopes in the amino terminus (N-terminal), the second extracellular loop (ECL2) or multiple domains (MD) of CCR5, and entry inhibition was determined by normalizing infection levels to those of untreated cells. As shown in Fig. 4.2, both mAbs that targeted CCR5 ECL2 (45531 and MC-1) inhibited entry by the R5X4 viruses. Infection levels were reduced 15-70% by mAb 45531 and 30-90% by MC-1. Treatment with 2D7, another ECL2-directed mAb, completely inhibited infection by all viruses, even at four-fold lower concentrations (data not shown). Importantly, R5X4 Envs that were better able to use lymphocyte CCR5 for

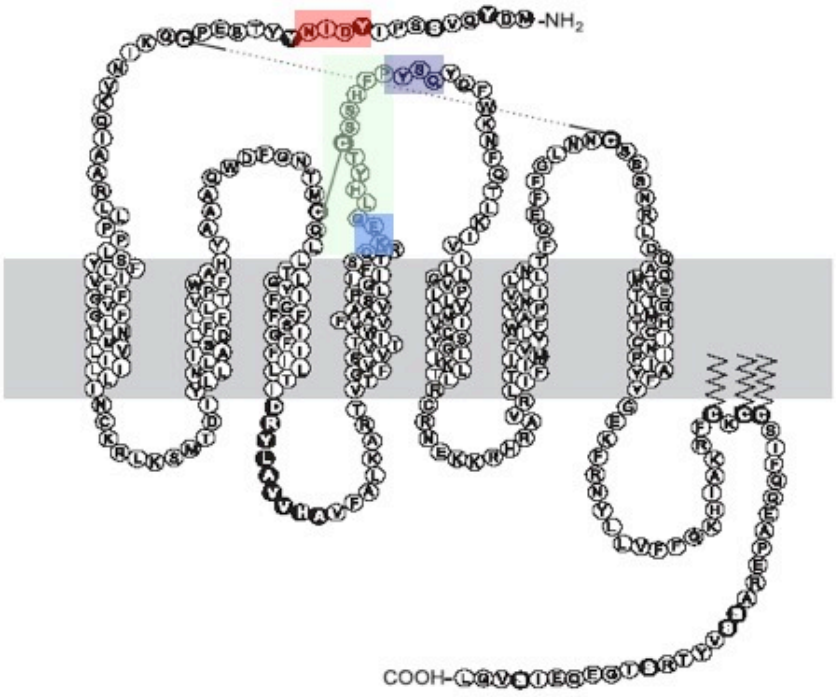

Figure 4.1. Antibody epitopes on CCR5.

The antibody binding sites for CCR5 antibodies CTC8 (red), 2D7 (blue), MC-1 (green) and 45531 (purple) are shown within shaded squares. The epitope for multi-domain antibody 45523 is discontinuous and is not shown.

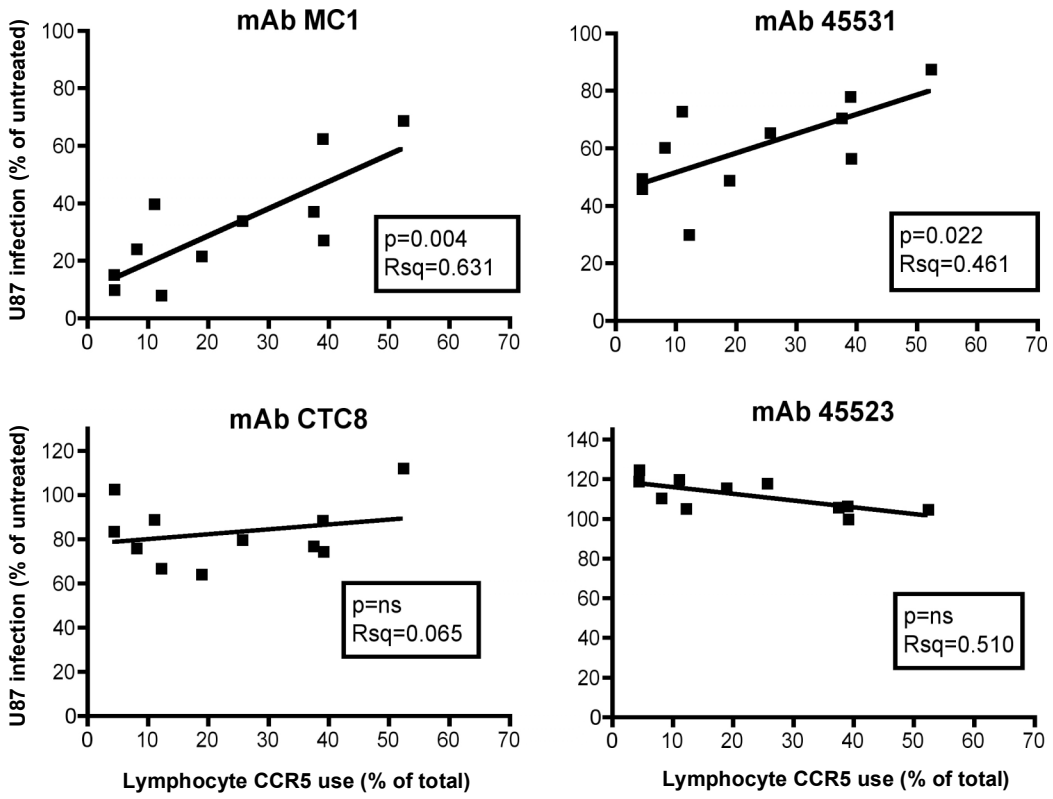

Figure 4.2. CCR5 use on CD4+ lymphocytes correlates with resistance to inhibition by ECL2 mAbs.
U87/CD4/CCR5 cells were treated for 1 hour with or without 10 µg/ml of CCR5-specific mAbs then infected with HIV-1 luciferase pseudotype viruses in the continued presence of mAb. Three days later, infection was determined by luciferase activity in cell lysates and normalized to luciferase activity in untreated cells. Each symbol represents an individual R5X4 virus, and results are presented as the proportion of total lymphocyte entry mediated by CCR5 (from Fig. 3.2b) on the X-axis versus the U87/CD4/CCR5 infection that occurred in the presence of CCR5 mAbs (A) on the Y-axis. Data are means for three experiments each carried out in duplicate.

entry were blocked less efficiently than those viruses that used the pathway poorly, as shown by the significant correlation between lymphocyte CCR5 use and infection in the presence of ECL2-directed mAbs 45531 and MC-1 (Fig. 4.2). In contrast to the ECL2-targeted antibodies, multidomain and N-terminal antibodies (45523 and CTC8, respectively) had a marginal or no effect on entry (Fig. 4.2).

Sensitivity to CCR5 antagonists but not a fusion inhibitor correlates with lymphocyte CCR5 use by R5X4 HIV-1

We then turned to small molecule CCR5 antagonists, which are frequently used as probes to assess Env-coreceptor interactions. Sensitivity to CCR5 blockers M657 and Maraviroc was determined by infecting U87/CD4/CCR5 cells in the presence of increasing concentrations of inhibitor. Infection by all viruses was extinguished at the highest concentrations of each antagonist (data not shown), but there was variation in sensitivity to both M657 and Maraviroc, exemplified by differences in antagonist EC50 values among the R5X4 Envs (Fig. 4.3). M657 and Maraviroc EC50 values were lower for R5X4 Envs with poor lymphocyte CCR5 use, compared to Envs such as C2-16 that are better able to use this coreceptor. Similar to inhibition by ECL2-directed CCR5 mAbs, M657 and Maraviroc EC50 values correlated with lymphocyte CCR5 use (Fig. 4.3). Concordant mAb and antagonist results show a link between lymphocyte CCR5 use and sensitivity to blocking among R5X4 HIV-1, and suggest that the variation in CCR5-mediated infection of lymphocytes results at least in part from differences among these strains in the efficiency of interactions with the coreceptor.

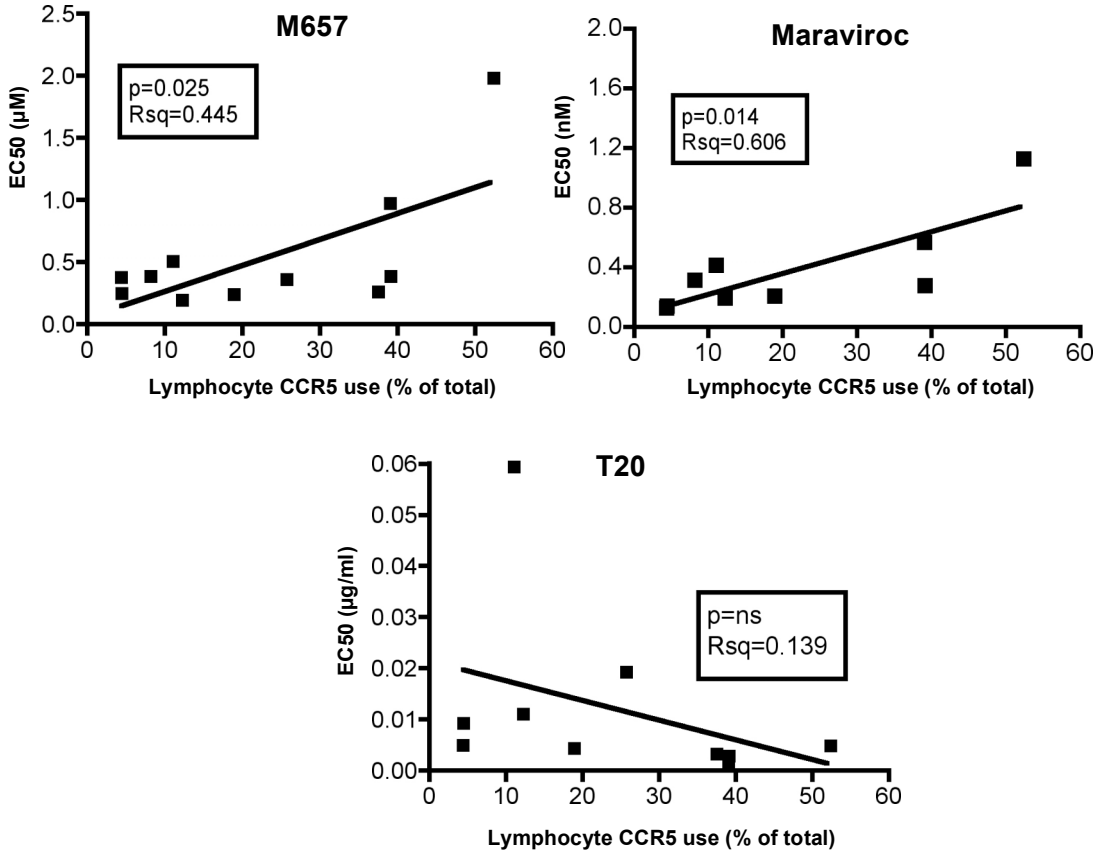

Figure 4.3. CCR5 use on CD4+ lymphocytes correlates with resistance to inhibition by CCR5 antagonists but not T20.

U87/CD4/CCR5 cells were treated for 1 hour with or without increasing concentrations of M657 or Maraviroc then infected with HIV-1 luciferase pseudotype viruses in the continued presence inhibitor. For experiments with T20 increasing concentrations of the drug were added with virus at the time of infection. Three days later, infection was determined by luciferase activity in cell lysates and normalized to luciferase activity in untreated cells. Each symbol represents an individual R5X4 virus, and results are presented as the proportion of total lymphocyte entry mediated by CCR5 (from Figure 3.2b) on the X-axis versus the entry inhibitor EC50 value on the Y-axis. Data are means for three experiments each carried out in duplicate**.**

The fusion inhibitor T20 binds to the HR1 region of gp41 and prevents the conformational changes that lead to membrane fusion. To determine whether R5X4 viruses differ in sensitivity to T20, U87/CD4/CCR5 cells were infected with HIV-1 in the presence of increasing concentrations of drug. Infection by all viruses was inhibited at the highest concentration of T20 (data not shown). While there was a range of T20 EC50 values that were similar for most viruses, DR-17 was substantially less sensitive to the drug with an EC50 value that was over two logs higher than the median EC50 value of the group (data not shown). Consequently, this virus was excluded from subsequent analysis. Unlike the CCR5 antagonists, viruses with greater lymphocyte CCR5 use were not less sensitive to inhibition by T20, and there was no correlation between T20 EC50 value and CCR5 use on lymphocytes by R5X4 viruses (Fig. 4.3). Thus, differences in the efficiency of fusion do not appear to impact lymphocyte CCR5 use by R5X4 viruses.

Greater lymphocyte CCR5 use is associated with reduced sensitivity to CCR5 density for infection of Affinofile cells

Increasing CCR5 expression on CD4+ lymphocytes increased the proportion of infection mediated by CCR5 for R5X4 viruses, suggesting inefficient use of endogenous CCR5 might be linked to sensitivity to coreceptor expression levels. To further address the interplay between CCR5 expression and R5X4 infection, we used 293 Affinofile cells (Fig. 4.4), a cell line in which expression of CD4 and CCR5 can be regulated precisely and independently by Minocycline and Ponasterone, respectively (Johnston et al., 2009; Lassen et al., 2009). The number of CD4 and CCR5 antibody binding sites (ABS) per cell can be determined for each inducer combination using quantitative FACS analysis.

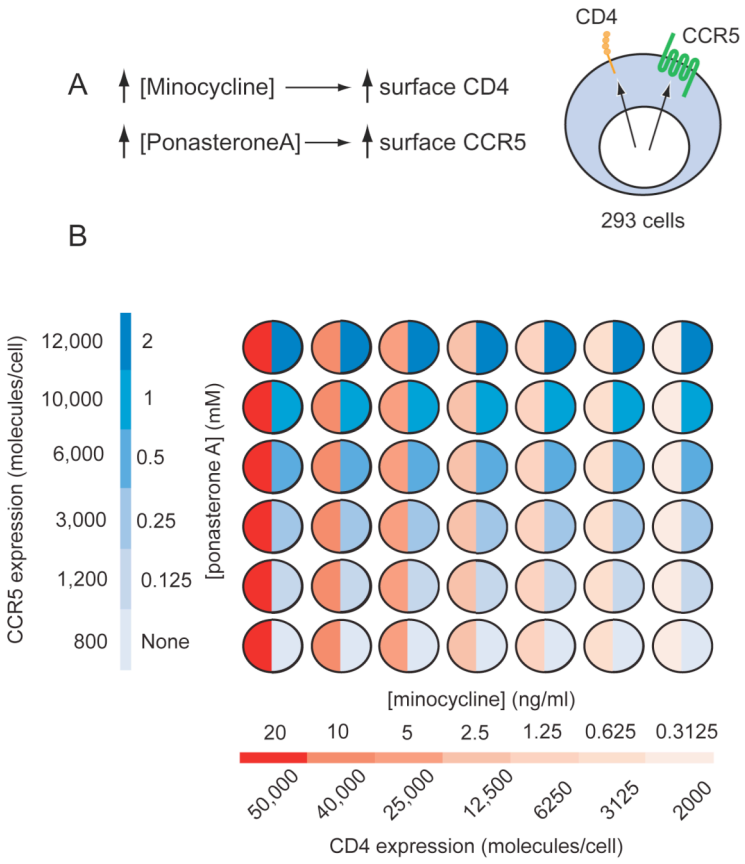

Figure 4.4. Inducible CD4 and CCR5 Affinofile expression system.

Affinofile cells were created by transfecting 293 cells with plasmids expressing CD4 and CCR5 along with separate plasmids expressing inducible transcription factors that are responsive to minocycline and ponasterone. Addition of increasing concentrations of minocycline or ponasterone increases CD4 or CCR5 expression, respectively (A). Addition of different combinations of each inducer allows independent regulation of CD4 or CCR5 levels, which can be quantitated by FACS (B). Figure courtesy of *Johnston, S. et al. J. Viro. 2010*

Induction of Affinofile cells resulted in CD4 expression levels that ranged from 3,000 to 200,000 ABS per cell and CCR5 levels from 1,000 to 50,000 ABS per cell. Importantly, these levels encompass the physiologically relevant range of CD4 and CCR5 levels reported on stimulated CD4+ lymphocytes of 65,000-100,000 and 500-7,000 molecules per cell, respectively (Lee et al., 1999b). Therefore, we chose to examine the sensitivity of R5X4 HIV-1 infection to CCR5 expression levels in Affinofile indicator cells by analyzing the effect of physiologically relevant CD4 expression levels (~83,000 molecules/cell). Cells were maintained with a stable concentration of CD4 inducer and different concentrations of CCR5 inducer, and infected with luciferase pseudotype viruses at the same time that receptor expression levels were confirmed by FACS. Luciferase levels produced at each CCR5 density were normalized to luciferase activity in cells with the highest density of CCR5.

As shown in Fig. 4.5a, at a stable level of CD4 expression, luciferase virus infection was reduced for all R5X4 viruses as CCR5 density decreased. However, there were marked differences in the response to declining CCR5 levels. R5X4 Envs C2-16 and NR10 were least affected by declining CCR5 expression, showing 60 to 80% of maximal infection even at the lowest CCR5 density. A second group of R5X4 viruses, R3A, DR17 and DH12, were more impaired by low CCR5 expression, achieving 35-45% of maximal infection at the lowest CCR5 expression level tested. 89.6 was the R5X4 virus most affected by decreasing CCR5 expression, at the lowest density of CCR5 expression reaching only 25% of its maximal level of infection (Fig. 4.5a). Thus, R5X4 HIV-1 display a spectrum in their ability to use diminishing levels of CCR5 on Affinofile cells in the presence of physiologically relevant CD4 levels.

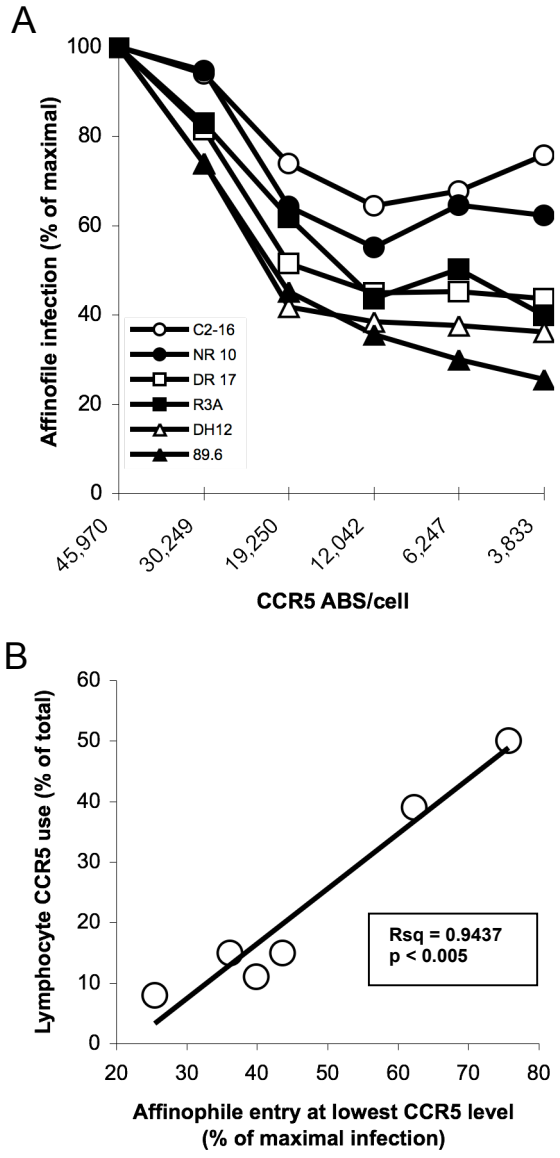

Figure 4.5. Efficiency of CCR5 use on Affinofile cells correlates with CCR5 use on CD4+ lymphocytes by R5X4 HIV-1.

Affinofile cells were induced to express a fixed level of CD4 (83,000 antibody binding sites (ABS)/cell) and varying levels of CCR5, then infected with HIV-1 luciferase pseudotypes in the presence of AMD3100 to block endogenous CXCR4. Infection was quantified 4 days later by luciferase activity in cell lysates. The results are shown as the luciferase levels at each CCR5 density normalized to infection levels on cells expressing the maximum density of CCR5 (A). The relationship between normalized infection of Affinofile cells expressing the lowest density of CCR5 and the proportion of total CD4+ lymphocyte infection that is mediated by CCR5 (from Fig. 3.2b) for each R5X4 Env pseudotype is also shown (B).

Ability to use CCR5 on CD4+ lymphocytes by R5X4 HIV-1 correlates with the use of low levels of CCR5 on 293 Affinofile cells

We then asked if there was an association between R5X4 use of lymphocyte CCR5 and entry into Affinofile cells expressing CCR5 at the lowest density (Fig. 4.5a). Overall, there was a strong correlation for R5X4 HIV-1 between the proportion of CD4+ lymphocyte entry mediated by CCR5 and the ability to infect Affinofile cells expressing diminishing levels of CCR5 (Fig. 4.5b). C2-16 and NR-10 used CCR5 on lymphocytes more efficiently than other R5X4 viruses and were least sensitive to decreasing levels of CCR5 on the Affinofile cells. Lymphocyte CCR5 use was lowest for 89.6 and infection of Affinofile cells was most impaired by decreasing CCR5 levels for this virus. DR17, R3A and DH12 showed an intermediate phenotype in both lymphocyte CCR5 use and infection of Affinofile cells expressing low levels of CCR5. These results confirm that there is biological heterogeneity among R5X4 HIV-1, and suggest that strains with marginal CCR5 use on lymphocytes are unable to effectively scavenge for CCR5 when it is expressed at low density.

Conclusions

Entry efficiency, which is dependent on Env-coreceptor interactions and coreceptor density on target cells, is an important determinant of replication capacity and pathogenesis. Previous work from our lab showed that R5X4 viruses were more sensitive to CCR5 antagonists than R5 viruses, and in chapter 3, I described how increasing CCR5 expression on CD4+ lymphocytes enhanced CCR5 use by viruses with poor use of this coreceptor. Thus, previous reports combined with my recent findings suggested inefficient Env-CCR5 interactions may be one mechanism that regulates lymphocyte CCR5 use by R5X4 strains.

In this chapter, I determined the relationship between the efficiency of CCR5 use by R5X4 strains and lymphocyte CCR5 use. Using sensitivity to entry blocking agents as a surrogate marker for the efficiency of CCR5 use, I found that R5X4 viruses were markedly inhibited by CCR5 antibodies with ECL2 epitopes but not by other CCR5 antibodies, and resistance to inhibition correlated with greater lymphocyte CCR5 use. In addition, I found a significant correlation between greater lymphocyte CCR5 use by R5X4 strains and resistance to inhibition by CCR5 antagonists, but not the fusion inhibitor T20. I also employed Affinofile cells with regulated levels of CD4 and CCR5 to examine the impact of CCR5 expression on infection by R5X4 strains. I discovered that decreasing CCR5 expression had the largest impact on infection by viruses that used lymphocyte CCR5 poorly, in that strains with greater lymphocyte CCR5 use could better exploit low levels of CCR5 on these cells. From these results, I conclude that the

spectrum of lymphocyte CCR5 use by R5X4 HIV-1 is determined by the efficiency of the Env-CCR5 interaction.

CCR5-mediated infection of CD4+ lymphocytes by R5X4 HIV-1 is relatively small in comparison to infection mediated by CXCR4 when that coreceptor is available, but the levels of lymphocyte CCR5 use vary among the R5X4 strains. The results outlined here indicate the efficiency of the Env-CCR5 interaction differs between viruses and is a principal determinant of the ability to use this coreceptor. Specifically, viruses with more efficient Env-coreceptor interactions are able to use low levels of CCR5 more efficiently, suggesting that these viruses require a lower threshold of CCR5 to facilitate membrane fusion. The density of CCR5 expressed on individual CD4+ lymphocytes varies (Reynes et al., 2000), and these findings indicate that R5X4 strains with a lower CCR5 density threshold can reach higher levels of infection by infecting lymphocytes that express minimal levels of CCR5. Thus, the percentage of CCR5+ CD4+ lymphocytes determines the potential levels of CCR5 use, but actual CCR5 use by an R5X4 variant is determined by how efficiently its Env interacts with the coreceptor.

The results of this study show that CCR5 mAbs with epitopes in ECL2 inhibit R5X4 infection, and the extent of this inhibition is associated with the ability to use CCR5 on lymphocytes. This suggests the efficiency of interaction with ECL2 contributes to determining lymphocyte CCR5 use by R5X4 strains. On the other hand, it is also possible that these mAbs are affecting interactions with other CCR5 domains, since inhibition by an antibody is not always dependent on competition for the epitope. For example 2D7 effectively blocks CCR5 use by R5 and R5X4 strains, yet in two studies, mutation of the CCR5 2D7 binding site had no impact on CCR5 use (Rabut et al., 1998;

Siciliano et al., 1999), indicating the 2D7 epitope itself is not required for infection by HIV-1. It has been proposed that 2D7 inhibits HIV-1 infection by blocking access to critical residues in the transmembrane core of CCR5 (Siciliano et al., 1999). The results presented in this chapter do appear to show a relationship between the proximity of an antibody epitope to the CCR5 core and the ability of that antibody to inhibit infection, although only a limited number of antibodies were used. Thus, these findings suggest the interactions with the ECLs, or possibly other residues near this region, make a significant contribution to regulating the efficiency of CCR5 use.

It is interesting that sensitivity to CCR5 antagonists correlated with lymphocyte CCR5 use but not sensitivity to the fusion inhibitor T20. CCR5 antagonists are allosteric inhibitors that are thought to prevent HIV-1 infection by binding within the pocket created by the extra cellular loops and altering the conformation of the coreceptor (Dragic et al., 2000). T20 is a steric inhibitor that binds to HR1 in gp41 and prevents formation of the 6-helix bundle that is required for membrane fusion (Rimsky, Shugars, and Matthews, 1998; Wild, Greenwell, and Matthews, 1993). Mutations in the coreceptor binding site that reduce Env binding invariably increase sensitivity to coreceptor antagonists, but the same mutations do not always have a similar effect on T20 sensitivity (Biscone et al., 2006; Reeves et al., 2002; Reeves et al., 2004). From these relationships, it appears that efficiency of the Env-coreceptor interaction may not be inextricably linked to the efficiency of the subsequent conformational changes in gp41. This also implies that sensitivity to coreceptor antagonists measures the efficiency of a single step in the fusion process. In light of that possibility, my findings suggest that Envs with more

efficient interactions with CCR5 likely have a higher affinity for the coreceptor, although this would have to determined by measuring affinity for CCR5.

The findings in this study indicate that R5X4 strains vary in the efficiency of interaction with CCR5, and the variation in Env-CCR5 interaction efficiency impacts coreceptor use on primary lymphocytes. Specifically, R5X4 HIV-1 with more efficient Env-CCR5 interactions infect CD4+ lymphocytes using CCR5 at much higher levels than viruses with inefficient interactions with the coreceptor. I also discovered that this variation in Env-CCR5 interaction may be, at least in part, determined by interactions with ECLs or residues proximal to this region, based on sensitivity to ECL2 antibodies and coreceptor antagonists.

Notably, 89.6, the virus with the least efficient interactions with CCR5, is also the virus that failed to respond to increased CCR5 expression on CD4+ lymphocytes in chapter 1. The barrier to CCR5 use by this strain and the factors in Env that regulate primary cell coreceptor usage and preference have not been identified, but this field necessitates further study if we are to fully understand pathogenesis by R5X4 HIV-1.

Chapter V - Molecular determinants within Env that regulate coreceptor use on CD4+ lymphocytes

Introduction

The spectrum of lymphocyte CCR5 use by the R5X4 viruses studied here is dependent on the efficiency of the Env-coreceptor interaction, and variation in this interaction is Env-dependent. Although many domains in Env influence interactions with coreceptor, the V3 domain is the primary determinant of coreceptor use, and it is thought to directly interact with the coreceptor (Choe et al., 1996; Cormier and Dragic, 2002; Cormier et al., 2000). The V3 domain is also a critical determinant of viral phenotype. In CXCR4/CD4+ T cell lines, X4 and R5X4 viruses are able to induce syncytia (SI variants) while R5 strains are nonsyncytium inducing (NSI) in these cells (Björndal et al., 1997). This phenotypic difference between strains is based on coreceptor use and is largely determined by the V3 domain (Björndal et al., 1997; de Jong et al., 1992b). Comparative analysis of the V3 sequence of HIV-1 viruses with distinct phenotypes has led to the identification of features within this domain that are linked to use of CCR5 or CXCR4. As outlined in chapter 1, a high net positive charge and the presence of positively charged amino acids at positions 11 and 24 or 25 (positions 306 and 319 or 320 using HXB2 Env numbering) are well-known features associated with CXCR4 use by HIV-1 (Cardozo et al., 2007; Fouchier et al., 1992; Milich, Margolin, and Swanstrom, 1993). Several position specific scoring matrices (PSSM) have been developed to predict coreceptor use from V3 sequences based on large databases of viruses characterized as R5 vs X4 (X4/R5 PSSM) or NSI vs SI (SI/NSI PSSM) (Jensen et al., 2003). The presence of these specific features within V3 and the related PSSM algorithm distinguish X4 from R5 viruses with reasonably high although imperfect accuracy; however, the V3

of R5X4 strains are more varied and do not always conform to this paradigm. Presently, it is unclear how variability in V3 relates to coreceptor use by R5X4 viruses, particularly on primary CD4+ lymphocytes.

The spectrum of lymphocyte CCR5 use by R5X4 strains includes variants with marginal use of this coreceptor, such as 89.6. Unlike the other R5X4 viruses in this category, increased CCR5 expression on CD4+ lymphocytes did not enhance CCR5 use by 89.6, and severely impaired lymphocyte CCR5 use by this virus correlated with inefficient interactions with CCR5 by a number of different measures. These findings together suggest restricted CCR5 use by 89.6 is dependent on the viral Env. Determinants within the 89.6 Env that impact coreceptor use have been identified in V3. Deletions on either side of the 89.6 Env V3 base abrogate CCR5 use, while deleting a section of stem that contains residue 306 abolished CXCR4 use by this virus (Nolan, Jordan, and Hoxie, 2008). Another study attempted to derive variants of 89.6 resistant to CXCR4 inhibitors on cell lines expressing both coreceptors and identified a change from arginine to serine at position 306 that conferred resistance through enhanced CCR5 use (Maeda, Yusa, and Harada, 2008). A recent study of other R5X4 viruses also suggests a role for amino acid 306 in modulating coreceptor use. The efficiency of CCR5 and CXCR4 use on primary cells for brain versus blood or spleen-derived R5X4 envelopes was linked to the presence of a serine or arginine, respectively, at position 306 in V3 (Gray et al., 2009). Thus, residue 306 within Env appears to be a critical determinant of coreceptor use for 89.6 and for other R5X4 viruses, but it is not known whether the amino acid at this position contributes to restricted lymphocyte CCR5 use by 89.6 or the spectrum of lymphocyte CCR5 use by the other R5X4 viruses.

The goals of this chapter are to assess whether elements in V3 regulate lymphocyte CCR5 use by R5X4 viruses, and to identify determinants in the 89.6 Env that are responsible for the severely impaired lymphocyte CCR5 use by this virus. To do this, the viral phenotype of each R5X4 virus was predicted using a V3-based position-specific scoring matrices (PSSM)-algorithm, and the relationship between predicted phenotype and lymphocyte CCR5 use by R5X4 viruses was evaluated. Eventhough all strains tested were R5X4 in indicator cells, I found that those with greater lymphocyte CCR5 use were predicted to be NSI viruses, while viruses with poor lymphocyte CCR5 use were predicted to be SI viruses. To determine the factors within the 89.6 Env that regulate lymphocyte CCR5 use, I mutated R306 in 89.6 to serine (89.6 R306S). In addition, the role of this residue in regulating coreceptor use by other R5X4 viruses was assessed by mutating the same residue in C2-16, the R5X4 Env with greatest lymphocyte CCR5 use, from serine to arginine (C2-16 S306R). I found that an arginine at this position was associated with increased sensitivity to CCR5 blocking agents for both 89.6 and C2-16. Moreover, viruses with an arginine at residue 306 were more dependent on the N-terminus of CCR5 for infection. My results also show the presence of a serine at position 306 dramatically enhanced CCR5 mediated infection of CD4+ lymphocytes by 89.6, and switched coreceptor preference from CXCR4 to CCR5 on these cells. However, arginine at this position had no impact on lymphocyte coreceptor use by C2-16. Thus, the amino acid at position 306 regulates interactions with CCR5 for 89.6 and C2-16; it also regulates coreceptor use on primary lymphocytes by 89.6.

Results

Relationship between V3 sequences and CD4+ lymphocyte coreceptor preference

The V3 region is a major determinant of coreceptor use, and position-specific scoring matrix (PSSM) algorithms based on variations in this domain are frequently used to predict viral phenotype. Two widely used algorithms are based on viruses phenotyped by syncytia induction in CXCR4/CD4+ cell lines (SI/NSI PSSM) or on coreceptor use in indicator cells (X4/R5 PSSM), which are highly related although not completely concordant features (Jensen et al., 2003). However, such algorithms are typically less able to identify dual-tropic R5X4 than single coreceptor R5 and X4 variants. More importantly, while used for indicator cell coreceptor use prediction, they have not been applied to selective use of coreceptors on primary cells. Therefore, we applied the PSSM algorithms to V3 sequences from Envs in our panel to determine whether primary CD4+ T cell coreceptor usage among R5X4 HIV-1 might be associated with sequences in V3 (Fig. 5.1a). Strikingly, we found that prediction of an NSI phenotype based on V3 sequence was associated with significantly more efficient use of lymphocyte CCR5 (Fig. 5.1a & b). Conversely, R5X4 viruses with more restricted entry through lymphocyte CCR5 were predicted to be SI. Thus, while unable to identify these R5X4 strains as a group, the SI/NSI algorithm appears to discriminate among R5X4 strains and predict relative efficiency of lymphocyte CCR5 use based on V3 determinants. In contrast, there was no link between primary lymphocyte coreceptor usage and predicted coreceptor phenotype based on V3 sequences using the X4/R5 coreceptor prediction algorithm.

A.

Strain	V3 sequence	CD4+ PBL %CCR5 use	V3 Net Charge	PSSM SI/ NSI	PSSM X4/R5
Bal	CTRPNNNTRK**S**IHIGPGRALYTT**GE**IIGDIRQAHC	100	3	0	0
C2-16	..........TM....VY..............	52	3	0	0
C2-22	..........TM....VY..............	39	3	0	0
NR10	..........A.....SF.A..**R**.....	39	5	0	0
NR8	..........A.....SF.A..**R**.....	35	5	0	0
NR11	..........A.....SF.A..**R**.....	26	5	0	0
DH12	.........**G**.TL....VF......V....K...	12	4	1	0
R3A	....G.....**R**VTL....VY....**Q**.....K...	11	5	1	1
DR1	.I.........MTL...KVF....-**V**T....K...	15	5	1	0
DR17	.I.........MTL...KVF....-**V**T....K...	8	5	1	0
DR8	.I.........MTL...KVF....-**V**T....K...	4	5	1	0
89.6	.........R**R**LS......F.AR**RN**.....	4	7	1	1

B.

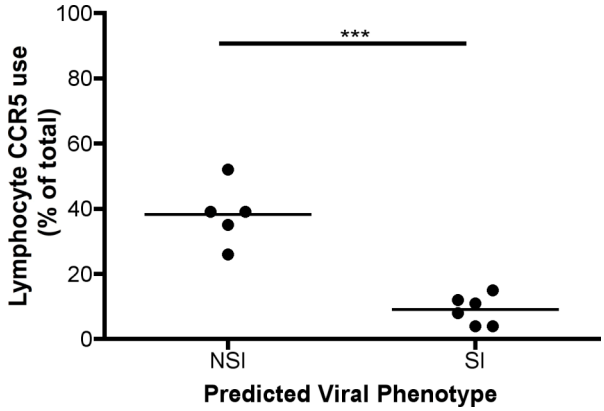

Figure 5.1. R5X4 HIV-1 CCR5 use on CD4+ lymphocytes is associated with the predicted viral phenotype.

V3 sequences from each virus were analyzed using the NSI/SI or X4/R5 PSSM algorithm. The table shows an alignment of V3 sequence from R5X4 HIV-1. Amino acids 11 (306) and 24/25 (319/320) are denoted in bold. The coreceptor use on primary lymphocytes for each R5X4 virus is shown (from Fig. 3.2). Predicted phenotypes are denoted by number with 1=SI or X4 and 0=NSI or R5 (A). Mean CCR5 use on CD4+ lymphocytes by R5X4 HIV-1 grouped by predicted viral phenotype. R5X4 viruses were grouped by SI or NSI phenotype from (A) with CCR5 use for each virus represented by black circles. The mean lymphocyte CCR5 use for each group was calculated, and the means were compared using a two-tailed, unpaired t-test in GraphPad Prism 4 software (***$p<0.0001$) (B).

Interestingly, most of these R5X4 Envs were predicted to be R5 by the X4/R5 PSSM matrix, which differed from the SI/NSI algorithm results. Thus, while the V3-based PSSM is an incomplete predictor of coreceptor use for R5X4 strains, it does appear to predict the relative coreceptor preference on lymphocytes suggesting an important role of V3 in regulating coreceptor interactions.

A high net positive charge and positively charged amino acids at position 306 and 319/320 of V3 are typically associated with CXCR4 use, whereas R5 strains typically lack these determinants. R5X4 strains can use CXCR4, but these viruses often lack many of the features common in the V3 of X4 strains. However, variability in V3, including the presence or absence of hallmarks of CXCR4 use, may be associated with coreceptor use on primary lymphocytes by R5X4 strains. To assess this, the V3 net charge, as well as the identity of the amino acids at positions 306 and 319/320, were evaluated to determine if they were linked to CCR5 use on CD4+ T cells by the R5X4 viruses (Fig. 5.1a). This analysis revealed most of R5X4 strains in this panel have V3 charges that are only slightly higher than the R5 strain, Bal. Positively charged amino acids were uncommon at residues 306 or 319/320, and there was no consistent connection between positive charges at these positions and lymphocyte CCR5 use by the R5X4 viruses. Interestingly, while the spectrum of CCR5 use could not be defined by the residues at 306 and 319/320 or the V3 net charge, the viruses on both extremes of the CCR5-use spectrum did adhere to these rules. C2-16 and C2-22, the viruses with the greatest CCR5 use on lymphocytes, had net positive charges equal to Bal, and these viruses did not have basic residues at either V3 stem position. Alternatively, 89.6, which had the poorest use

A.

```
89.6              CTRPNNNTRRRLSIGPGRAFYARRNIIGDIRQAHC 35
89.6 R306S        ••••••••••S••••••••••••••••••••••••• 35

C2-16             CTRPNNNTRKSITMGPGRVYYTTGEIIGDIRQAHC 35
C2-16 S304R       ••••••••••R••••••••••••••••••••••••• 35
```

B.

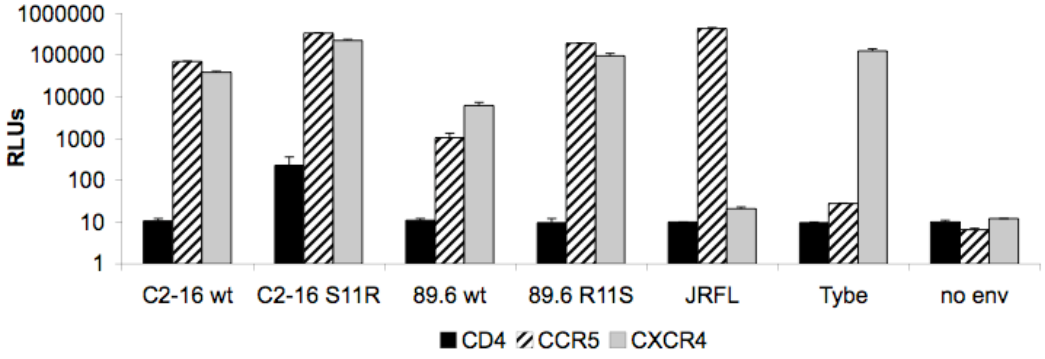

Figure 5.2. The amino acid at position 306 in Env does not affect coreceptor use on U87 cells by 89.6 or C2-16.

The amino acids of envelope V3 regions of 89.6 and C2-16 are shown, along with the 89.6 R306S and C2-16 S306R mutations, which correspond to position 11 of the V3 domain (A). U87/CD4, U87/CD4/CCR5 and U87/CD4/CXCR4 were infected with wild-type and mutant 89.6 and C2-16 luciferase pseudotype viruses. The results are presented as the RLUs measured from cell lysates 3 days after infection (B).

of lymphocyte CCR5, had a much higher V3 charge than any other R5X4 strain, and it was the only clone with basic amino acids at both positions 306 and 319 in this study. This result also suggests that elements within V3 contribute to regulation of coreceptor use by R5X4 viruses on lymphocytes.

R306 is associated with increased sensitivity to inhibition by 2D7 and Maraviroc and less efficient interactions with CCR5

While CCR5 use on CD4+ lymphocytes by R5X4 envelopes was bolstered by increasing CCR5 expression, this measure had little effect on lymphocyte CCR5 use by 89.6. Therefore, for this virus, we wished to address the Env side of the Env-CCR5 interaction by determining if specific changes within V3 might alter the efficiency of CCR5 use and affect lymphocyte entry. I speculated that the amino acid at position 306 within Env might be involved in determining the efficiency of 89.6 Env-CCR5 interaction, since several studies have shown that this residue can influence coreceptor use for 89.6 and other R5X4 strains (Gray et al., 2009; Maeda, Yusa, and Harada, 2008; Nolan, Jordan, and Hoxie, 2008). To test this notion, R306 in 89.6 was mutated to serine (89.6 R306S; Fig. 5.2a). In contrast to 89.6, the R5X4 Env with greatest lymphocyte CCR5 use, C2-16, has a serine at position 11 in V3. Therefore, we also mutated the serine at this position in C2-16 to arginine in order to assess the converse effect (C2-16 S306R) (Fig. 5.2a). Both Envs remained R5X4 in U87/CD4/coreceptor cells (Fig. 5.2b). Sensitivity to CCR5 antagonists and mAbs are one method used to probe Env-coreceptor interactions, so I employed these inhibitors to determine whether mutations at residue 306 within the 89.6 and C2-16 V3 domains had an effect on interactions with CCR5.

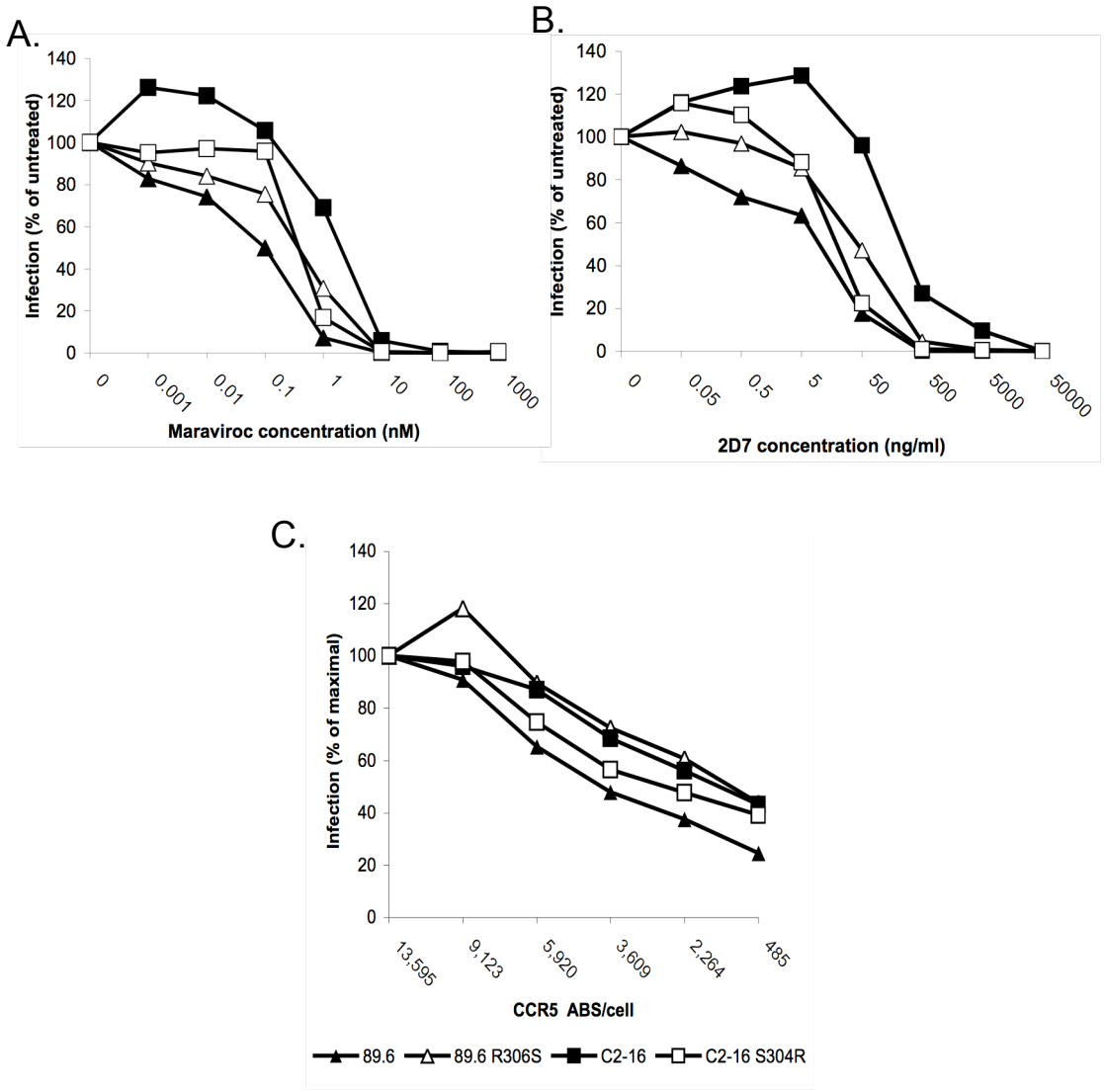

Figure 5.3. Residue R306 in Env is associated with less efficient interactions with CCR5.

U87/CD4/CCR5 cells were not treated or pretreated with increasing concentrations of Maraviroc (A) or 2D7 (B), then infected with wt and mutant 89.6 and C2-16 in the continued presence of blocker. Results are presented as the luciferase activity in the presence of drug normalized to luciferase activity in the cell lysate of untreated cells. Affinofile cells expressing a fixed level of CD4 and differing levels of CCR5 were infected with wild-type and mutant 89.6 and C2-16 luciferase pseudotype viruses. The results are shown as the luciferase levels at each CCR5 density normalized to infection levels on cells expressing the maximum density of CCR5 (C).

Infection of U87 cells by wt and mutant 89.6 and C2-16 was inhibited at the highest Maraviroc concentrations (Fig. 5.3a). As seen in earlier studies, wt 89.6 was substantially more sensitive to inhibition by Maraviroc than wt C2-16, which required approximately one log higher concentration of the drug to completely inhibit entry. The R306S mutation in 89.6 resulted in a decrease in sensitivity to Maraviroc, while the S306R mutation in C2-16 increased sensitivity to this CCR5 antagonist (Fig. 5.3a). Infection of U87 cells in the presence of mAb 2D7 resulted in a similar inhibition pattern, with wt 89.6 and C2-16 being the most and least sensitive to inhibition by the mAb, respectively (Fig. 5.3b). The 89.6 mutant R306S showed reduced sensitivity to 2D7 compared to wt 89.6, and the reciprocal S306R mutation in C2-16 lead to increased 2D7 sensitivity for this virus relative to wt C2-16. These findings suggest that the residue at position 306 in Env modulates Env-CCR5 interactions. In particular, the presence of arginine at residue 306 decreases the efficiency of the Env-CCR5 interaction while serine at this position enhances interaction.

The changes in sensitivity to CCR5 blockers caused by mutations at position 306 suggested that the identity of the amino acid at this position could also impact how efficiently viruses used CCR5 to infect target cells. To address this question, we infected 293 Affinofiles expressing a fixed level of CD4 and varying levels of CCR5 to test the effect of the Env mutations on the efficiency of CCR5 use in that system. As noted in chapter 4, infection by 89.6 decreased markedly as CCR5 density on Affinofiles dropped from maximal to minimal levels. Replacing the arginine at position 306 with serine resulted in a two-fold enhancement of entry at the lowest CCR5 levels, reaching a relative entry efficiency comparable to that for C2-16 (Fig. 5.3c). In contrast, the C2-16

S306R mutation had some effect on entry efficiency at intermediate CCR5 levels, but not at the lowest CCR5 density; which most closely mimics those on primary CD4+ lymphocytes (Fig. 5.3c).

Impaired CCR5 use on CD4+ lymphocytes by 89.6 is reversed by the R306S mutation within the V3 region

Since residue 306 modulates the efficiency of CCR5 use, it might also regulate the ability or inability of 89.6 to use CCR5 on lymphocytes. This question was addressed by evaluating coreceptor use on primary CD4+ T cells by wild type and mutant 89.6 and C2-16. As shown in Fig. 5.4a, the R306S mutation dramatically enhanced use of lymphocyte CCR5 for 89.6. In addition, the R306S change also decreased CXCR4 mediated infection for the 89.6 mutant. Importantly, this dramatic shift in lymphocyte coreceptor use occurred eventhough the mutant virus, like parental 89.6, remained R5X4 in indicator cells (Fig. 5.2b), albeit with some increase in relative CCR5 use associated with the mutation. In fact, 89.6 R306S showed a preference for CCR5 use on lymphocytes, in contrast to wild type 89.6, which was essentially restricted to CXCR4 for entry into these cells (Fig. 5.4b). Unlike 89.6, CCR5 use on CD4+ lymphocytes by strain C2-16 was not affected by the converse S306R mutation (Fig. 5.4a & b).

Thus, replacing the positively charged arginine with serine at residue 306 in the 89.6 Env increased the efficiency of CCR5 use, and resulted in preferential infection of CD4+ lymphocytes through CCR5, reversing its otherwise strict dependence on CXCR4

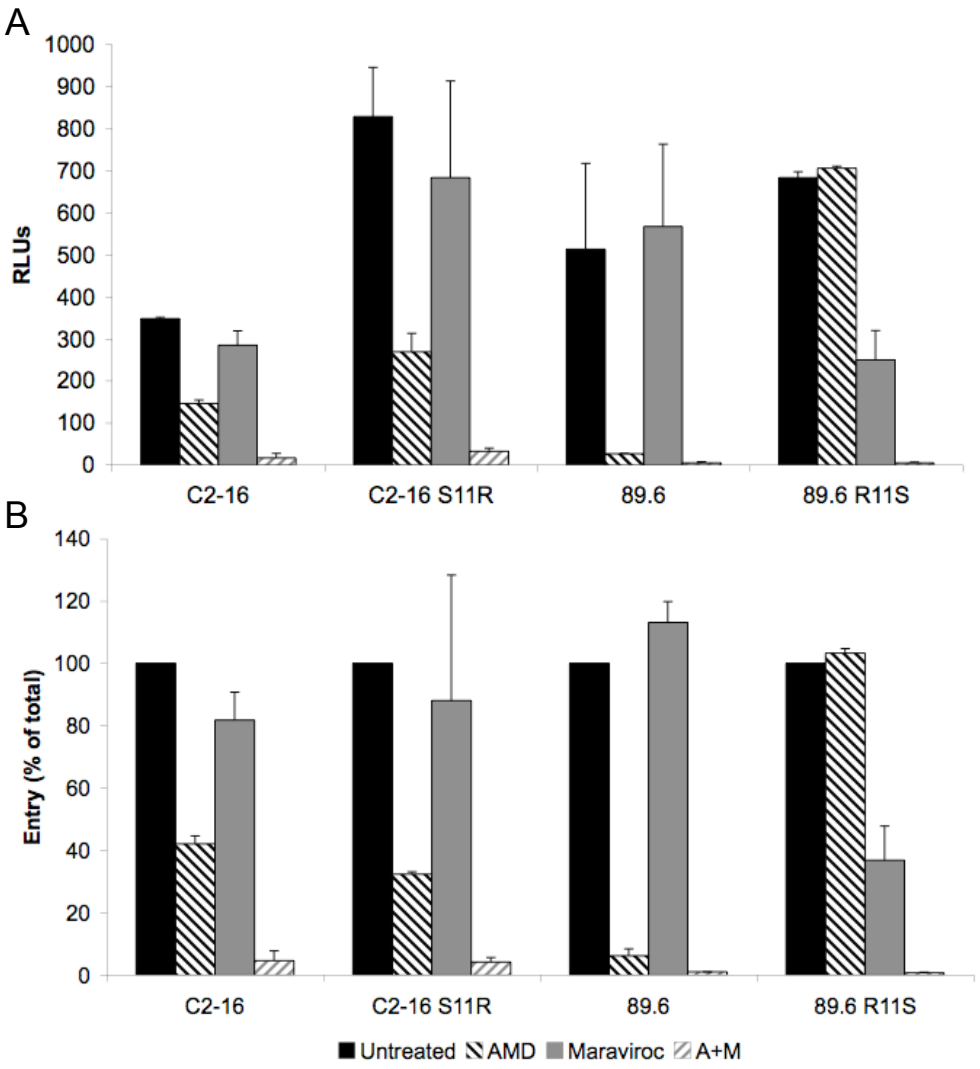

Figure 5.4. Residue 306 in Env regulates CCR5 use on CD4+ lymphocytes for 89.6 but not C2-16.

PHA stimulated CD4+ lymphocytes were infected with wild-type and mutant 89.6 and C2-16 luciferase pseudotype viruses in the presence or absence of AMD3100, Maraviroc or a combination of both inhibitors. The results are representative of the average of three experiments on cells from different donors each performed in duplicate. The results are presented as the RLUs measured from cell lysates 4 days after infection (A) or the RLUs for each treatment condition normalized to infection of untreated cells (B).

in those cells. However, the amino acid at this position is not a critical determinant for all R5X4 viruses, since converting serine to arginine at that residue did not notably impact the efficiency of CCR5 use or coreceptor preference on primary lymphocytes by the C2-16 Env, indicating that other context-dependent factors are involved as well.

Residue R306 increases Env dependence on the CCR5 amino terminus

Residue R306 is located in the stem of the V3 loop. This region of the V3 loop is thought to interact with the CCR5 ECLs, and mutations or deletions at this site in other Envs has been associated with changes in interaction with this coreceptor (Cormier and Dragic, 2002; Gray et al., 2009; Nolan et al., 2009). To determine how mutations at position 306 in Env impact interactions with CCR5, wt and mutant 89.6 and C2-16, along with the R5 virus JRFL, were used to infect 293 cells that expressed CD4 and wt CCR5 or versions of this coreceptor containing deletions of the N-terminus. All coreceptors were expressed at similar levels on the cell surface based on staining using the ECL2 mAb 2D7 (data not shown).

As shown in figure 5.5, infection by wt 89.6 was substantially reduced on cells expressing CCR5 that lacked four N-terminal residues (Δ2-5), and deletion of eight amino acids from this domain (Δ2-9) completely abolished infection by this virus. Conversely, infection of cells expressing Δ2-5 by 89.6 R306S was two fold greater than infection by wt virus, and unlike wt 89.6, the mutant virus used Δ2-9 to infect 293 cells (Fig. 5.5a). For C2-16, the Δ2-5 deletion had minimal effect on infection by wt or mutant virus, and neither virus efficiently used Δ2-13 for infection. However, infection of cells

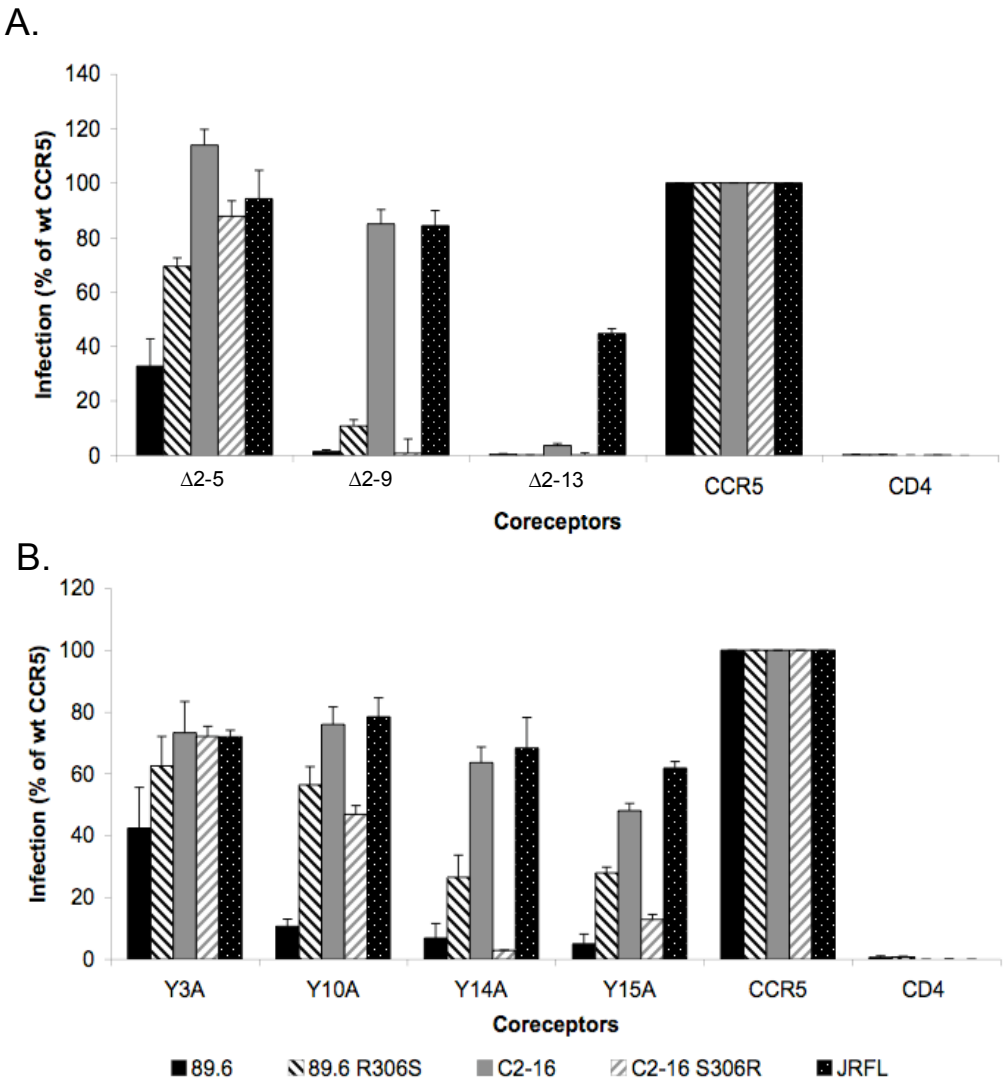

Figure 5.5. Envs with R306 are more dependent on the N-terminus of CCR5 for infection.

293 cells expressing CD4 and mutant CCR5 with either N-terminal deletions (A) or N-terminal Y to A substitutions (B). Following transfection, cells were infected with 5ng of wt and mutant 89.6 and C2-16 luciferase pseudotypes. Results are shown as the luciferase activity in cell lysates from cells expressing mutant coreceptor normalized to infection of 293 cell expressing wt CCR5 after 3 days in culture. Results are representative of two infections each performed in triplicate.

expressing Δ2-9 by wt and mutant C2-16 revealed stark differences in how these viruses are affected by changes in the CCR5 N-terminus. While infection of cells expressing Δ2-9 by wt C2-16 was only 10% lower than infection using wt CCR5, the S306R conversion resulted in a virus incapable of using this mutant coreceptor (Fig. 5.5a). Unlike the R5X4 viruses, JRFL was able to use all three truncated coreceptors for infection (Fig. 5.5a). Thus, the identity of the residue at position 306 in Env is associated with different requirements for the CCR5 N-terminus. Viruses with arginine at this position are more dependent on the N-terminus of CCR5 for infection, and serine at this position is associated with the ability to use CCR5 despite truncation of this domain.

There are four tyrosines in the N-terminus of CCR5, and these tyrosines can be sulfated (Farzan et al., 1999). Tyrosine sulfation at residues 10, 14 and 15 in the CCR5 N-terminus are thought to be critical for gp120 binding and HIV-1 infection (Cormier et al., 2000; Farzan et al., 1998; Farzan et al., 1999; Rabut et al., 1998). Since the residue at position 306 regulates dependence on the N-terminus of CCR5, I thought it might also regulate dependence on the tyrosines in this domain. Therefore, I sought to determine whether altering the tyrosines in the CCR5 N-terminus had a larger impact on infection by wt or mutant Env clones of 89.6 and C2-16, and if an arginine or a serine at position 306 was associated with increased sensitivity to tyrosine mutation. This was done by infecting 293 cells that expressed CD4 and wt CCR5 or CCR5 mutants in which an individual tyrosine is mutated to alanine. All coreceptors were expressed at similar levels on the cell surface based on staining using the ECL2 mAb 2D7 (data not shown).

As shown in Fig 5.5b, infection by 89.6 on cells expressing the Y3A coreceptor was less than half that mediated by wild-type CCR5, and infection of cells expressing the

three other mutant coreceptors only approached 10% of wt levels (Fig. 5.5b). In contrast, the 89.6 mutant carrying the R306S change was less affected by tyrosine mutation, consistently reaching infection levels on each mutant at least 20% higher than infection by wt virus, and use of the Y10A coreceptor was five fold greater for 89.6 R306S compared to 89.6 (Fig. 5.5b). In contrast to 89.6, C2-16 was only modestly affected by the individual tyrosine mutations, whereas changing serine to arginine (C2-16 S306R) resulted in a virus with markedly reduced use of the Y14A and Y15A coreceptors, and that was also less able to use the Y10A coreceptor. Interestingly, CCR5-mediated infection by JRFL was only slightly affected by any of these individual tyrosine mutations, which was similar to C2-16 and markedly different from 89.6 (Fig. 5.5). Thus, the presence of arginine at position 306 is associated with increased dependence on tyrosine residues in the N-terminus of CCR5, while serine at this position results in less dependence on these residues.

Conclusions

CCR5 use on CD4+ lymphocytes varies among the R5X4 strains, but it is generally low, and includes one virus, 89.6, that is almost completely unable to use lymphocyte CCR5 even after CCR5 expression is increased on these cells. The V3 domain in Env is a principal determinant of coreceptor use and interacts directly with the coreceptor. In this chapter, I sought to determine the contribution of V3 to the spectrum of CCR5 use by R5X4 strains.

Using a PSSM algorithm, I found that viruses with greater lymphocyte CCR5 use were predicted to have an NSI phenotype while viruses that used this coreceptor poorly were classified as SI viruses. Comparative analysis of V3 revealed that viruses at the extremes of the lymphocyte CCR5 use spectrum, C2-16 and 89.6, could clearly be distinguished by the absence or presence, respectively, of common hallmarks of CXCR4 use, including the charge of the residue at position 306 in Env. I mutated the amino acid at this position in 89.6 (R306S) and C2-16 (S306R) to determine its role in controlling CCR5 use by these viruses. My findings show that the R306S change in 89.6 dramatically enhanced lymphocyte CCR5 use and changed coreceptor preference on these cells from CXCR4 to CCR5. However, the reciprocal mutation had no affect on lymphocyte coreceptor use by C2-16. The presence of an arginine at position 306 was associated with increased sensitivity to CCR5-specific blockers and less efficient infection of Affinofile cells when compared to viruses with serine at this same position. Having an arginine instead of a serine at residue 306 was also associated with greater dependence on the CCR5 N-terminus for infection. These results indicate that elements

within V3 determine the spectrum of lymphocyte CCR5 use by R5X4 strains, the amino acid at position 306 in Env regulates the efficiency of CCR5 use for 89.6 and C2-16, and R306 is responsible for the impaired use of lymphocyte CCR5 by 89.6.

The bridging sheet and base of V3 are thought to interact with the N-terminus of CCR5, while the tip and stem interact with the ECLs (Cormier and Dragic, 2002; Cormier et al., 2001; Huang et al., 2007). The data presented here imply that the greater dependence on the CCR5 N-terminus displayed by R306 compared to S306 Envs results from reduced interactions between V3 containing R306 and the CCR5 ECLs. Amino acids 306 and 319/320 in V3 are thought to create a surface in Env that is positively charged for X4 viruses or negatively charged for R5 strains (Cardozo et al., 2007). While the charges of the residues at these positions for R5X4 strains do not always conform to this rule, 89.6 has positive charges at both positions, and C2-16 has a neutral residue at position 306 and a negatively charged residue at positions 320. Differences in the charge of the surface created by amino acids 306 and 319/320 between 89.6 and C2-16 regulate interactions with residues in the ECLs of CCR5, and the strength of this interaction contributes to regulation of CCR5 use.

Residue 306 in Env regulated CCR5 use by both 89.6 and C2-16, with serine at this position associated with more efficient Env-CCR5 interactions. Most of the R5X4 strains, including those with poor lymphocyte CCR5 use, had a neutral or acidic amino acid at this position, suggesting other residues may be responsible for the pattern of CCR5 use by these strains. Positions 306 and 319/320 likely associate in space, and perhaps the amino acid at either of the later positions could contribute to regulation of lymphocyte CCR5 use by some of these strains. The majority of the variability between

the V3 sequences of these R5X4 Envs is on either side of the tip, in residues that contact the ECLs. For some viruses, basic amino acids are found at these residues, and perhaps these amino acids negatively contribute to Env-CCR5 interactions. However, some of the R5X4 viruses with poor lymphocyte CCR5 use had no positive charges at either of these positions, and for these Envs, other domains may play an important role. For instance, the V1-V2 region of DH12 can confer CXCR4 use to an R5 virus, and N-glycosylation in this domain in the DH12 Env also influences the efficiency of CCR5 or CXCR4 use (Cho et al., 1998; Ogert et al., 2001). Thus, while I show that regulation of lymphocyte CCR5 use by the R5X4 strain 89.6 is influenced by interactions between the ECLs and the residue at position 306, additional factors exist likely, both within and outside V3, that regulate this interaction.

The R306S change in the 89.6 Env was also associated with a decrease in the contribution of CXCR4 to infection of CD4+ lymphocytes. V3 loops of X4 viruses have higher net positive charges than R5 viruses, and it has been proposed that this higher charge allows X4 viruses to utilize the negatively charged surface of CXCR4 (Chabot et al., 1999). Deletion of residues 304-307 in the R3A and 89.6 Env abrogates CXCR4 use by these viruses, suggesting this region is critical for interactions with the CXCR4 ECLs (Nolan et al., 2009). Thus, it is plausible that the amino acid at position 306 also contributes to regulation of CXCR4 use for some R5X4 viruses and that S306 is associated with reduced interactions with the CXCR4 ECLs.

It is interesting that changing the amino acid at position 306 had an impact on every measure of how efficiently 89.6 and C2-16 mutants used CCR5, but the S306R mutation did not alter CCR5 use on primary lymphocytes by C2-16. The V3 loop of C2-

16 has features that are very similar to R5 viruses, including a low net positive charge (+3) and the lack of positively charged amino acids at the two key residues in the V3 stem. The S306R mutation introduces a positive charge into Env, which should decrease interactions with the CCR5 ECLs. It is possible that other residues within C2-16 Env continue to contact critical determinants in the ECLs, and, as a result, CCR5 use by the C2-16 mutant might be less affected. An alternative possibility is that the density of CCR5 expressed on lymphocytes is above the threshold required by both wt and mutant C2-16, and this would make it is difficult to observe a difference in lymphocyte CCR5 use by the two strains. Presently, it is unclear what accounts for the robust use of lymphocyte CCR5 by the C2-16 mutant.

PSSM predictive algorithms are not highly accurate for distinguishing R5X4 from single coreceptor-tropic viruses, but we found that among R5X4 variants, the SI/NSI matrix showed a strong correlation between "NSI-like" characteristics and greater lymphocyte CCR5 use, and "SI-like" and poorer lymphocyte CCR5 use. These results further support the role of V3 in determining coreceptor preference in the context of primary cells and, in addition, further emphasize the limitations of indicator cells in predicting primary cell coreceptor use. In contrast, the X4/R5 predictive matrix, based on coreceptor use in indicator cell lines, did not distinguish between R5X4 strains with greater or lesser lymphocyte CCR5 use. Future studies will be needed to characterize the strains for which phenotype predictions differ between the scoring matrices, and determine whether these Envs may exhibit other distinguishing features in their interactions with primary cells.

The role of particular Env domains in determining CCR5 or CXCR4 use has been well studied, however, the exact mechanisms underlying the discrimination of CCR5 and CXCR4 are still obscure. Small changes in the viral Env can impact interactions with different domains of the coreceptor, and this can affect viral entry. However, it is currently not known whether the structural determinants of CCR5 are equally exposed on different primary cell types or cells from different donors. These questions have important implications for the design of coreceptor antagonists and the study of drug resistance. Thus, understanding how changes in the HIV-1 Env alters interactions with the CCR5 structure and impacts primary cell coreceptor use is an extremely important topic.

Chapter VI - Discussion

R5X4 strains are usually the first CXCR4-using viruses to emerge, and these variants appear late in disease with varying frequency in people infected with HIV-1 from many of the group M subtypes (van Rij et al., 2000; Zhang et al., 1997; Zhang et al., 1998). During the acute and early phases of disease, R5 strains predominate (Liu et al., 1996; Samson et al., 1996). However, R5X4 strains also use CCR5 but fail to establish infections in recipients. Selection against CXCR4 using variants may be the reason R5X4 viruses rarely seed new HIV-1 infections. Alternatively, R5X4 strains may fail to transmit because CCR5 use by these variants is distinct from coreceptor use by R5 strains. The study of coreceptor use by R5X4 viruses may provide key insights into this important aspect of HIV-1 infection.

Coreceptor use by HIV-1 strains is typically determined on cell lines that have been engineered to express CD4 and a coreceptor, and infection of these cell lines indicates which coreceptors a virus can use. However, viruses may fail to infect primary cells using a coreceptor that it uses in indicator cell lines, raising questions of whether coreceptor use on cell lines accurately models coreceptor use on primary cells (Tokunaga et al., 2001). The primary cell targets of HIV-1 express CCR5 and CXCR4, and a previous study from our lab demonstrated that prototype R5X4 viruses use CCR5 and CXCR4 to infect macrophages. However, infection of CD4+ lymphocytes occurred predominately through CXCR4, whereas CCR5 was used poorly, if at all (Yi, Shaheen, and Collman, 2005). For this dissertation, I utilized a variety of dual-tropic isolates representing a broad spectrum of phenotypes, to assess whether R5X4 strains vary in the ability to use CCR5 on lymphocytes, and what factors determined the use of this pathway.

Limited lymphocyte CCR5 use: implications for the evolution and transmission of R5X4 HIV-1

Results presented in this dissertation indicate limited CCR5-mediated infection of CD4+ T cells by the R5X4 strains tested, which did not substantially contribute to overall infection levels when CXCR4 was available. However, blocking CXCR4 use revealed a spectrum of lymphocyte CCR5 use among the R5X4 viruses. CCR5 is expressed on a small percentage of CD4+ T cells, and increasing CCR5 expression noticeably improved CCR5 use for all R5X4 strains, except 89.6. If CCR5 use was determined strictly by the percentage of cells expressing this coreceptor, then infection by R5X4 strains should be equivalent to that of R5 viruses. CCR5 density varies within the population of CD4+ lymphocytes, and the variability in lymphocyte CCR5 use by R5X4 strains suggested that coreceptor density could be a factor in regulating lymphocyte CCR5 use. However, other additional factors likely exist, since CCR5-mediated infection of CD4+ lymphocyte by 89.6 was not enhanced by increasing CCR5 expression. R5X4 and R5 strains with the greatest CCR5 use were proficient at detecting this coreceptor than antibody specific for CCR5, suggesting Env might also regulate use of this coreceptor on CD4+ lymphocytes.

The R5X4 strains in this report exhibited a spectrum in their efficiency of lymphocyte CCR5 use, but none preferentially infected CD4+ T cells using this coreceptor. On one hand, this is not surprising given the difference in number of cells positive for each coreceptor. On the other hand, R5X4 strains emerge *in vivo* from R5 strains in memory lymphocytes that express CCR5 and CXCR4, while later in infection, R5X4 along with X4 viruses are found mostly in the CCR5-/CXCR4+ naïve subset (van Rij et al., 2000). Thus, it seems likely that some R5X4 strains would use CCR5 as the

predominant coreceptor to infect CD4+ T cells. The fact that the R306S mutation in 89.6 changes coreceptor preference on lymphocytes from CXCR4 to CCR5 indicates that this type of R5X4 Env can exist and that only minor changes may be required to shift between these two phenotypes. Perhaps R5X4 viruses that predominately use CCR5 to infect lymphocytes emerge but then rapidly disappear from the viral quasispecies. Alternatively, evolution of R5X4 strains might never transit through a phase in which CCR5 use is dominant. In a study of *in vitro*-derived R5X4 switch variants, the earliest CXCR4-using Envs were more sensitive to CCR5 antagonists and less efficient at using CCR5 for entry when compared to the parental R5 virus (Pastore, Ramos, and Mosier, 2004), and the mutations conferring CXCR4 use were detrimental except when introduced with compensatory changes elsewhere in the envelope (Pastore et al., 2006). *In vivo*, R5 strains showed reduced ability to infect CCR5-low cells prior to the emergence of R5X4 viruses (Coetzer et al., 2008). Thus, CXCR4 use may only emerge after compensatory mutations occur that have the effect of decreasing CCR5 use, suggesting that R5X4 evolution may not involve a phase of predominate lymphocyte CCR5 use.

R5 viruses are responsible for establishing the majority of new HIV-1 infections, but it is still not clear why. R5 strains are typically more macrophage-tropic than X4 variants, and CCR5-dependent macrophage tropism was classically proposed as a possible reason (Liu et al., 1996; van't Wout et al., 1994; Zhu et al., 1993). However, R5X4 strains also use CCR5 to infect primary macrophages, yet this strain is rarely transmitted (Liu et al., 1996; van't Wout et al., 1994; Yi, Shaheen, and Collman, 2005). Furthermore, recent studies using transmission pair isolates obtained near the time of

transmission enabling donor/recipient comparison, or PCR amplification and phylogenetic reconstruction of the successfully transmitted virus, reveal that these founder viruses are restricted to CCR5 and readily infect lymphocytes but typically infect macrophages inefficiently and/or no better than the donor variants (Isaacman-Beck et al., 2009; Keele et al., 2008b; Salazar-Gonzalez et al., 2009). The fact that R5X4 variants generally use lymphocyte CCR5 poorly relative to R5 viruses raises the possibility that these strains do not establish infections in recipients because use of CCR5 on lymphocytes is the critical determinant of transmission. Alternatively, it remains possible that neither R5X4 nor X4 strains efficiently transmit because CXCR4 use is selected against during transmission or establishment of new infections in recipients (Cornelissen et al., 1995).

Efficiency of CCR5 use by R5X4 HIV-1: implications for pathogenesis

The variability in lymphocyte CCR5 use among the R5X4 strains, coupled with the observations that Envs at the extremes of this spectrum appeared to use CCR5 with different efficiencies, suggested that Env-coreceptor interactions influenced lymphocyte CCR5 use by R5X4 strains. Experiments with CCR5-specific mAbs showed a significant correlation between greater lymphocyte CCR5 use and reduced sensitivity to ECL-2, but not N-terminal or multi-domain mAbs. When sensitivity to entry inhibitors was assessed, reduced sensitivity to CCR5 small molecule antagonists, but not fusion inhibitor T20, also correlated with greater lymphocyte CCR5 use. The link between coreceptor use and the efficiency of the Env-CCR5 interaction was further evaluated using cell lines with

regulated levels of CD4 and CCR5. At physiologically relevant CD4 levels, R5X4 strains with greater lymphocyte CCR5 use were less sensitive to reductions in CCR5 density. Moreover, there was a significant correlation between the ability to exploit low levels of CCR5 on this cell line and higher levels of lymphocyte CCR5 use. These results suggest that variation in the efficiency of Env interactions with the ECLs of CCR5 has an effect on efficiency of coreceptor use and contributes to the spectrum of lymphocyte CCR5 use by R5X4 strains. These findings underscore the importance of the ECLs, in particular ECL2, in infection by R5X4 viruses, and raise the possibility that determinants in Env ultimately regulate lymphocyte CCR5 use by these viruses.

The limited CCR5-mediated infection of lymphocytes by R5X4 HIV-1 raises questions of whether the efficiency of CCR5 use by R5X4 viruses has any impact on viral pathogenesis. Although rare, R5X4 strains have been transmitted and were able to establish infection in recipients (Meissner et al., 2004; Yu et al., 1998). Given the importance of CCR5 use in transmission, these viruses might be expected to utilize lymphocyte CCR5 more efficiently than R5X4 viruses that arise later. Strain R3A was obtained from an unusual R5X4 acute infection (Meissner et al., 2004), but R3A was not among the R5X4 Envs with more efficient lymphocyte CCR5 use. Paradoxically, the two variants with greatest relative efficiency in lymphocyte CCR5 use were NR10, from a late stage patient, and C2-16, from a CCR5-null subject; neither are clinical scenarios in which one might expect more efficient lymphocyte CCR5 use from the isolated virus. Macrophage infection is thought to be the major source of virus in tissue, a critical component of neuropathogenesis, and responsible for sustaining virus replication at very late stages of disease (Igarashi et al., 2001; Koenig et al., 1986; Schuitemaker et al.,

1992). Prototype and some primary R5X4 strains readily infect macrophages using CCR5, which may be explained by the higher density of CCR5 expressed on these cells compared with primary CD4+ lymphocytes (Lee et al., 1999b; Yi, Shaheen, and Collman, 2005). However, use of this coreceptor may be dispensable since CXCR4 also serves as a viable coreceptor for R5X4 entry into these cells (Yi, Shaheen, and Collman, 2005). Thus, it remains to be determined how the efficiency of lymphocyte CCR5 use among R5X4 variants is linked to specific aspects of pathogenesis. In addition, while X4 use is common in subtype B HIV-1, it is less frequent among subtype C strains and, furthermore, the factors that regulate coreceptor interactions may differ for different subtypes (Lynch et al., 2009). Further studies will be needed to determine the extent to which these findings apply to other subtypes

V3 determinants that regulate CCR5 use by R5X4 HIV-1: implications for structural interactions with coreceptor

The repeated correlation between measures of Env-CCR5 interaction efficiency and CCR5 use on lymphocytes suggested that elements within Env regulate lymphocyte CCR5 use. Since V3 is the principal determinant of coreceptor use, I focused on how variation in this domain might regulate lymphocyte CCR5 use by R5X4 strains. I discovered a relationship between the viral phenotype predicted using a V3-based PSSM algorithm and lymphocyte CCR5 use, with viruses that used this coreceptor well predicted to be NSI viruses and those that use it poorly identified as SI variants. Subsequent analysis of V3 sequences revealed that the two variants at the extremes of the

spectrum of CCR5 use, 89.6 and C2-16, differed in the presence of hallmarks associated with CXCR4 use, including the presence or absence, respectively, of a basic amino acid at position 306 in V3. Evaluation of mutant compared to wt viruses showed that 89.6 R306S was less sensitive to blocking CCR5 and was less dependent on the CCR5 N-terminus for infection. Conversely, the C2-16 S306R mutant was more sensitive to agents directed at CCR5 and was more dependent on the N-terminus of the coreceptor. The impact of these mutations on coreceptor use was determined by infecting primary lymphocytes, and the results indicated that the R306S mutation in 89.6 changed its coreceptor preference from CXCR4 to CCR5, while the reciprocal S306R change in C2-16 did not alter coreceptor use. These studies revealed that features within V3 likely determine the spectrum of lymphocyte CCR5 use, that the residue at position 306 in Env modulates the efficiency of CCR5 use for 89.6 and C2-16, and that R306 is responsible for impaired lymphocyte CCR5 use by 89.6.

The V3 domain is a primary determinant of coreceptor use, however, the effect of subdomains within this region on primary cell coreceptor use has not been previously addressed. Here, I show that a serine at position 306 is associated with greater lymphocyte CCR5 use by 89.6. This occurs because serine at this residue enhances the efficiency of CCR5 use, likely through enhanced interactions with the CCR5 ECLs. Most of the R5X4 strains have serine at this position, but not all use CCR5 efficiently. PSSM results indicate that elements within V3 likely regulate lymphocyte CCR5 use by R5X4 viruses. The majority of the variation in the V3 region is in stem and tip residues other than 306. Thus, these stem and tip residues may regulate lymphocyte CCR5 use for the other R5X4 strains. This would presumably occur through interactions with the

ECLs, since the V3 stem and tip are thought to interact with this domain of the coreceptor. Interestingly, the C2-16 S306R mutation had an impact on the efficiency of CCR5 use by C2-16, but did not alter its coreceptor use on primary lymphocytes. The reasons for this are unclear, but may involve other regions of Env or of V3. Additionally, mutations at this position also affected CXCR4 use by 89.6, suggesting that residue 306 may regulate interactions with CXCR4 for some viruses.

The ability to use CCR5 and CXCR4 must hinge on the ability of R5X4 strains to recognize conserved regions of each coreceptor, but it is unclear exactly how this is regulated. Numerous studies indicate that the underlying architecture of CCR5 and CXCR4 are similar. R5, X4 and R5X4 viruses are capable of using CCR5/CXCR4 chimeras for infection, even when multiple discontinuous domains are exchanged (Karlsson et al., 2004; Pontow and Ratner, 2001). Removal of an N-linked glycosylation site in the N-terminus or a substitution that removes a negatively charged residue in ECL-2 of CXCR4 permits use of this coreceptor by R5 viruses (Chabot and Broder, 2000; Chabot et al., 2000; Chabot et al., 1999). As mentioned, deletion of amino acids surrounding the V3 loop of R3A and 89.6 abrogated CXCR4 use; the resulting virus retained CCR5 use but was resistant to CCR5 antagonists (Nolan, Jordan, and Hoxie, 2008). These findings indicate this segment of the V3 stem interacts with the ECLs of CCR5 and CXCR4. Residue 306 is located in this region of V3 and it modulates Env-CCR5 and potentially Env-CXCR4 interactions for some viruses. Perhaps position 306 serves as a pivot point in Env, and the identity of the amino acid at this position allows the virus to favor one coreceptor over another by recognizing a critical ECL determinant that is similar in both coreceptors but distinct enough to allow the virus to distinguish

CCR5 from CXCR4. For other R5X4 strains, in which amino acid 306 may not regulate the efficiency of CCR5 use, another stem or tip residue in V3 may play this role.

Summary and final conclusions

In summary, using a panel of primary and prototype R5X4 HIV-1 variants, this study confirms that these strains are highly skewed towards CXCR4 use for entry into CD4+ T cells. However, among the isolates tested, there is a range in the relative ability to use CCR5 on primary lymphocytes, although we did not identify an evident relationship between relative lymphocyte coreceptor use and the clinical context in which the R5X4 Envs were derived. The ability to use lymphocyte CCR5 by R5X4 variants was linked to greater efficiency in CCR5 interactions based on antibody and small molecule antagonist blocking studies, and this was dependent on residues within V3. Furthermore, the barrier to CCR5 use could be overcome by CCR5 over-expression in lymphocytes for most of the R5X4 isolates or, by mutating Env to enhance coreceptor interaction efficiency.

These finding lead to the conclusion that potential CCR5-mediated infection of CD4+ lymphocytes is limited by the proportion of cells that express CCR5, but actual CCR5 use by a specific R5X4 virus is regulated by the threshold of CCR5 it requires for infection. The differences in CCR5-Env interactions identified among the R5X4 strains here, which are dependent on determinants within the Env V3 that interact with the CCR5 ECLs, regulate the threshold of CCR5 required for entry. Thus, the efficiency of the Env-CCR5 interaction is the principal factor underlying differences in CCR5-mediated target cell availability and, ultimately, lymphocyte CCR5 use for each isolate.

Better understanding of the viral and cellular factors that control coreceptor use by HIV-1 strains is critical to understanding the impact of viral evolution on target cell tropism.

Chapter VII - Bibliography

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