Molecular Determinants of CCR5-Tropic Human Immunodeficiency Virus 1 Entry Into Low-CCR5 Expressing Cells

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Molecular determinants of CCR5-tropic Human Immunodeficiency Virus 1 entry into low-CCR5 expressing cells

A dissertation presented
by
Nicole Joy Espy
to
The Program in Biological Sciences in Public Health
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
Biological Sciences of Public Health

Harvard University
Cambridge, Massachusetts
December 2016
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Abstract

Over 80 million people have been infected with Human Immunodeficiency Virus 1 (HIV-1) worldwide. Despite advances in the treatment of HIV-1 infected individuals, practical cure or prevention of HIV-1 infections has not been achieved. Basic research on HIV-1 infection is imperative to better understand and prevent new infections. It is currently known that the HIV-1 envelope glycoprotein (Env) binds immune cell receptors CD4 and CCR5, sequentially. Binding to each receptor triggers conformational changes in Env that promote viral fusion and entry. Researchers seek to gain insight into the CCR5-induced transition from the Env pre-hairpin intermediate to downstream fusion-active conformations, as CCR5 plays an essential role in infection. The structures assumed by Env after CCR5 binding and before membrane fusion, in which the protein assumes a six-helix bundle conformation, remain unknown.

In my work, I tested the essential role of CCR5 by attempting to adapt HIV-1 to infect cells with reduced levels of CCR5. To do so, I generated cells with titratable cell surface CCR5 expression levels using a tet-promotor system, and passaged virus in these cells in the presence of doxycycline. In doing so, I obtained an HIV-1 capable of infecting cells expressing ~1300 CCR5 molecules/cell. Changes in Env mapped to gp120 and gp41, the surface and transmembrane subunits of Env. These changes conferred either
enhanced replication capacity or allowed Env to expose receptor-binding sites in the absence of receptor. Functional analysis of the adapted HIV-1 helped to identify new regions in Env critical to CCR5-mediated entry kinetics and viral fusion. This work will also help us to understand the pathways of HIV-1 escape from CCR5 inhibitors and provide information about the vulnerability of Env intermediate conformational states to inhibition by drugs and antibodies.

New tools to handle CCR5 for biochemical studies could help researchers isolate these intermediate conformational states. I therefore generated lipid bilayer nanodiscs containing human CCR5 molecules derived from mammalian CHO cells. The reconstituted CCR5 maintained its capacity to bind the anti-CCR5 monoclonal antibody 2D7 and HIV-1 gp120. Optimization of CCR5 nanodiscs can allow this tool to be used in future studies of CCR5-bound HIV-1.
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Appendix 1 Formation of a gp120-sCD4-sCCR5 complex

Bibliography
Dedication
My father and mother, my sister and brother.
Chapter 1

Overview of CCR5 dependence in HIV-1 Entry
Section 1.1 Overview and Attributions

Overview

This chapter is composed of a review of the field on the role of CCR5 in HIV-1 Envelope binding to target cells and initiating fusion of the virus to the target cell.

Attributions

I researched and wrote the manuscript. Dr. Sodroski provided editorial input.
Section 1.2: Beyond binding: Reviewing the role of CCR5 in HIV-1 Infection

Nicole Espy¹ and Joseph Sodroski²

¹Department of Immunology and Infectious Diseases, Harvard School of Public Health
²Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA

Abstract

Human immunodeficiency virus 1 (HIV-1) requires coreceptor binding to mediate cell attachment and membrane fusion. CCR5, the primary coreceptor for HIV-1, is essential for viral infection. In this review, we summarize the role of CCR5 in HIV-1 infection, characterize the regions in the HIV-1 Envelope glycoprotein required for CCR5 binding and summarize the Env conformational changes induced by the receptor binding. We identify gaps in knowledge about the structure of Envelope after CD4 binding.

Introduction

The human immunodeficiency virus-1 (HIV-1), the causative agent of the acquired immunodeficiency syndrome (AIDS) epidemic, has affected the lives of millions and changed the practice of medicine and global health over the past 30 years (1). The Joint United Nations Programme on HIV/AIDS has estimated over 80 million infections and 40 million deaths since the start of the epidemic. In 2015 alone, there were 36.7 million (34.0-39.8 million) adults living with HIV, 2.1 million (1.8 million-2.4 million) new infections, most of which are in adults, and 1.1 million (0.94 million-1.3 million) deaths. The biomedical response to this epidemic produced highly active antiretroviral therapies
(HAART) that evolved from the first anti-HIV-1 drug zidovudine, or AZT, discovered in 1986 (2). The consistent use of HAART in HIV-1 infected individuals has greatly extended disease-free survival (3, 4). In some settings, treatment of HIV-1 infected individuals decreased the rate of transmission (5). Additionally, science has gained a more sophisticated understanding of the human immune system (6-8). Despite all of the tools and knowledge gained over the past 30 years of the epidemic, the cure of HIV-1 infected individuals or the generation of an effective vaccine remains elusive.

Part of the challenge of curing an HIV infection is due to the complexity of the virus, the establishment of a reservoir of latently infected cells, and the constant adaptation of the virus to evolve resistance to anti-HIV drugs and neutralizing antibodies (9, 10). HIV is a positive-stranded RNA virus that generates a provirus that is ~9.8 kb in length. The two HIV subtypes in humans, HIV-1 and HIV-2, originated as a result of independent cross-species transmission events from African primates (11). Further, HIV-1 strains vary in genetic sequence significantly, such that they are categorized into the M (in which there are subtypes A-D, F-H, J, and K), N, or O groups. The cause of this genetic diversity is the high mutation rate of HIV-1, which is estimated to be 0.1-0.2 mutations/genome/generation (12). Although many mutations are deleterious, the high rate of replication of the virus (1.2-day generation time with $10^{10}$ virions produced daily) produces a sufficient number of viruses that do not contain a deleterious mutation, and thus can stably reinfect cells, and continue to evolve (13). The result is a population of HIV-1 (the viral “quasispecies”) that varies in genetic sequence within an infected person and across geographic regions. This genetic diversity necessitates the understanding of mutations in a virus within the context of its distinct genetic
background, and a nuanced approach to generating anti-HIV drugs or vaccines that is either specific for the strains within the patient or broadly effective across all strains.

The most effective HIV vaccine generated thus far was tested in the RV144 trial, in which the vaccine demonstrated an efficacy of 31.2% in preventing HIV-1 infections (14). Many of the antibodies generated targeted the HIV-1 Envelope protein (Env), the glycoprotein on the surface of the virus responsible for viral entry into target cells. Specifically, the antibodies were directed against the first and second variable loops (V1/V2) of the gp120 surface unit Env. These variable loops become more exposed after gp120 binding to human CD4 expressed on T-cells and macrophages. These antibodies do not neutralize HIV-1, but can mediate antibody-dependent cell cytotoxic (ADCC) responses that result in the killing of virus-infected cells (15-17). The RV144 vaccine also raised antibodies directed against the third variable loop (V3), which binds to human CCR5 (18). Env interaction with CD4 and CCR5 represents two critical interactions in the HIV-1 entry process. Further emphasizing the role of Env binding to its cellular receptors, the only person who has been functionally cured of HIV-1 infection was Timothy Ray Brown, who received a hematopoietic stem cell transplantation from a donor that was homozygous for CCR5Δ32/Δ32 as treatment for his acute myeloid leukemia (19, 20). The result was the expression of the CCR5Δ32 gene that generates an N-terminally truncated CCR5 unusable by HIV-1 for entry. Mr. Brown demonstrated a clearance of all detectable viremia for over 20 months without HAART, and the reestablishment of CD4+ T cells and a functioning immune system. Therefore, a better understanding of the HIV-1 entry process and the Env interaction with its receptors is
necessary to identify critical steps in infection and for a more rational design of anti-HIV therapies.

**The HIV Entry Pathway**

HIV-1 infects T-cells and macrophages that express the viral receptors, CD4 and CCR5, on their surface. HIV-1 entry is mediated by the metastable, trimeric Env glycoprotein spike, which consists of a receptor-binding surface unit (gp120) and a membrane-fusing transmembrane unit (gp41). Viral entry begins when an HIV-1 gp120 subunit binds target cell-expressed CD4. CD4 binding induces significant conformational changes in Env to form [1] a pre-hairpin intermediate state, in which the N-terminal helix of gp41 forms a coiled coil at the trimer axis, and [2] a CCR5-binding site (Figure 1.1). The CCR5-binding site is composed of an extended V3 loop that binds the second extracellular loop (ECL2) of CCR5 and a bridging sheet composed of a beta-stranded sheet and the base of the V3 loop that binds the CCR5 N-terminus (21-30). Env binding to CCR5 is thought to allow further conformational transitions in the extracellular heptad repeats (HR1 and HR2) in gp41 to convert the pre-hairpin

![Figure 1.1: HIV entry schematic.](image-url)

intermediate to the six-helix bundle, in which HR1 folds back onto HR2. The result is the fusion of the viral and target cell membranes to allow the viral core to enter the target cell cytoplasm.

There are significant gaps in our knowledge of CCR5-induced transitions. Structural information from the unliganded Env trimer and the CD4-bound gp120 core, in addition to previous research on the molecular dynamics of CD4-gp120 binding, provides information about the transition between unliganded and CD4-bound conformations during viral entry. Knowledge about the transition from the CD4-bound Env trimer to the fusion-active conformation, which includes the CCR5-bound Env conformation, is limited.

**CCR5 as a critical receptor in HIV-1 entry**

CCR5 is a seven-helix transmembrane G-protein coupled receptor protein (GPCR). CCR5 is a receptor for the chemokines macrophage inflammatory protein 1 alpha and 1 beta (MIP-1a and MIP-1b) and regulated on activation normal T expressed and secreted protein (RANTES). CCR5 binding of these beta-chemokines promotes chemotaxis as well as other inflammatory responses. The observation that these chemokines also suppress HIV infection in cells expressing CD4 indicated that CCR5 functions as a necessary coreceptor with CD4 for HIV entry (31-35). CCR5 can be expressed on the cell surface as a monomer, a homodimer, and a heterodimer with other GPCRs (36). CCR5 has been found to naturally exist as a dimer in the microvilli of T-cells and macrophages (37, 38), and dimerization is promoted by chemokine binding.
Additionally, the N-terminus of CCR5 is posttranslationally modified by sulfation of tyrosine residues that are necessary for gp120 binding (40-44).

CCR5 is expressed in dendritic cells, T-cells, and macrophages at levels varying from ~45,000 molecules per cell in mature dendritic cells to ~2,875 in monocytes (6). The level of CCR5 required for HIV entry has not been quantified, as it is a function of the method of infection, the level of CD4 available on the cell surface, and the HIV-1 Env sequence. Platt, et al, attempted to measure the entry kinetics of viruses pseudotyped with a JR-CSF Env on Hela cells expressing wild-type CCR5 or CCR5 with an N-terminal change that reduced the affinity for Env (45). While wild-type HIV-1 JR-CSF could infect cells with less than ~10,000 CCR5 molecules per cell, the virus demonstrated an EC$_{50}$ of ~150,000 mutant CCR5 molecules/cell for infection. This suggests an increased availability of CCR5 molecules can overcome a reduced affinity of the Env-CCR5 interaction to result in a productive infection. To further test this assumption, they evaluated the entry kinetics by measuring over time the HIV-1 JR-CSF infection of Hela-CD4-CCR5 cells with ~6000 or ~190,000 CCR5 molecules/cell after spinoculation, in which cell-free virus is made to adhere to the cell by centrifugation in order to increase the chance of infection. By comparing the time to infection and the number of particles that infected, these researchers were able to estimate the rate of infection and viral inactivation. For JR-CSF on low-CCR5 expressing cells, the rate of infection was 0.09/hour and the rate of viral inactivation was 0.47/hour, resulting in a 3% chance that a virus infects a low-CCR5 cells. The chance of infection on high-CCR5 expressing cells was 15%, suggesting that infection competes with viral decay and
further demonstrating that HIV-1 Env entry kinetics is positively correlated with expression levels of CCR5.

There are a number of rare alternate coreceptors that mediate HIV-1 infection. CXCR4 is the most common alternate coreceptor to CCR5 for some strains of HIV-1 (46). HIV-1 Env uses CXCR4 in a similar manner to CCR5 for viral entry, but the physical interactions between Env and the molecule require molecular changes in V3 that include changes in V3 length and an increase in positively charged residues (47, 48). In this manner, CCR5-tropic HIV-1 may evolve to become CXCR4 tropic or dual-tropic (49). Expression of CXCR4 is similarly high on mature DC cells (~35000 molecules/cell, and low on monocytes (~2,500 molecules/cell) (6). Since the majority of HIV-1 strains that initiate an infection in a patient (ie, Transmitted/Founder viruses) use CCR5, we will focus on the Env-CCR5 interaction (50).

**CCR5 independence**

The vast majority of Transmitted/Founder viruses are CCR5-tropic (50). These viruses also require high levels of CD4, and therefore infect T-cells better than macrophages (51). While the role of CD4 in HIV entry has been studied extensively, Envs that permit infection in cells expressing only CCR5 (i.e. CD4-independent viruses) have been generated in laboratory settings or found in simian immunodeficiency viruses (42, 52-55). CD4 independence in HIV-1 often involves an N197K change to remove an N-linked glycosylation site in the V1/V2 loop or the deletion of the V1/V2 loop altogether. Although the CD4-independent phenotype is not seen in natural strains of HIV-1, as they are readily neutralized by the host immune system, we can deduce that the use of
CD4 is important for immune evasion but not absolutely required for HIV-1 entry or membrane fusion.

An early clinical observation indicating the importance of CCR5 for HIV-1 infection was that patients that were homozygous for CCR5Δ32/Δ32 were not infected with HIV-1 when exposed (56-59). Further, mice with a hematopoietic stem cell transplant treated with zinc-finger nucleases inhibiting CCR5 expression were cleared of HIV-1 infection (60). And as mentioned above, the only patient that functionally cleared an HIV-1 infection received a CCR5Δ32/Δ32 hematopoietic stem cell transplant. The only cases of HIV-1 infection in CCR5Δ32/Δ32 individuals involve CXCR4-tropic HIV-1 (61).

It is curious that no HIV-1 strain yet discovered has demonstrated a CCR5, CXCR4, or alternate coreceptor-independent phenotype. The limited information we have about the role of CCR5 binding in viral infection indicates that CCR5 binding by Env is required for both attachment to the target cell and the initiation of viral fusion, and that these events are essential for infection. Experiments in which V3 was truncated have demonstrated attenuated viral infection (29, 30, 62, 63). Attempts to rescue the replication capacity of these mutant viruses by passaging them on CD4+ CCR5+ cells failed to recover viral fitness (64). Interestingly, Envs with V1/V2 deletions that exhibit a CD4-independent phenotype acquired changes that involve changes around the V3 domain, perhaps due to an enhanced reliance on CCR5 for infection, and changes throughout gp41, suggesting the necessity of improved triggering of viral fusion after CCR5 binding (64). This experiment emphasizes the essentiality of V3 binding to CCR5 for infection and highlights the role of CCR5 in inducing gp41-mediated viral fusion.
Attempts to block Env binding to CCR5 with small molecule inhibitors have reemphasized the essentiality of CCR5 engagement for infection. Maraviroc, an FDA-approved HIV-1 entry inhibitor, binds to CCR5 and disrupts the conformation of the N-terminus and extracellular loops to prevent Env binding and infection (65, 66). After years of use, Maraviroc-resistant viruses were identified in HIV-1 infected individuals (67). Interestingly, when screening HIV-1 subtype C viruses in patients that had not been treated with antiretroviral therapy, researchers discovered that these strains exhibit some natural resistance to Maraviroc (68, 69). These viruses were not CCR5-independent and in fact still required CCR5 for infection, unless they were already CXCR4-tropic to begin with. Instead, they were capable of using the drug-bound CCR5 to mediate viral infection (67, 70-72). Changes in the resistant viruses resulted in more efficient CD4 binding, altered CCR5 engagement by the V3 loop, and enhanced fusion capacity. Adaptation experiments that generated HIV-1 resistant to vicriviroc, a derivative of Maraviroc, identified changes in both V3 and gp41 that allowed the virus to utilize the drug-bound CCR5 (70). These changes conferred altered CCR5 binding by the V3 loop and changes in the fusion peptide within gp41. These results again emphasize that the role of CCR5 in HIV-1 infection goes beyond attachment and that enhanced fusigenicity by gp41 can compensate for inefficient receptor binding.

**Improved models of HIV-1 infection in a setting of low CCR5 expression**

HIV-1 replication in an individual undergoing Maraviroc treatment should in principle mimic the conditions of a CCR5Δ32/Δ32 patient. Instead, the virus adapted to use Maraviroc-bound CCR5, essentially equivalent to a shift in receptor tropism. This
phenomenon requires a reevaluation of how the conditions of a CCR5Δ32/Δ32 patient (i.e., low or absent CCR5) could be met, and whether any of the changes observed in Maraviroc-resistant viruses are due to scarce CCR5 or to the acquisition of a Maraviroc-bound CCR5 tropism.

In an adaptation experiment in which HIV-1 AD8 was passaged in SupT1 suspension cells expressing CD4 and low levels of CCR5, an N302Y change in the V3 loop conferred improved replication capacity. Concomitant with this change was an enhanced resistance to Maraviroc, suggesting that the N302Y change improved the physical interaction with CCR5 (73). These results are in accordance with the requirement for V3 binding to CCR5 for infection, and suggest that the virus adapted to strengthen this interaction.

To further test the effect of scarce CCR5 on HIV-replication, Aarons, et al, adapted HIV-1 JR-CSF to replicate in adherent U87-CD4-CCR5 cells in the presence of 2D7, a monoclonal antibody that binds the N-terminus and ECL2 of CCR5, thereby blocking Env binding with CCR5 (74). Virus was still able to replicate in these conditions at low levels. The mutant virus did not change its receptor tropism (i.e. still required CCR5 for entry) but acquired a D164N change within the gp120 V1/V2 loop and the R525A, R549K, and G666D changes within the first and second heptad repeats of gp41. The mechanism of resistance was not identified in this study, leaving open questions about the role of CCR5 in gp41 activation. These results, however, suggest that by whatever mechanism, the virus has enhanced the efficiency of entry in order to maintain growth in this system. The fact that there were no changes in V3 suggest that the adaptation
mechanism is not related to a stronger binding to CCR5, and that other steps in the entry pathway can overcome the rarity of interaction with CCR5.

The above studies point to gp41 activity in the entry pathway, but leave open the question of whether the changes seen in gp41 are related to CCR5 binding, or are a generic mode of improving viral fusion by promoting the formation of the six-helix bundle. Assays testing resistance to fusion inhibitors have also implicated changes in gp41. For example, Wang, et al, passaged HIV-1 JR-CSF in suspension PM-1 lymphoid cells in the presence of T-20, a fusion inhibitor that binds HR1 to prevent the formation of the six-helix bundle (75, 76). An E560K in HR1 and an E648K in HR2 was observed, among other changes throughout Env, and these changes increased the stability of the six-helix bundle. These changes did not have an altered effect in the presence of varying levels of CCR5, or improve entry kinetics (76). Additional research testing the effect of the T-20 resistance change D547G in HIV-1 LAI found that the observed increased fusion activity was independent of receptor-binding activity (77). It is therefore possible to distinguish changes that influence gp41 HR1 and HR2 activation upon CCR5 binding and those that enhance the formation of the gp41 six-helix bundle.

The aforementioned adaptation experiments hinted at a pattern such that changes in HIV-1 replicating in adherent cells were more likely to be in gp41 and changes in HIV-1 replicated in suspension cells were in gp120. More specifically, changes in V3 were more likely to occur when cell-cell contact is rare and changes in gp41 were more likely to occur when cells are in contact, permitting cell-cell transmission of the virus. The mode of infection is an important dynamic to consider when evaluating the entry kinetics of a virus. In a review of cell-cell transmission of viruses, Mothes and Zhong, et al.
described a few conditions for which cell-cell transmission of a virus would be favored over cell-free infection (78, 79). Conditions include instability of the cell-free virus and inefficient binding to cellular receptors. Cell-cell transmission is also favored when the cellular receptors or viral budding is polarized on the cell surface, creating a microenvironment of high density.

HIV-1 encounters challenges during infection when CCR5 is scarce. First, HIV-1 entry kinetics are a function of the balance between the ability to induce fusion and the rate of viral inactivation (45). Second, adaptive changes to conditions of scarce CCR5 might attempt to enhance the overall efficiency of receptor binding. When considering models of HIV-1 infection when CCR5 is scarce, virus replicating in adherent cells has ample opportunity to interact with the target cell but limited opportunity to promote viral fusion; the adapted virus must therefore overcome this by altering its gp41 fusion machinery. Virus replicating in suspension cells, however, must optimize engagement with CCR5 to facilitate the attachment of viruses in the media; this requirement might be met by strengthening Env-CCR5 binding by V3 changes. Third, it has been previously shown that CCR5 and CD4 interact in primary T cells, HIV3T3, HeLa and HOS cells; that CXCR4 interacts with CD4 in Cf2Th cells (80-82); and that CD4 and CCR5 form clusters on the cell surfaces (83, 84). These observations suggest that a localization of HIV-1 receptors may occur. Further, evidence of a virologic synapse in vivo has been demonstrated via the visualization in multiphoton images of clusters of cells infected by the same fluorescently tagged HIV-1 in spleens from hu-PBL mice (85, 86). The opportunity for HIV-1 infection across a viral synapse, when available, would likely result
in the promotion of adaptive changes to promote cell-cell transmission over changes that alter receptor-binding affinity.

**Implications of gp41 changes in the model of Env binding to CCR5**

The above studies indicate that CCR5 engagement by Env might involve a number of activities that include CCR5 binding by V3, alteration of the fusion peptide conformation, uncharacterized gp41 activity mediated by the HR1 and HR2 regions, and preparation for the formation of the six-helix bundle. All of these activities can overcome barriers to CCR5 engagement by altering receptor tropism, increasing the exposure of the fusion peptide, and stabilizing the six-helix bundle or improving that yet-to-be characterized gp41 activity.

This uncharacterized gp41 activity could influence the energetic barriers to assuming a fusion-ready conformation in Env. Analysis of CD4-independent viruses using neutralizing sensitivity assays demonstrated the spontaneous exposure of the CCR5 binding site in Env in the absence of CD4, thereby naturally overcoming the energetic barriers typically mediated by CD4 binding (53, 54). The neutralizing sensitivity of the HIV-1 adapted to replicate in any of the experiments described above was not tested, so the general conformation of Env in these viruses is unknown. Research in this area could shed more light on the relationship between changes in gp41 activation and conformations Env assumes.

Other possibilities to describe this uncharacterized gp41 activity include altered gp120 shedding or improved cooperativity between gp120 and gp41 domains, which could be measured by structural analysis of Env before and after CD4 or CCR5 binding.
This information is needed to define the role gp41 in CCR5 binding and viral fusion and could indicate why CCR5 independence has been achieved in the experimental conditions previously used. The multiple functions precipitated by CCR5 engagement insinuate that it may require many Env changes for HIV-1 to achieve complete CCR5 independence.

**Conclusion**

This review of current literature concerning HIV-1 engagement with CCR5 argues for a new model for CCR5 engagement and further investigation of the role of gp41 in viral infection. Such research might lead to improved antibodies or small molecules that target critical steps in viral infection and can mimic the conditions in a CCR5Δ32/Δ32 patient. In doing so, we can perhaps design therapies to which the virus is not already naturally resistant, or cannot develop resistance over time. Additionally, identification of these regions can inform the monitoring and surveillance of viral strains in a patient or population that are naturally resistant to possible drugs or vaccines. As more information is gathered about HIV-1 viral infection at the molecular level, we get closer to elucidating how to stop HIV-1 infections at the population level.
Chapter 2

Generation of a cell line that modulates CCR5 expression and adaptation of HIV-1 to infect cells with diminishing CCR5.
Section 2.1 Overview and Attributions

Overview

This chapter is comprised of a manuscript that I wrote to describe the generation of HIV-1 adapted to infect cells with low levels of CCR5 expression on the cell surface. We will use this as supplementary materials for a manuscript to be submitted for publication.

Attributions

I performed the experiments in all figures and wrote the manuscript. Dr. Sodroski provided guidance in the experimental design and editorial input. Beatriz Pacheco assisted in the development of the adaptation method. Alon Hershhorn provided technical assistance in the use of the TetOn system (Clontech).
Section 2.2 Molecular determinants of Human Immunodeficiency Virus 1

Envelope glycoprotein-mediated entry into cells expressing low levels of the CCR5 coreceptor

Nicole Espy¹, Beatriz Pacheco, Joseph Sodroski¹²³.

¹Department of Immunology and Infectious Diseases, Harvard School of Public Heath
²Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA
³Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA

Abstract

The Human Immunodeficiency Virus 1 (HIV-1) envelope glycoprotein (Env) trimer mediates viral infection of cells expressing the receptors CD4 and CCR5. The V3 variable loop in the gp120 surface unit of Env directly binds CCR5. Modulating the expression levels of CD4 and CCR5 affects the infection capacity of virus and leads to molecular changes in Env to enhance viral entry. In this study, we generated CD4-positive Cf2Th cells that regulate CCR5 expression in a tetracycline-dependent expression system and adapted HIV-1 NL4.3(JR-FL) to infect these cells with diminishing levels of CCR5 expression. Viral replication was observed at levels >1300 CCR5 molecules per cell. While molecular changes throughout the gp120 and gp41 subunits of Env were identified, no changes were observed in V3. These changes emphasize the relationship between gp41 and gp120 in promoting CCR5 binding and viral fusion.
Introduction

The human immunodeficiency virus 1 (HIV-1) envelope glycoprotein (Env) is a metastable trimer of a gp120-gp41 heterodimer that is present on the surface of HIV-1 virions. HIV-1 infects T-cells and macrophages through a series of steps mediated by Env that result in entry into the target cell. Initially, cell-free virions interact with the target cell membrane through weak interactions with adhesion factors. Productive infection then begins when the gp120 surface unit of Env sequentially binds CD4 and the chemokine receptor CCR5. Binding of each target cell molecule triggers conformational changes in gp120 and the gp41 transmembrane domain that promote viral fusion with the target cell membrane followed by entry of the viral core. Further rounds of viral infection may occur from cell-free virus binding to cells or via cell-to-cell transmission. Cooperativity within the different subunits of Env promotes movement through the viral entry cascade upon CD4 and CCR5 engagement.

The variables influencing the dependency of HIV-1 on receptor CD4 or coreceptor CCR5 binding are determined by genetic and structural characteristics of Env. For example, viruses with a deletion in the variable loops V1 and V2 in gp120 or an N197K change in the HIV-1 ADA strain do not rely on CD4 for infection (53). These changes eliminate structural barriers to assuming an infection-capable conformation, such as the exposure of gp120 variable loop V3, which binds to the extracellular loop 2 of CCR5, and the formation of the coreceptor-binding site (53). Currently, there are no known coreceptor-independent HIV-1 strains. HIV-1 strains resistant to entry inhibitors, such as the CCR5 antagonist Maraviroc or the fusion peptide binding T-20, have been
characterized; these studies indicate that the V3 and gp41 regions undergo structural changes before and after CCR5 binding that are dependent upon the molecular background of Env (87). These changes include the formation of a three-stranded coil by gp41 that extends through the Env core, the exposure of the fusion peptide at the N-terminus of gp41, and the triggering of gp41 into a six-helix bundle concomitant with viral membrane fusion with the target cell membrane.

The relative abundance of CD4 receptor and CCR5 coreceptor on the target cell surface influences the viral infection kinetics (45, 88, 89). Affinofile cells regulate CD4 and CCR5 expression on HeLa cells upon doxycycline and minocycline regulation. Relative infectivity of viral strains at each ratio of CD4 to CCR5 expression levels demonstrates a varied dependency on these molecules for infection. Of particular interest is the relationship between CCR5 expression levels and infection efficiency, and the identification of a minimal level of CCR5 expression required for infection.

This study intends to adapt HIV-1 to use progressively lower levels of CCR5 for infection to determine the lowest limit of CCR5 molecules per cell required for viral entry, and to identify the molecular determinants of CCR5 binding and viral fusion. In this study, we generated Cf2Th cells that stably express CD4 and express CCR5 in a doxycycline-regulated manner at the lowest levels of CCR5 of physiological relevance. We then infected these cells with HIV-1 NL4.3(JR-FL) and passaged cells with diminishing concentrations of doxycycline to generate a virus capable of infecting cells with ~1300 CCR5 molecules per cell. The provirus was sequenced from the genomic DNA from infected cells at each round of adaptation to identify molecular changes that
facilitated the low-CCR5 infection phenotype. Genetic changes were identified in gp120 and gp41 at sites previously unknown to be influenced by CCR5 binding.

**Materials and Methods**

*Generation of tTA-expressing cells*

Cells expressing the tetracycline transactivator protein (tTA) for which gene expression is induced (Tet-On) or repressed (Tet-Off) in the presence of doxycycline was generated according to the Clontech Tet-On and Tet-Off manual (Takara). In summary, Cf2Th cells were transfected with the pTet-On vector using Effectene (Qiagen) or transduced with the pTet-Off plasmid using the Lenti-X™ Tet-Off © Advanced Inducible Expression System (Clontech) and single cells were selected via limiting dilution in 96-well plates in the presence of 0.6 μg/mL puromycin. Cells were expanded and tested for activity by an 18-hour incubation with doxycycline and infection with a single-round recombinant HIV-1 pseudotyped with VSV-G and expressing luciferase under a tet promoter (ptet-luc). The recombinant viruses were generated by transient transfection in 293T cells. Cells were incubated in DMEM-10 for 2 days and lysed with 30 μL passive lysis buffer (Promega). Luciferase activity was measured with a Mithras LB 940 luminometer (Berthold Technologies).

Cells demonstrating dynamic tTA activity were transfected with pGL422-tetO-CCR5, selected for single clones via limiting dilution in 96-well plates, and expanded in the presence of 0.6 μg/mL G418. CCR5 expression in cell clones was measured via flow cytometry by incubating cells for 18 hours in the presence of increasing concentrations of doxycycline, labeling with R-Phycoerythrin-conjugated mouse anti-
human CCR5 (PE-anti-CD195, BD Biosciences), and analyzing a million cells in a BD FACSCanto II flow cytometer (BD Biosciences).

Cells demonstrating tight regulation and a dynamic range of CCR5 expression were transfected with pcDNA-CD4, selected for single cells via liming dilution in 96-well plates, and expanded in the presence of 0.025 μg/mL zeocin. Cells labeled with PE-anti-CD195 and fluorescein isothiocyanate-conjugated mouse anti-human CD4 were analyzed via flow cytometry to identify clones that attained stable CD4 expression and retained tet-regulation of CCR5.

Quantification of CCR5 expression levels as measured by flow cytometry was estimated via BD QuantiBrite™ (BD Biosciences).

*Generation of replication-competent HIV-1 and adaptation of virus to low levels of CCR5 in R5-Low cells*

Replication-competent HIV-1 was generated by transfecting 2 million 293T cells with 20 μg of a plasmid containing the pNL4.3 provirus with the HIV-1 JR-FL env (between the SalI and BamHI sites) using Effectene transfection reagent (Qiagen). The supernatant was harvested after two days and frozen in aliquots. Reverse transcriptase (RT) activity in the supernatant was measured using ³H-labelled nucleotide triphosphates for initial viral production assays and ³²P-labelled nucleotide triphosphates for all viral adaptation assays, in a method previously described (90). To produce the virus in the Cf2Th cell line, 100,000 Cf2-T4R5 cells were infected with 3000 cpm (³H) of HIV-1 NL4.3(JR-FL) virus made in 293T cells as above. Cells were passaged every 3-4 days by lifting them from the plate using 5 mM EDTA in PBS and diluting them 1:5.
Supernatants were collected at each passage to measure RT. Additional studies of virus replication in Cf2Th-CD4/CCR5 cells used 6600 cpm \(^{32}\text{P}\) of virus from the time point with peak RT in the presence of 2 μg/mL polybrene.

For all subsequent rounds of adaptation, 25,500 cpm \(^{32}\text{P}\) RT units of supernatant, collected at the time point with peak RT activity, was used to infect R5-High cells and then R5-Low cells at decreasing expression levels of CCR5 (ie, lower concentrations of doxycycline). Serial passage of the virus was continued until 3 independent assays with triplicate samples containing cells expressing the lowest amount of CCR5 yielded no detectable RT activity.

**Sequencing of proviral HIV-1 env genes**

HIV-1 proviruses present in samples throughout the adaptation were sequenced from the genomic DNA of infected cells. Genomic DNA from cells frozen at peak RT was extracted using the QIAamp DNA blood minikit (Qiagen). The gag and env regions of HIV-1 were amplification using a protocol adapted from Gall, et al. (91). In summary, the HIV-1 genome was PCR-amplified by primers SK145 and OFM19 using the PrimeSTAR GXL DNA polymerase (Clontech). The PCR reactions were then used to amplify the viral gag and env using the Pan-HIV-1_2 or Pan-HIV-1_2_4 forward and reverse primers, respectively. PCR fragments were purified using the QIAquick PCR Purification kit (Qiagen) and sequenced by the Sanger method. Alternatively, PCR fragments were purified and cloned into the pCR®4blunt-TOPO plasmid (Invitrogen), transformed into TOP10 competent cells (Thermo Fisher), and sequenced from the amplified plasmid. The sizes of gDNA and PCR fragments were estimated in reference to a 1kb plus
ladder (Thermo Fischer Scientific) by electrophoresis on a 1% agarose gel stained with ethidium bromide.

**Results**

**Generation of cells expressing tetOn-regulated CCR5**

Canine Cf2Th thymus cells are CD4/CCR5/CXCR4-negative, are susceptible to stable transfection and transduction, and serve as efficient host cells for HIV-1 infection when expressing human CD4 and CCR5 (92). Cf2Th cells were sequentially transfected with plasmids encoding the tetracycline transactivator protein (tTA), human CCR5 under a tTA-regulated promoter (pGL422-tetO-CCR5), and human CD4 and selected for stable transfection of the three genes. To generate cells that stably express, tightly regulate, linearly reduce, and/or completely shut off CCR5 expression, two cell lines were made using either tTA that induces (TetOn) or inhibits (TetOff) CCR5 expression in the presence of the tetracycline analogue, doxycycline.

To generate Tet-On cells, we stably transfected Cf2Th cells with pTetOn and selected and cultured single cells by serial dilution. TetOn activity was measured in clones by incubating cells with 1 μg/mL doxycycline and infecting them with HIV-1 pseudotyped with VSV G glycoprotein and expressing the luciferase gene under a tet-promoter (Figure 2.1a). Determining the ratio of luciferase activity with or without doxycycline identified clones that exhibited high tTA activity in response to doxycycline. Clone pTetOn21 demonstrated the highest relative increase in luciferase activity following incubation with different amounts of the recombinant VSV G pseudotyped virus. We therefore stably transfected pTetOn21 with ptet-CCR5 and selected single
clones (ie, CCR5-TetOn21 cells). We performed FACS analysis of these cells after treatment with 0-250 ng/mL doxycycline for 18 hours, using R-phycoerythrin-conjugated
2D7 antibody, which recognizes the second extracellular loop of CCR5 (93). Figure 2.1b shows different patterns of CCR5 expression for clone 4 (4-CCR5-pTetOn21) and clone 8 (8-CCR5-pTetOn21). The majority of 4-CCR5-pTetOn21 cells express no CCR5, but a small portion of the cells express a broad range of CCR5 levels in response to doxycycline. The majority of 8-CCR5-pTetOn21 cells, instead, express increasing levels of CCR5 in response to increasing concentrations of doxycycline. The levels of CCR5 molecules per cell, estimated by the quantification of PE/cell (see Materials and Methods), indicate a difference in the range of CCR5 expression on 4-CCR5-pTetOn21 and 8-CCR5-pTetOn21 cells that could be useful in adapting HIV-1 to cells expressing incrementally lower levels of CCR5 (Figure 2.1c).

4-CCR5-pTetOn21 and 8-CCR5-pTetOn21 cells were stably transfected with a plasmid expressing human CD4. Selection for single clones resulted in 7CD4-4R5-TetOn (R5-Low) and 11CD4-4R5-TetOn (R5-High) (Figure 2.2). These cells demonstrate high CD4 expression by FACS analysis of cells labeled with PE-2D7 and FITC-conjugated RPA-T4 antibody. Both cells demonstrated doxycycline regulation of CCR5 expression that could be controlled experimentally. Although both cell lines exhibited a slight growth delay compared to the parental Cf2Th cells, this did not prevent the use of these cells for HIV-1 adaptation.

**Generation of cells expressing tetOff-regulated CCR5**

To obtain a wide range of regulated CCR5 expression, we also attempted to generate cells that reduced CCR5 expression in response to increasing concentrations
of doxycycline. We utilized the same method to generate TetOff cells as that used to generate the TetOn cells (Figure 2.3).

In short, we stably transfected Cf2Th cells with a plasmid expressing TetOff and screened infected clones with a recombinant HIV-1 pseudotyped with VSV G and containing the luciferase gene under a tet-promoter (Figure 2.3a). Cells that had the greatest difference in luciferase activity with and without doxycycline were stably
transfected with ptet-CCR5. FACS analysis of clones labeled with PE-2D7 in the presence of increasing doxycycline concentrations demonstrated that there existed a small range between 0-250 ng/mL in which CCR5 expression was regulated (Figure 2.3b). Although this limitation makes regulation of CCR5 expression difficult experimentally, we proceeded with stable transfection of clones 6R5-TetOff, 11R5-TetOff, and 18R5-TetOff with a plasmid expressing human CD4. The majority of the single clones lost the ability to regulate CCR5 in a dynamic range, were not able to express CD4, or both (Figure 2.3c). Therefore, we did not pursue further the generation of TetOff cells as a means of regulating CCR5 expression.

**HIV-1 JR-FL infects cells with low levels of CCR5 after serial rounds of adaptation**

HIV-1 JR-FL is a macrophage-tropic viral strain derived from the frontal lobe of an HIV-1 infected patient; HIV-1 JR-FL naturally uses low levels of CD4 for infection (94). The JR-FL env gene was cloned into the NL4.3 provirus (derived from a T-cell tropic strain). The proviral DNA was transfected into 293T cells. The cell supernatant, containing recombinant replication-competent HIV-1 NL4.3(JR-FL) virus, was incubated in triplicate with canine thymocyte cells expressing human CD4 and CCR5 (Cf2Th-CD4/CCR5), and the cells were passaged for 40 days (Figure 2.4a). Supernatants from each passage were collected in order to measure the reverse transcriptase activity, as
an indicator of viral concentration. Low levels of viral replication were observed after 14 days and did not peak until days 30-37. To enhance infection, Cf2Th-CD4/CCR5 cells were again incubated with 6600 cpm of cell supernatant from peak RT in the presence of 2 ug/mL polybrene (Figure 2.4b). High levels of replication were observed 10 days after infection. R5-High and R5-Low cells were also infected in the same manner. R5-High cells incubated with 1 μg/mL doxycycline exhibited viral replication activity at levels similar to that of HIV-1 JR-FL in Cf2Th-CD4/CCR5. R5-Low cells in 1 μg/mL doxycycline supported low levels of viral replication, approximately 10-fold lower than the peak replication activity in the Cf2Th-CD4/CCR5 cells.

Cell supernatants from the Cf2Th-CD4/CCR5 in this replication assay were used to initiate infection of R5-High cells, a mixture of R5-High and Low cells, and R5-Low cells in the presence of decreasing levels of doxycycline. Table 1 outlines the cells and doxycycline concentrations used in subsequent adaptations. In total, twelve rounds of adaptation resulted in virus that infected R5-Low cells in 12.5 ng/mL doxycycline. Based on quantification of CCR5 expression on R5-Low cells by QuantibritePE prior to adaptation, the adapted virus failed to replicate detectibly in cells expressing <1300 CCR5 molecules per cell.
Figure 2.4: HIV-1 JR-FL infection of Cf2Th cells expressing CD4 and different levels of CCR5. (A) Cf2Th cells were transfected in triplicate with NL4.3(JR-FL) and passaged over 60 days. A $^{32}$P RT assay was performed on medium removed at each passage. Results are displayed as cpm of $^{32}$P-labeled nucleotides reverse transcribed. (B) Cf2Th cells, R5-High, and R5-Low cells were infected with ~6600 cpm NL4.3(JR-FL) collected from Cf2Th-CD4/CCR5 medium in (A). Cells were incubated in 1 μg/mL doxycycline, as indicated, and 2 μg/mL polybrene. In parenthesis is the concentration of doxycycline in ng/mL.
The viral pools from each passage that were used for subsequent infections were sequenced using the protocol developed by Gall, et al. (91) to analyze genetic mutations that may influence viral fitness (Figure 2.5, Table 1). Three Env changes, S115N, R564H, and E662K, appeared early in the adaptation. These Env changes became fixed by the 3rd round of adaptation in which R5-High and R5-Low cells were mixed in the presence of 1 μg/mL Doxycycline (4000-10000 CCR5/cell). These Env changes have not previously been observed in other HIV adaptations in Cf2Th cells (95). The S115N and E662K changes have been observed in viruses

![Figure 2.5: Sequencing of the HIV-1 JR-FL env provirus from genomic DNA of CCR5-expressing cells during viral adaptation.](image-url)

The NL4.3(JR-FL) virus was adapted to replicate on R5-Low cells treated with 1000 μg/mL doxycycline or on 50/50 mixtures of R5-High cells and R5-Low cells (50/50). The reverse transcriptase (RT) activity in the culture supernatants is shown in triplicate cultures. (B) The genomic DNA (gDNA) of a Cf2Th and 50/50 culture sample was extracted and the HIV-1 env gene was amplified by nested PCR using the indicated primers.
resistant to a fusion inhibitor SC34EK in combination with other Env changes (96). R564H is a reversion to the most common residue among HIV-1 strains (www.hiv.lanl.gov).

By the ninth adaptation, the S164N and E831D changes appeared in viruses replicating in 25 ng/uL Dox (~2500 CCR5/cell). The S164N change is located between the gp120 V1 and V2 loops, and potentially could influence CD4 binding or CD4-induced conformational changes in Env. E831D is within the putative alpha-helix in the gp41 cytoplasmic tail associated with trafficking Env to lipid rafts, and could hypothetically influence incorporation of Env into virions. The last viruses to replicate in cells with 12.5 ng/mL doxycycline (~1300 CCR5/cell) lost the E831D change.

The gag gene was also sequenced from the provirus in infected cells from rounds 3, 9, and 11 of the adaptation. No mutations in gag were observed until round 11, at which time Gag changes S126N and M228I were observed. These changes are not known to influence viral replication.
<table>
<thead>
<tr>
<th>Round 1: Cf2Th-CD4/CCR5</th>
<th>Wild Type</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Round 6: R5-Low(250)</strong></td>
<td>PCR Seq: S115N, R564H, E662K</td>
</tr>
<tr>
<td><strong>Round 11: R5-Low(17.5)</strong></td>
<td>PCR Seq: S115N S274F E429K R564H, E662K</td>
</tr>
<tr>
<td><strong>Round 12: R5-Low(12.5)</strong></td>
<td>PCR Seq: S115N, S164N, R564H E662K</td>
</tr>
</tbody>
</table>

**Table 2.1: Changes in HIV-1 NL4.3(JR-FL) Env at each round of adaptation.** HIV-1 NL4.3(JR-FL) was passaged in R5-High and R5-Low cells. Envs were PCR amplified from genomic DNA. PCR fragments were either sequenced directly (PCR seq) or cloned into a pCR®4blunt-TOPO plasmid, cloned into TOP10 competent cells, and sequenced from the amplified plasmid (Colony PCR). Changes in the adapted Envs as compared to wild-type JR-FL were numbered relative to HXB2CG. In parenthesis is the concentration of doxycycline in ng/mL.
Discussion

The development of tetracycline-regulated expression of proteins in cell lines has provided a great opportunity for testing the role of protein expression levels on protein signaling (89, 97-102). In this study, we adopted the tetracycline-regulated expression system to create cell lines in which CCR5 could be progressively decreased to obtain an HIV-1 strain that can replicate in cells with low levels of CCR5.

In previous work, the Affinofile system was generated to assess the CD4 and CCR5 levels utilized by HIV-1 strains (89). These cells regulate CD4 via minocycline and CCR5 via doxycycline, so that a matrix of CD4/CCR5 expression levels can be obtained for viral infection. For example, the ZP6248 transmitted/founder virus requires equally high levels of CD4 and CCR5 expression for infection, while the ZP6246/E312G mutant more efficiently infects cells with high levels of CD4 and any level of CCR5 expression above a threshold, indicating an improved engagement with CCR5 (103). These studies have illustrated that there is a range in which CD4/CCR5 expression could be titrated and HIV-1 strains have a characteristic profile of receptor expression levels that are necessary for efficient infection.

In this study, we generated R5-High and R5-Low cells in which HIV-1 JR-FL was passaged and CCR5 expression levels were down-regulated using a tetracycline-regulated expression system. This TetOn system permitted a dynamic range of CCR5 expression levels to be attained with an undetectable background level of CCR5 expression in the absence of doxycycline. The levels of CCR5 expression achieved in this system varied within the physiological range of CCR5 expression in immune cells. This was ideal in that HIV-1 is taken up by or infects high CCR5-expressing dendritic
cells and effector memory T cells early in natural infection, and low CCR5-expressing macrophages and naive cells later in infection as the former cells become depleted (6). Our virus selection experiments may mimic the course of infection with respect to decreasing levels of CCR5 expression by adapting the virus over many rounds of decreasing doxycycline concentrations.

Interestingly, the Env changes associated with the low-CCR5 phenotype arose by the second round of adaptation. Subsequent rounds of adaptation essentially purified the virus population for these mutations, with no demonstrable enhancement of the phenotype or replication efficiency. A few additional changes arose throughout the adaptation, which were lost in the subsequent round of adaptation. For example, I604K and E429K were only detected in a single round and were absent in the following round. These changes are unlikely to have arisen as compensatory changes to assist in viral evolution, since no new change occurred immediately before or afterwards. The S164N and E831D mutations arose together in the final rounds of adaptation, which could suggest that these changes are essential to replication in the lowest amounts of CCR5 expression. These changes do not, however, permit the virus to become CCR5 independent, as the virus does not replicate in cells expressing lower than ~1300 CCR5 molecules per cell. Further interpretation of these changes in the context of protein cooperativity and viral fitness would be required to confirm the relevance of these changes.

The limitations of this study are that these mutations arose in the absence of a humoral immune response. It is known that laboratory-cultured viruses (eg., HXB2) are more sensitive to neutralizing antibodies than primary isolates (104, 105). Whether the
Env changes that we observed would arise in the presence of selective immune pressure is unknown. This system may, however, mimic a physiological compartment like the central nervous system in which cell-to-cell transfer of virus occurs, levels of neutralizing antibodies are low, and CCR5 expression in immune cells (microglial cells) is low. Noting that the parental virus, JR-FL, was derived from the frontal lobe of an HIV-1 positive patient, this study might simulate some of the conditions in which this virus was replicating in vivo.

In this work, we elucidate a new evolutionary trajectory of HIV-1 infection in the presence of decreasing levels of CCR5. This study identified changes N-terminal to gp120 V1 and in gp41 HR1 and HR2 that affect the Env response to CCR5. As these may represent regions in which potential resistance mutations could occur to CCR5-directed antiviral agents, we now have new information that can improve the development of therapeutic molecules to block CCR5 binding by HIV-1.

Conclusion

HIV-1 requires a minimal expression level of CD4 and CCR5 for a productive infection of target cells. Here, we were able to reduce the levels of CCR5 expression needed for JR-FL Env-mediated infection by adapting the HIV-1 NL4.3(JR-FL) to infect adherent cells with diminishing levels of CCR5. Changes in JR-FL Env arose throughout the adaptation in gp120 and gp41. These changes emphasize the relationship between molecular interactions within and between gp120 and gp41 that maintain the replication capacity of HIV-1.
Acknowledgements

We would like to thank Vlad Novitsky for the protocol for proviral sequencing from genomic DNA.
Chapter 3

Biochemical analysis of Envs of HIV-1 adapted to replicate in cells expressing low levels of CCR5
Section 3.1 Overview and Attributions

Overview

This chapter is comprised of a manuscript that I wrote to describe the analysis of HIV-1 adapted to infect cells with low levels of CCR5 expression on the cell surface. This manuscript is to be submitted for publication.

Attributions

I performed the experiments and wrote the manuscript. Dr. Sodroski provided guidance in the experimental design and editorial input. Beatriz Pacheco assisted in the development of the adaptation method. Navid Madani, Amy Princiotto and Alon Herschhorn provided technical assistance in performing the neutralization assays (Figure 3.7 and Table 3.2).
Section 3.2 Adaptation of HIV-1 to Cells with Low CCR5 Expression

Espy N1, Pacheco B, Sodroski J123.

1Department of Immunology and Infectious Diseases, Harvard School of Public Heath
2Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA
3Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA

Abstract

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) mediates virus entry into CD4+ T-cells. Env binding to host cell receptors CD4 and CCR5 typically triggers HIV-1 entry. CCR5-tropic HIV-1 has been adapted to infect cells in the presence of CCR5 antagonists, but fails to replicate in cells that do not express CCR5. To identify regions in Env that respond to CCR5 binding in the absence of inhibitors, HIV-1 was serially passaged on a CD4-expressing canine cell line expressing progressively reduced levels of CCR5, which were controlled using a doxycycline-regulated system. HIV-1 replication was observed in cells expressing ~1300 CCR5 molecules/cell. We identified changes that confer a low-CCR5 replication phenotype outside the V3 variable region of the gp120 Env subunit, which is already known to contribute to CCR5 binding. These changes were located in the gp120 V1/V2 variable regions and in the heptad repeat, HR1, and membrane-proximal external region (MPER) of the gp41 transmembrane domain of Env. These changes enhanced HIV-1
replication in cells at all levels of CCR5 expression. In cell-free virus infections, these changes reduced overall infection efficiency and exposed conserved regions near the CD4 and CCR5 binding sites. These results expand our understanding of coreceptor engagement and HIV-1 entry.

**Introduction**

The HIV-1 envelope glycoprotein (Env) trimer mediates entry into target cells by sequentially binding surface-expressed CD4 and a coreceptor, most often CCR5 (31-33). Previous research on HIV-1 Env binding to CD4 has provided important insights into the HIV-1 entry process and Env structure. Specifically, structural and biochemical studies demonstrate that CD4 binding induces conformational changes that expose the CCR5-binding site and form the pre-hairpin intermediate (106-110). Generation of CD4-independent HIV-1 variants in vitro and analysis of viruses derived from patient brain tissue that have a reduced dependence on CD4 have elucidated the role CD4 binding plays in Env conformational change and HIV-1 entry (52, 111). These viruses are more able than laboratory-adapted viruses to infect macrophages and microglia that express very low levels of CD4 by more efficiently binding to CCR5 and further transitioning into a fusion-competent conformation (112-117). There are only incomplete data, however, on the CD4- or CCR5-bound Env trimer conformations, and many gaps exist in our understanding of these later conformational transitions in HIV-1 entry.

CCR5 binding is an essential step in viral entry and infection. CCR5 binding brings the pre-hairpin intermediate closer to the target cell membrane and triggers Env to form the post-fusion six-helix bundle conformation. CCR5 tropism is a characteristic
of most of the viruses that initiate an HIV-1 infection (50) and the majority of the world’s HIV+ patients harbor CCR5-tropic viruses (118, 119). Clinical observations suggest that blocking Env-CCR5 binding will suppress HIV-1 infection. In 2009, an HIV+ patient in Berlin received a stem cell transplant from a CCR5Δ32 homozygous donor, in which 32 N-terminal residues in CCR5 are absent in the expressed protein, to treat acute myeloid leukemia. Since then, this individual has had an undetectable viral load and exhibited a sustained reconstitution of his immune system (19, 20).

Small-molecule CCR5 antagonists, like Maraviroc, have been used to treat HIV-1 infection, but drug resistance has limited their efficacy and, in turn, elucidated the extent to which HIV-1 will adapt to resist inhibition of coreceptor binding (67, 70-72). Maraviroc is an FDA-approved HIV-1 entry inhibitor that binds in the hydrophobic pocket formed by the CCR5 transmembrane helices and stabilizes a CCR5 conformation that resists efficient gp120 binding. HIV+ patients treated with MVC showed reduced viral loads, followed by selection for previously undetected viruses that use CXCR4 or the evolution of CCR5-tropic viruses capable of using MVC-bound CCR5 (70). These latter MVC-resistant viruses have altered residues in the V3 stem to enhance binding affinity to the CCR5 N-terminus and altered affinity for the drug-bound extracellular loops, despite overall decreases in entry and replication capacity (67, 72). Studies of HIV-1 adapted to the presence of vicriviroc, an investigational compound and CCR5 antagonist, displayed altered residues in gp41 that have been proposed to lead to increased triggering of the fusion peptide as well as enhanced affinity for the CCR5 N-terminus (70). These studies indicate that HIV-1 can evolve to use conformationally altered CCR5 for viral entry.
Despite our understanding that CCR5 binding is a useful target to block HIV-1 infection, there still are gaps in our scientific knowledge about the structures of the CCR5-bound Env trimer and downstream intermediates, the Env determinants of these conformational transitions, and the molecular dynamics required for viral entry. We hypothesized that we can generate HIV-1 isolates that infect cells with little or no CCR5 and that molecular changes in Env will identify regions that are critical for CCR5 binding or CCR5-triggered membrane fusion. This project evaluates the extent to which HIV-1 can evolve to adapt to limiting amounts of CCR5. We progressively reduced the levels of CCR5 expressed on target cells and monitored the compensatory changes in the adapted HIV-1 Env. We utilized a chimeric HIV-1 JR-FL, since it is a macrophage-tropic virus that uses fairly low levels of CCR5; these isolates may already have Env polymorphisms apart from the changes that facilitate adaptation to even lower CCR5 expression. We were able to generate viruses that can infect cells with ~1300 CCR5 molecules per cell. These viruses demonstrate enhanced infectivity compared to the wild-type virus in the context of cell-to-cell replication. These viruses were more sensitive to neutralizing antibodies, suggesting that lack of immune pressure permitted the exposure of coreceptor-binding regions to promote viral fusion. New insights from this study will be applied to our understanding of the Env structure, coreceptor binding, and the role the above has on HIV-1 pathogenesis. This work may influence the development of new clinical strategies (eg. small molecule antagonists, vaccines, etc) to block HIV-1 entry by identifying critical Env targets.

**Materials and methods**
Generation of cell lines

293T and Cf2Th cells were obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium with 10% tetracycline-free fetal bovine serum (DMEM-10, Clontech). Cf2Th cell lines stably expressing human CD4 and CCR5 (Cf2Th-CD4/CCR5) were grown in DMEM-10 in the presence of 400 μg/mL G418 and 200 μg/mL hygromycin (Invitrogen).

Cf2Th cell lines with regulatable CCR5 expression were generated in the following manner: Cf2Th cells were transfected with Tet-On® Advanced Vector (Clontech), passaged in DMEM-10 and 0.6μg/mL puromycin, and single cells selected by limiting dilution in 96-well plates. Single-cell clones were expanded and tested for expression of the tTA protein after treatment with 0-1 μg/mL doxycycline by Western blotting of whole cell lysates with a monoclonal antibody against TetR (Clontech). tTA-expressing cells were transfected with pGL422-tetO-CCR5, passaged in the presence of 0.6 μg/mL G418, and selected for single clones via limiting dilution. Cells were expanded and tested for doxycycline-regulated CCR5 expression via flow cytometry as follows: after incubation with R-Phycoerythrin-conjugated mouse anti-human CCR5 (PE-anti-CD195, BD Biosciences), a million cells were analyzed with a BD FACSCanto II flow cytometer (BD Biosciences). Clones with the least background and greatest dynamic range of induced CCR5 expression were then transfected with the pcDNA-CD4 plasmid expressing human CD4, passaged in the presence of 0.025 μg/mL zeocin, and selected for single clones via limiting dilution. Cells were tested for doxycycline regulation of CCR5 expression and stable expression of CD4 using flow cytometry with
PE-anti-CD195 and fluorescein isothiocyanate-conjugated mouse anti-human CD4, clone RPA-T4 (FITC-anti-CD4, BD Biosciences), respectively.

Two clones that express high levels of CD4 and low or high ranges of cell-surface CCR5 expression upon doxycycline treatment, herein called R5-Low and R5-High, were selected for HIV-1 adaptation to low levels of CCR5. Levels of CCR5 were measured by QuantiBritePE (BD Biosciences) and vary within the physiological range reported for different types of human immune system cells (Figure 3.1c).

*Generation of replication-competent HIV-1 and adaptation of virus to low levels of CCR5 in R5-Low cells*

Replication-competent HIV-1 was generated by transfecting 2 million 293T cells with 20 μg of a plasmid containing the pNL4.3 provirus with the HIV-1 JR-FL env (between the Sall and BamHI sites) using Effectene transfection reagent (Qiagen). After two days, the cell supernatants were harvested and frozen at -80°C in aliquots. Reverse transcriptase (RT) activity in the supernatant was measured using $^3$H-labelled nucleotide triphosphates for initial viral production assays in a method previously described (90).

To initiate viral replication in Cf2Th-CD4/CCR5 cells, 100,000 Cf2Th-CD4/CCR5 cells were infected with 3000 cpm ($^3$H) of HIV-1 NL4.3(JR-FL) virus made in 293T cells as above. Cells were passaged 1:5 every 3-4 days using 5 mM EDTA in PBS to lift cells. Supernatants were collected at each passage and frozen to measure RT.

For all subsequent rounds of viral replication, RT activity was measured using $^{32}$P-labelled nucleotide triphosphates. Initiation of viral adaptation in Cf2Th-CD4/CCR5

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cells used 6600 cpm ($^{32}\text{P}$) of virus from the time point with peak RT in the initial viral replication assay in the presence of 2 μg/mL polybrene. All subsequent rounds of adaptation were initiated with 25,500 cpm ($^{32}\text{P}$) RT units of supernatant, collected at the time point with peak RT activity in previous rounds, was used to infect R5-High cells and then R5-Low cells at decreasing expression levels of CCR5 (ie, lower concentrations of doxycycline). Serial passage of the virus was continued until 3 independent assays with triplicate samples containing cells expressing the lowest amount of CCR5 yielded no detectable RT activity.

Quantification of p24 levels in cell supernatants were performed using a p24 ELISA kit (Advanced BioScience Laboratories).

*Sequencing env from genomic DNA of infected cells*

Proviruses were sequenced from the genomic DNA of infected cells. Genomic DNA was isolated from cells frozen at peak RT using the GIAamp DNA blood minikit (Qiagen) followed by genome amplification by a protocol from Gall, et al. (91). In summary, the HIV-1 genome was PCR-amplified by primers SK145 and OFM19 using the PrimeSTAR GXL DNA polymerase (Clontech). The viral *env* was then amplified from the PCR reaction using the Pan-HIV-1_4 forward and reverse primers. PCR fragments were purified using the QIAquick PCR Purification kit (Qiagen) and sequenced by the Sanger method.

*Env cloning and mutagenesis*
Changes in the HIV-1 JR-FL env that arose in the replication assays were introduced into the pNL4.3-JRFL proviral DNA or the Env-expressor plasmid pE7-JRFL (HIV-1 JR-FL env in pSVIIenv) (120, 121) by site-directed mutagenesis using the PfuUltra II Hotstart PCR Master Mix (Agilent). Mutagenesis was confirmed by automated DNA sequencing.

**Viral replication assay**

To test the replication capacity of NL4.3 viruses expressing wild-type JR-FL or the adapted Envs, approximately $4 \times 10^4$ Cf2Th-CD4/CCR5 or R5-Low cells preincubated with 50 or 100 ng/mL doxycycline were transfected with 100 μg proviral DNA and passaged for 3 weeks. Supernatants of each cell sample were collected throughout cell culture for the detection of RT activity.

**Generation and purification of single-round recombinant virus expressing luciferase**

Single-round recombinant HIV-1 expressing firefly luciferase was generated by transfecting 293T cells with pE7-JR-FL constructs, the firefly luciferase-expressing vector, and the pCMVΔP1ΔenvpA HIV-1 Gag-Pol packaging construct at a ratio of 1:2:1 micrograms of DNA using Effectene transfection reagent (122). The supernatant was harvested after 3 days and used in all assays without freezing.

To test levels of Env expression on virions, viral particles were pelleted by ultracentrifugation through a 20 percent sucrose cushion at 4°C for 2 hours in a Beckman SW55 rotor at 30,000 rpm. Pellets were lysed in 4x NuPAGE LDS Sample Buffer plus β-mercaptoethanol and frozen. Samples were run on SDS-PAGE,
transferred to Hybond ECL nitrocellulose membranes (GE Healthcare Life Sciences) using a Trans-blot semi-dry transfer cell (Bio-rad), and blotted with 1:5000 patient serum and secondary HRP-Protein A/G. Blots were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fischer) and imaged on the GelDoc XR+ System (Bio-Rad).

Cell-fusion assays

To test the capacity of wild-type JR-FL Env and the adapted Envs to initiate cell fusion by measuring syncytium formation, we used an alpha complementation assay (123). In this assay, COS-1 cells were plated in a 96-well plate and transfected the next day with 0.1 μg/well pSVIIIenv-JR-FL, 0.01 μg/well of a Tat-expression vector, and 1.1 μg/well of a plasmid containing the N-terminal fragment of beta-galactosidase using the Effectene protocol (124). Cf2Th-CD4/CCR5 cells were plated in a T-75 flask and transfected with 10 μg of a plasmid containing the C-terminal fragment of β-galactosidase using the Effectene protocol (123). After 2 days of incubation at 37°C, Cf2Th-CD4/CCR5 cells were lifted with 5 mM EDTA in PBS and diluted to 2x10^5 cells per mL DMEM-10. To each well of COS-1 cells preincubated with increasing concentrations of Maraviroc was added 100 μL Cf2Th-CD4/CCR5 cells. Cells were incubated at 37 degrees for 8 hours, washed in PBS, and lysed with 20 μL Galactostar Lysis Buffer (ThermoFisher). Plates were frozen and thawed three times at -20°C to improve cell lysis. Plates were then warmed to room temperature and incubated with 100 μL 1:100 Galactostar substrate in Reaction Buffer Diluent. β-galactosidase enzymatic activity was measured in the luminometer, reading 1 second per well.
Cell-to-cell HIV-1 transmission assay

293T cells producing single-round luciferase recombinant virus were lifted with 5 mM EDTA in PBS and added to cultured Cf2Th-CD4/CCR5 cells. Cocultures were passaged every 3-4 days. At passaging, the remaining cells were diluted in DMEM-10 on a 96-well plate and incubated overnight. At various time points, cells were lysed with 30 μL passive lysis buffer (Promega) and luciferase activity was measured with a Mithras LB 940 luminometer (Berthold Technologies).

Viral neutralization assays

Single-round luciferase-expressing recombinant viruses were incubated with antibodies for 1 hour at room temperature and then incubated with Cf2Th-CD4/CCR5 cells in a 96-well plate for 2 hours at 37°C. The virus and antibody mixtures were incubated with the cells in DMEM-10 for 3 days in a 37°C, 5% CO₂ incubator. Cells were lysed with 30 μL passive lysis buffer (Promega) and luciferase activity was measured with a Mithras LB 940 luminometer (Berthold Technologies).

Data representation and statistical analysis

Microsoft Excel and Prism 6.0 (GraphPad Software, L1 Jolla Ca) was used to graph and analyze numerical data. Statistical tests as reported in the figure legends were performed using Prism software.

Results
**HIV-1 JR-FL can adapt to replicate in cells expressing low levels of CCR5**

We adapted HIV-1 JR-FL to infect cells with low levels of CCR5 by serially passaging the virus in cells in which the level of CCR5 expression was gradually decreased. Cf2Th canine cells constitutively expressing human CD4 and expressing human CCR5 in a Tet-regulated fashion were used as target cells for the adaptation. An initial stock of the HIV-1 NL4.3(JR-FL) was prepared by transfecting 293T cells with the proviral plasmid and harvesting the cell supernatant three days later. Cf2Th-CD4/CCR5 cells, which constitutively express human CD4 and human CCR5, were incubated with the 293T cell supernatants and cultured. Reverse transcriptase activity was detected in the supernatants by 30 days of culture (data not shown). Cell supernatants with RT activity were used to reinfect Cf2Th-CD4/CCR5 in the presence of 2 μg/mL polybrene to enhance infection. Viruses in the supernatants of these cells were used for adaptation of HIV-1 to CD4-expressing cells with low levels of CCR5.

Cf2Th cells expressing human CD4 constitutively and human CCR5 in a Tet-regulated manner (Figure 3.1) were incubated with the HIV-1 NL4.3(JR-FL) virus and then passaged. By three rounds of passage, viruses that replicated on a mixture of 70% R5-High cells and 30% R5-Low cells in 1 μg/mL doxycycline emerged. By 9 rounds of passage, viruses that replicated on R5-Low cells in 75 ng/mL doxycycline were obtained.

After additional rounds of virus passage, we observed decreasing RT activity in cell supernatants as the levels of CCR5 expression decreased (Figure 3.2a). Viral
replication was not detected beyond round 12, in which we measured RT activity in the supernatants of R5-Low cells in 12.5 ng/mL doxycycline. Cell-free viruses in the supernatant of the round 12 cultures did not detectably replicate in R5-Low cells in the
same levels of doxycycline (data not shown). Quantibrite PE was used to estimate the number of CCR5 molecules/cell by quantifying the 2D7 epitopes per cell (BS Biosciences). Replication of HIV-1 NL4.3(JR-FL) virus was not detected in the CD4-expressing cells with less than ~1300 CCR5 molecules per cell (Figure 3.1).

To confirm that the viruses generated by these rounds of adaptation could replicate in cells expressing low levels of CCR5, we infected R5-Low cells at 50 and 100 ng/mL doxycycline, as well as Cf2Th-CD4/CCR5 cells, with cell supernatants from round 3 (J3), 9 (J9) and 11 (J11) that were normalized for the level of p24 Gag protein. Figure 3.2b shows the enhanced replication capacity of the J3 and J9 viruses in each cell type and concentration compared with that of the parental HIV-1 NL4.3(JR-FL) virus. In Cf2Th-CD4/CCR5 cells, J3 and J9 viruses infect 2-fold and 4-fold more efficiently than JR-FL, respectively. The J3 virus produced RT levels of virus in R5-Low(100) cells that were comparable to that of the HIV-1 NL4.3(JR-FL) in Cf2Th-CD4/CCR5 cells; by contrast, the HIV-1 NL4.3(JR-FL) replicated only marginally in the R5-Low(100) cells. Both J3 and J9 viruses replicated in R5-Low(50) cells, whereas no HIV-1 NL4.3(JR-FL) RT activity was detected in these cells. These observations indicate that the J3 and J9 viruses have adapted to replicate better than the parental HIV-1 NL4.3(JR-FL) virus on cells expressing low levels of CCR5.

The J11 viral pool did not replicate in any cell type, suggesting that some adaptive changes after round 9 were deleterious to cell-free infection in this context.

**Figure 3.1:** Characterization of CCR5-expressing cells. (A) FACS analysis of doxycycline-regulated Cf2-CD4-CCR5(TetOn) cells. Cell clones were incubated with 0-1000ng/mL doxycycline for 18h, stained with anti-CCR5 (2D7-PE) or anti-CD4 (RPA-T4-FITC) fluorescent antibodies, and analyzed by FACS. One set of cells was stained with labeled mouse IgG_1, κ isotype control. (B) Average of PE-anti-CD195 epitopes/cell for 2 thaws of cell stocks as estimated by QuantiBrite PE quantification. (C) Representation of the number of 2D7 epitopes/cell compared with that on immune cells (6).
Adaptive changes in JR-FL Env map to regions near V1/V2 and in gp41

We isolated the genomic DNA of the cells collected at the time point of peak RT activity in the supernatant at each round of adaptation, PCR amplified the integrated HIV-1 provirus, and sequenced the env gene (Table 2.1). The wild-type JR-FL env
sequence was maintained throughout multiple rounds of replication in Cf2Th-CD4/CCR5 cells (data not shown). In the viruses adapted to replicate in R5-Low cells, multiple changes were observed. Changes that were retained through multiple rounds of adaptation are shown in Figure 3.3A. Three changes, S115N, R564H, and E662K, were found in all three adapted viruses, J3, J9, and J12. Both J9 and J12 had, in addition, an S164N change, and J9 had an E831D change.

None of these changes has been previously implicated in the interaction of gp120 with CCR5. Based on crystal structures of gp120 bound to a CD4-induced antibody which binds gp120 near the coreceptor-binding site (107), serine 115 is located in the membrane-distal end of the α1 helix, not far from the coreceptor-binding region. Arginine 564 and glutamic acid 662 are located in the HR1 region and the MPER of gp41, respectively. The arginine 564 residue faces the N-terminal peptide of gp120 in the soluble gp140 SOSIP.664 structures (125). S115N and E662K, in combination with other Env mutations, have been observed in viruses resistant to the fusion inhibitors SC34EK (76, 96). Serine 164 is between the gp120 V1 and V2 regions; the S164G change has been observed along with other Env alterations to confer resistance to the entry inhibitor BMS-378806 (126). A D164N change in HIV-1 JR-CSF along with other Env alterations has been associated with viral replication in cells expressing CD4 and CCR5 despite a block in CCR5 binding caused by monoclonal antibody 2D7 (74). Additionally, E831D is located within the cytoplasmic tail of Env, which, among other functions, has been implicated in trafficking of Env into lipid rafts (Table 3.1).

Because the HIV-1 Gag polyprotein is involved in the incorporation of Env into viral particles, we sequenced the gag gene to evaluate whether these changes alter the low-
Figure 3.3: Changes in adapted Env permit viral replication in low-CCR5 expressing cells. (A) Map of JR-FL Env changes by round of adaptation. V: Variable loop, F: Fusion peptide, HR: Heptad repeat, M: Membrane Proximal External Region, TM: Transmembrane region, CT: Cytoplasmic Tail. (B) Cells were transfected with the proviral vector with the wild-type JR-FL Env or with the J3, J9, or J12 Envs and passaged for 30 days. A $^{32}$P RT assay was performed on medium removed at each passage. Each point represents the average of duplicate samples of a representative replication kinetics assay. The dashed line represents average RT activity of supernatants from Cf2Th cells transfected with proviral vectors, and represents the background of the assay.
CCR5 usage phenotype. No changes in \textit{gag} were observed until round 11 and the observed alterations (Gag S126N and M228I) have not been reported to affect HIV-1 infection. We therefore focused on the contribution of changes in Env to the low-CCR5 usage phenotype.

To do so, we generated proviruses with the changes observed in J3, J9, and J12. We transfected 100 ng of the proviral plasmids into R5-Low cells in 50 or 100 ng/mL doxycycline, or Cf2Th-CD4/CCR5 cells (Figure 3.3b). The viruses with adaptation-associated changes demonstrated more efficient replication in cells at all levels of CCR5 expression. The \textit{env} gene was sequenced from genomic DNA from all cells on the final day of the assay. No changes in \textit{env} sequence were noted for samples in which viral replication was observed. A similar test of viral replication in which cells were transfected with twice as much proviral DNA demonstrated enhanced replication of J3, J9, and J12 as compared to parental JR-FL in samples in which viral replication was observed. When sequencing samples in which no replication was observed, there were changes in wild-type JR-FL and multiple changes in J3 and J12, suggesting that defective proviruses may have arisen under these circumstances. The E831D change was absent in one J9 sample.

\textbf{Adaptive changes in JR-FL Env attenuate cell-free viral infectivity}

Changes that are associated with increased Env availability on the viral membrane or improved Env processing may improve viral infectivity in low CCR5 conditions, as they allow more available functional glycoprotein per virus, therefore increasing the opportunities to bind receptors. To measure Env levels on the viral
surface, we generated single-cycle recombinant HIV-1 pseudotyped with the wild-type JR-FL Env, the adapted Envs, and JR-FL Env containing the E831D change in 293T cells. The viral particles in the 293T cell supernatants were concentrated by ultracentrifugation through a sucrose cushion. The lysed viral pellet was run on SDS-PAGE and Western blotted for gp120, p55, and p24 (Figure 3.4a). Although the E831D mutant may have higher gp120 levels, the viruses containing the Envs with adaptation-associated changes displayed similar levels of gp120 relative to viral proteins, p55 and p24, as the parental virus.

<table>
<thead>
<tr>
<th>Env amino acid change</th>
<th>Location</th>
<th>Frequency in natural HIV-1 isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>S115N</td>
<td>N-terminal to V1/2 loop, at the C-terminus of the α1 helix (gp120)</td>
<td>S: 95.9%, 4.1% other, no Ns. Identified with E662K in SC34EK-resistant HIV-1 (95)</td>
</tr>
<tr>
<td>S164N</td>
<td>Between V1 &amp; V2 (gp120)</td>
<td>E: 65.3%, 34.1% other, no Ns. S164G seen with other mutations in BMS-378806 resistance (120). D164N seen in JR-CSF resistance to 2D7 (73)</td>
</tr>
<tr>
<td>R564H</td>
<td>Within HR1 (gp41)</td>
<td>H: 92.9%, 7.1% other, no Rs</td>
</tr>
<tr>
<td>E662K</td>
<td>MPER (gp41)</td>
<td>E: 22.9% / A: 66.5% / other: 10.6%/K in HIV-2 &amp; SIV. Identified with S115N in SC34EK-resistant HIV-1. E662A found in patients with resistance to Enfurvitide (95)</td>
</tr>
<tr>
<td>E831D</td>
<td>Cytoplasmic tail, Hydrophilic surface of LLP-1 (gp41)</td>
<td>E: 92.4%, E831D in strain KSM4001</td>
</tr>
</tbody>
</table>

To evaluate whether these viruses retain replication competence, we infected Cf2Th-CD4/CCR5 and R5-Low cells with equivalent RT units of recombinant virus (Figure 3.4b). We observed that the adapted Envs exhibited approximately a 5-fold reduced infectivity compared to the parental JR-FL Env. Viruses with single and double-residue changes introduced into the wild-type JR-FL Env did not demonstrate as great an attenuation in infectivity as the adapted Envs. The low levels of infectivity of single-round viruses with adaptation-associated changes contrast with the enhanced infection of Cf2Th-CD4/CCR5 or R5-Low cells by replication-competent viruses with the same changes. This result suggests that although the parental JR-FL Env mediates better infection in a cell-free, single-round context (Figure 3.4b), the adapted Envs display an
enhanced replication capacity in a context in which cell-free and cell-cell infection occurs.

**Adaptation-associated changes in JR-FL Env do not result in coreceptor switching or affect sensitivity to entry inhibitors**

The attenuated infectivity of the adapted Envs for CD4-positive, CCR5-positive target cells could result from an altered coreceptor tropism, e.g. with a switch to CXCR4 or to coreceptors present in Cf2Th cells. We tested this by incubating single-cycle recombinant virus with the parental Cf2Th cell line, and with Cf2Th cells stably expressing human CCR5 alone, CD4 and CXCR4, or CD4 and CCR5. Detectable levels of infection were observed only in Cf2Th-CD4-CCR5 cells (Figure 3.5a).

To interrogate the interaction between our adapted Envs and CD4 and CCR5 further, we tested their sensitivity to small-molecule entry inhibitors. Previous adaptation experiments using CCR5 antagonists or suspension cells that regulate CCR5 expression have observed changes in HIV-1 Env that confer resistance to entry inhibitors (73, 87, 127). We therefore measured the sensitivity of our adapted Envs to the CCR5 antagonist Maraviroc, the fusion inhibitor T-20, and BMS-806, which blocks CD4-induced conformational changes in Env (65, 128-130). Interestingly, viruses with the parental JR-FL Env and the adapted Envs were inhibited comparably by all three antiviral agents (Figure 3.5b). These results indicate that viruses adapted to low CCR5 expression retain dependence on CD4 and CCR5, and infect cells using Env conformational transitions inhibited by BMS-806 and T-20.
Figure 3.5: Cellular tropism of viruses containing wild-type or mutant Env and sensitivity to entry inhibitors. (A) Infection capacity of recombinant, luciferase-expressing viruses containing the wild-type JR-FL Env or the Envs of the adapted viruses, as measured by the average relative luciferase units (RLU) in lysates of cells infected with virus in duplicate. Data is representative of two independent assays. (B) Sensitivity of viruses with the indicated Envs to entry inhibitors, as a percentage of the infectivity observed in the absence of inhibitor. Shown are the means and standard deviations from duplicate samples in a typical experiment.
Changes in gp41 are sufficient for the ability of HIV-1 JR-FL to replicate in cells with low CCR5 expression

We sought to identify the changes responsible for the low-CCR5 usage phenotype by transfecting Cf2Th-CD4-CCR5 and R5-Low cells with proviral DNA containing single and double mutations observed in the adapted Envs (Figure 3.6). The R564H change alone was able to allow detectable replication in cells expressing different levels of CCR5 expression. The addition of the E662K change to the R564H change enhanced infection in Cf2Th-CD4/CCR5 cells to levels as high as those seen for the adapted Envs (Figure 3.3b). S115N alone conferred a low level of replication in R5-Low(100) cells but replication was enhanced at each level of CCR5 tested when both S115N and E662K changes were present. The S115N/R564H double mutant exhibited very low but detectable levels of infection. The sequences of the env gene determined at the end of a similar infectivity assay did not demonstrate any additional changes.

The adaptation-associated changes enhance cell-to-cell transmission of HIV-1

None of the observed single or double Env changes conferring the low-CCR5 usage phenotype support enhanced single-cycle infection (Figure 3.4b). To determine if the observed changes enhance Env-mediated cell-cell fusion, we evaluated the adapted Envs in an alpha-complementation assay (123). In this assay, COS-1 cells expressing Env are cocultivated with Cf2Th-CD4/CCR5 cells for 8 hours before measuring reporter activity (Figure 3.7a). The J3, J9, and J12 Envs exhibited between 25-33% of the cell-cell fusion capacity of the wild-type JR-FL Env. The reduced fusion capacity of the
adapted Envs with respect to the JR-FL Env mimics what we observed in the single-cycle viral assays. No difference in the inhibition of cell-cell fusion by Maraviroc was
observed between the JR-FL Env and the adapted Envs, consistent with the results seen in the single-cycle viral assays (Figure 3.7b).

![Graphs](image)

**Figure 3.7: Syncytium-forming ability and cell-to-cell transmission of Env variants.** (A) Cos-1 cells expressing the wild-type JR-FL or the adapted Envs were incubated with Cf2Th-CD4/CCR5 cells in an alpha complementation assay, as described in the Materials and Methods. The β-galactosidase activity of each sample reflects the degree of cell-cell fusion (syncytium formation) mediated by the Env. The ∆Env is a negative control. (B) Sensitivity of the alpha complementation assay to Maraviroc, a CCR5 inhibitor. The % fusion for each Env is shown relative to the value seen in the absence of inhibitor. (C) 293T cells transiently expressing single-round recombinant HIV-1 with the indicated Envs were cocultivated with Cf2Th-CD4/CCR5 cells and passaged for approximately 6 days. Means and standard deviations derive from 2 or more coculture samples, normalized to the RLU of wild-type JR-FL. The ∆Env is a negative control virus lacking a function Env. Student t-test as compared to JR-FL: * P ≤ 0.05 ** P ≤ 0.01 *** P ≤ 0.001 **** P ≤ 0.0001.
The above results indicate that, in contrast to the presumed advantage of the adaptation-associated Env changes to HIV-1 replication in Cf2Th cells expressing CD4 and low levels of CCR5, these changes do not improve cell-free infection or cell-cell fusion mediated by Env. We therefore devised an assay in which cell-to-cell transmission of HIV-1 occurs, mimicking the conditions under which the viruses were adapted to the lower level of CCR5 expression. We cocultured Cf2Th-CD4/CCR5 cells with 293T cells expressing single-round luciferase-expressing viruses (Figure 3.7c). In this assay, the Cf2Th-CD4/CCR5 target cells could be infected by single-cycle recombinant virus via cell-free infection or by cell-to-cell transfer. Because luciferase expression is initially high in the transfected 293T cells, the assay is conducted for at least 6 days, by which time luciferase expression in the transfected cells diminishes; this is monitored by using an Env-deleted virus that cannot initiate new infections. If the cotransfected Env can support a new round of either cell-free or cell-to-cell transmission from the transfected 293T cell to the Cf2Th-CD4/CCR5 cells, the expression of luciferase from the integrated provirus is sustained for long periods of time. Thus, we measured luciferase activity in the cocultures when the luciferase activity associated with the ΔEnv control returned to background levels.

In this assay, the adapted Envs supported cell-to-cell infection better than the parental JR-FL Env. The S115N, S164N and E662K mutants, as well as some of the combined mutants, also were very efficient in this assay. The activity of the single and double mutant Envs did not correlate with the level of enhancement seen for these Envs in the virus replication assay. Thus, although this single-round cell-to-cell infection assay does not fully reproduce all of the features of the virus replication assay, the
results suggest that the adaptation-associated Env changes enhance HIV-1 cell-to-cell transmission.

Adaptation-associated changes in Env increase HIV-1 sensitivity to neutralization by particular antibodies

Studies of CD4-independent HIV-1 demonstrated an increased sensitivity to antibodies targeting the CD4-binding and coreceptor-binding sites, such as F105 and 17b, respectively (25, 53, 109). Therefore, we measured the neutralization sensitivity of our adapted Envs by infecting Cf2Th-CD4/CCR5 cells with single-round luciferase-expressing virus that had been pre-incubated with a panel of antibodies.

We observed no significant difference in the inhibition of viruses containing the parental JR-FL and adapted Envs by soluble CD4 and the broadly neutralizing antibodies VRC01, PGT121, and 35O22 (Table 3.2). The viruses with the adapted Envs exhibited significant increases in sensitivity to neutralization by the F105, 17b, 830A, 19b, and 1.4E antibodies, which did not inhibit the viruses with the wild-type JR-FL Env. It has been recently shown that these anti-gp120 antibodies against the CD4-binding site (F105), CD4-induced gp120 epitopes (17b), V2 region (830A), and V3 region (19b and 1.4E) recognize an intermediate Env conformation (State 2) that is on the HIV-1 entry pathway (131). The adapted Envs apparently sample conformations similar to State 2 more readily than the parental JR-FL Env.

To identify the specific Env changes responsible for this phenotype, we assayed the sensitivity of single and double Env mutants to VRC01, F105, 17b, and 19b. The mutants R564H, E662K, and R564H/E662K exhibited a neutralization profile similar to
The S115N mutant was more sensitive to F105 and 19b neutralization than the wild-type JR-FL virus, although not as sensitive as the viruses with the adapted Envs. The virus with the S115N and E662K changes was as sensitive as the viruses with the J3, J9, and J12 Envs to neutralization by F105 and 19b. These results indicate that the S115N change makes a major contribution to the sampling of State 2-like conformations, in which the CD4-binding site and V3 region of gp120 is more exposed. Other changes, like E662K in gp41, contribute to the degree of this phenotype. Ultimately, multiple changes in Env such as those seen in the adapted J3 virus, result in greater sampling of a full State 2-like conformation with
increased exposure of two epitopes (V3 and CD4-induced) near the CCR5-binding region.

**N302Y does not enhance a low-R5 replication phenotype in JR-FL**

Adapting HIV-1 to infect cells expressing low levels of CCR5 has been recently reported (73). In this system, SupT1 T lymphocytes (L23 cells) stably expressing low levels of
human CCR5 were infected with HIV-1 YU-2, followed by passage for 70 days. The resulting virus contained an N302Y change in the gp120 V3 region that conferred 5-fold resistance to Maraviroc and enhanced infection and replication in L23 cells.

We introduced the N302Y change into the wild-type and J9 mutant Envs in the HIV-1 NL4.3(JR-FL) provirus to determine whether the V3 change would enhance replication in R5-Low cells. The N302Y change did not enhance the replication of the JR-FL virus (Figure 3.9). We observed no significant improvement in the replication of the J9/N302Yvirus in R5-Low cells, compared with that of the J9 virus. The replication of the J9/N302Y virus in Cf2Th-CD4-CCR5 cells was attenuated relative to that of the J9 virus. These results indicate that the N302Y change is not sufficient to confer the ability to replicate efficiently on cells with low CCR5 to HIV-1 JR-FL.

Introduction of adaptation-associated changes into HIV-1 AD8

The R564H change in the HIV-1 JR-FL Env contributed to the replication of the virus in cells with low levels of CCR5. HIV-1 AD8 and 92.7 percent of all HIV-1 strains have a histidine residue at position 564. Since the wild-type sequence of AD8 already has a histidine at position 564, we hypothesized that the addition of the other single changes that arose in the adapted JR-FL Env would allow HIV-1 AD8 to infect cells expressing low CCR5 levels. The addition of these single-residue changes to the Env of the proviral HIV-1 AD8 did not result in any detectable replication in Cf2Th-CD4/CCR5 or R5-Low cells (data not shown). Introduction of S115N in single-cycle recombinant HIV-1 AD8 did result in increased infectivity in Cf2Th-CD4/CCR5 cells and an increased sensitivity to the 17b and 19b antibodies (Figure 3.10). Apparently, some but not all of
Figure 3.9: Replication kinetics of viruses with the wild-type JR-FL Env or mutant Envs. Cells were transfected with proviruses with the wild-type JR-FL Env or mutant Envs, and passaged for 15 days. A $^{32}$P RT assay was performed on medium removed at each passage. Each point represents an average of duplicate samples.
Figure 3.10: Sensitivity of viruses with the wild-type AD8 Env or mutant Envs to antibody neutralization. (A) Recombinant, luciferase-expressing viruses with the wild-type AD8 Env or mutant AD8 Envs were incubated with antibody prior to incubation of the virus-antibody mixtures with Cf2Th-CD4/CCR5 cells for 2 days. For comparison, the infectivity of recombinant, luciferase-expressing virus containing the JR-FL Env J9 mutant Env is shown. The infectivity is expressed as the ratio of relative luciferase units (RLU) of infected cells normalized to the reverse transcriptase activity (cpm). (B) Viral infection is measured as a percentage of the luciferase activity observed in the absence of antibody. Shown are means and standard deviations from duplicate samples in a typical experiment.
the phenotypes resulting from the adaptation-associated changes in the HIV-1 JR-FL Env can be reproduced in the context of the HIV-1 AD8 Env.

Discussion

New structural data and adaptation experiments have necessitated a more nuanced understanding of the HIV-1 entry pathway. Previous research has emphasized the importance of understanding Env binding to CD4 for the development of interventions. CD4-independent HIV-1 that can infect CD4-negative, CCR5-expressing cells arise in laboratory tissue culture systems (52, 132). The study of CD4-independence has taught us important lesions about the role of CD4 binding in virus entry, the mechanisms of small-molecule entry inhibitors, and the relationship between CD4 usage and neutralization sensitivity. It is notable that there is no recorded coreceptor-independent HIV-1, although some primary isolates use low levels of CCR5 (133). Additionally, viral replication was not observed in cells with less than ~1300 CCR5 molecules/cell in this study, indicating that we were unable to generate a completely CCR5-independent virus when titrating down CCR5 expression over many rounds of passaging. This result is not surprising, since the functional cure of the Berlin Patient, the patient infected with R5-tropic HIV-1 who received CCR5Δ32/CCR5Δ32 bone marrow transplant, has emphasized the fact that CCR5 plays a critical role in HIV-1 infection (20). Understanding the mechanism of conformational changes in Env upon and beyond CCR5 binding is required for a complete understanding of HIV-1 infection.

Current knowledge about the role of CCR5 in viral entry is limited. Available Env structures provide little information about the CCR5-bound state due to the necessary
Env modifications for obtaining the structural model. Inferences about the structural influence of CCR5 on Env have centered on the physical interactions of CCR5 with gp120. Crystal structures of gp120 monomers bound to sCD4 and 17b demonstrate the formation of a structure composed of the bridging sheet and V3 loop of gp120 that is thought to interact with the N-terminus and ECL2 of CCR5 (107). This model provides no information about the structure of gp41, so no inferences about the influence of gp41 on CCR5 binding could be made. Tomograms of the 17b-bound Env trimer in a cell membrane have provided an indication that there are conformational changes in gp41 compared to the unliganded or b12-bound Env trimer (134). The resolution of these structures at 23 angstrom, however, does not provide the detail necessary to suggest a structural model of gp41. The BG505 SOSIP trimer, whose structure has been solved by crystallography and cryo-electron microscopy, provides some information on gp41 structure in a cleaved Env (125). However, the BG505 SOSIP gp41 has been modified such that it is in a conformation of uncertain relevance to the HIV-1 entry pathway.

Lastly, a crystal structure of the 6-helix bundle, the conformation gp41 adopts after membrane fusion, provides the most information about how residues in gp41 interact with each other, but relatively little about its conformation in the unliganded Env trimer. Because the changes identified in this study often involve gp41 regions that are either omitted or highly modified in structural models, it is difficult to infer their influence on Env structure based on these models; interpretations of our data take advantage of the results of multiple biochemical assays.

Information regarding the mechanism of HIV-1 Env engagement with CCR5 has mostly arisen from biochemical analysis of HIV-1 using inhibitors of CCR5 binding and
fusion. Adaptation of HIV-1 to infect cells in the presence of Maraviroc and other CCR5 antagonists have reconfirmed the role of V3 as essential to binding CCR5, and additionally identified supplementary changes in the fusion peptide (G516V, M518V, F519I in the primary HIV-1 isolate D1/85.16) that enhance viral fusion with the target cell membrane (127). The development of HIV-1 capable of infecting cells treated with the 2D7 anti-CCR5 antibody also identified changes in HR1 and HR2 (R525A, R549L, and G666D in JR-CSF), along with the D164N change, similar to the S164N change identified in this study. The identification of these residues suggests that a V3-independent conformational change in gp41 upon CCR5 binding can overcome the blockade to infection imposed by 2D7.

Another adaptation experiment, which followed a similar method of passaging HIV-1 YU-2 in cells expressing low levels of CCR5, resulted in changes in V3 that permitted growth in those conditions (73). These results are not surprising because the target cells were grown in suspension, and due to the rarity of cell-cell contact, most infection events in this system probably must derive from cell-free virus. The limiting step would therefore be optimizing binding of HIV-1 Env with whatever scarce CCR5 is available in each chance encounter of the virus with the cell. In our system, adherent cells provide a greater surface area for cell-to-cell contact. While CCR5 is scarce, the viruses budding from the cell surface have a greater opportunity to interact with target cells. The rate-limiting step in this case may be to ensure that each encounter with CCR5 actually triggers a functional infection.

This study identified changes in Env that confer efficient replication in cells expressing low CCR5. These changes do not alter the sensitivity of the virus to entry
inhibitors, Maraviroc, T-20, or BMS-806. The R564H change was responsible for this replication phenotype and its effect was enhanced by the presence of the E662K change. The arginine 564 in the HIV-1 JR-FL Env structure is in an alpha-helix, and faces away from other gp41 residues towards the alpha-1 helix of gp120. Noting that a histidine is conserved across HIV-1 strains in this position, we can assume that this region is integral to HIV-1 Env function; the presence of an arginine at this position in JR-FL may alter the local structure of this region of gp41. While the E662K mutant did not generate detectable RT activity in the replication assays, it did demonstrate cell-to-cell transmission and the R564H/E662K mutant replicated efficiently in both assays. Similar to the results found in the 2D7 adaptation performed by Aarons et al, these HR1 and HR2 changes are sufficient to overcome scarce CCR5 and trigger fusion with target cells (74).

Adapting viruses in a lab setting allows the virus to evolve without immune pressure and may promote the exposure of conserved regions (53). The R564H and R564H/E662K mutants exhibited an antigenic profile comparable to that of the wild-type JR-FL virus. The adapted Envs, however, contained an S115N change that promoted the exposure of the F105, 19b, and 17b epitopes on gp120. The S115N/E662K mutant is also capable of more efficient replication in Cf2Th-CD4/CCR5 cells than the parental JR-FL in our study. The S115N and E662K changes were also identified in studies of HIV-1 resistant to fusion inhibitors (76, 96). This implicates these residues in contributing to viral fusion. Similar to the findings in 2D7-resistant viruses, perhaps these changes allow a more efficient triggering of gp41 to downstream fusion conformations. Noting that these changes also permit the exposure of epitopes
recognized by broadly neutralizing antibodies, there must also be a relationship between maintaining CCR5 binding capacity and avoiding immune response. Perhaps adopting a more open, State 2-like conformation reduces the energetic barrier of these Envs to move through the viral entry cascade.

The adapted Envs generated here show no change in inhibition by sCD4, Maraviroc, or T-20, or a change in immune cell tropism, as has been observed in Maraviroc-resistant strains (135). The rate-limiting step must therefore be independent of CD4 binding, V3-specific CCR5 binding, and the interaction of the gp41 HR1 and HR2 regions. The adaptation-associated changes may influence the overall structure of the gp41 ectodomain. In addition to the proposed role of CCR5 in promoting virus attachment to the target cell and insertion of the fusion peptide into the target cell membrane, CCR5 binding may signal a conformational change in gp41 to adopt a fusion-active state.

Future studies should elucidate the role of the observed changes in the HIV-1 entry process. Crystallographic studies of these intermediary states or FRET measuring the movement between multiple states can assist in our mapping of the appropriate sequence of events. Additionally, investigating the role of these changes in a natural infection might elucidate the advantage of enhanced cell-to-cell replication in a highly neutralization-resistant HIV-1 strain. The strains resistant to CCR5 antagonists or fusion inhibitors in the published literature have not been tested for antibody neutralization, which provides an opportunity to further elucidate the relationship between CCR5 binding, viral fusion, and immunogenicity. This will result in a better understanding of the mechanics of HIV-1 viral entry and new opportunities to block it at multiple stages.
Conclusion

Adaptation of wild-type JR-FL to infection of low-R5 expressing cells has uncovered residues in gp120 and gp41 that improve or facilitate receptor-triggered fusion with the target cell. These changes do not affect the sensitivity of Env to Maraviroc, indicating no alteration of Env’s CCR5 binding capacity. These changes do alter the neutralization sensitivity to monoclonal antibodies targeting epitopes exposed in the CD4-bound Env structure. Changes in gp41 have been observed in viruses resistant to some R5-antagonists and fusion inhibitors (76, 87, 96). Future studies will focus on the generalizability of these changes to other retroviruses in order to establish complete models of Env binding, fusion, and infection.

Acknowledgements

We would like to thank Vlad Novitsky for the protocol for proviral sequencing from genomic DNA.
Chapter 4

Analysis of CCR5 generated in CHO cells and reconstituted into nanodiscs
Section 4.1 Overview and Attributions

Overview

This chapter is comprised of a manuscript that I wrote to describe the generation of nanodiscs containing CCR5.

Attributions

I performed the experiments and wrote the manuscript. Castillo-Menendez L designed the human CCR5 expression plasmid and assisted in the experiments involving Multiangle Light Scattering and Cryo-Transmission Electron Microscopy. The CHO cell line expressing CCR5 was generated by Dai Q and Kappes JC. Dr. Sodroski provided editorial input.
Section 4.2 Stable overexpression of human CCR5 in CHO cells and 
Reconstitution in Nanodiscs

Espy N¹, Castillo-Menendez L², Dai Q, Kappes JC, Sodroski J¹²³.

¹Department of Immunology and Infectious Diseases, Harvard School of Public Heath
²Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA
³Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, USA.

Abstract

CCR5 is a 7-helix transmembrane G-coupled protein receptor that is a ligand for chemokines and gp120, the soluble unit of the human immunodeficiency virus type 1 envelope protein (Env). Solublization of CCR5 in lipid nanodiscs is a potentially useful tool to investigate the biochemistry and structural biology of the interaction of CCR5 with Env. In this study, we overexpressed CCR5 in CHO cells and incorporated them into brain lipid nanodiscs. We observed that overexpression results in incomplete post-translational modification of CCR5 and that coexpression with tyrosylprotein sulfotransferase 1 was not sufficient to restore complete CCR5 maturation. CCR5 incorporated into nanodiscs maintained stability and binding capacity to gp120 and monoclonal antibody 2D7 as measured by immuneprecipitation, transmission electron microscopy and multiangle light scattering.
Introduction

CC chemokine receptor 5 (CCR5) is a 7-helix transmembrane G-coupled protein receptor (GPCR) that is expressed on the cell surface membrane of immune cells. Its primary function is chemotaxis and cell signaling in response to binding of chemokines MIP-1\(\alpha\), MIP-1\(\beta\) and RANTES. CCR5 is also a receptor for human immunodeficiency virus 1 type 1 (HIV-1) that permits binding of the virus to target cells and triggering of conformational changes in the HIV-1 envelope glycoprotein (Env) to promote fusion of the cell and viral membranes (31-35).

Manipulation of CCR5 for biochemical analysis is challenging, as CCR5 assumes a native conformation in cholesterol-rich lipid membranes and after post-translational modification. Bacterial and insect cell lines are not ideal systems for CCR5 production because this protein is both sulfated and glycosylated, among other modifications, in a manner that affects function (40, 136). Tyrosine-sulfation of residues in the N-terminus of CCR5 is required for Env binding (40, 42-44). Commonly available mammalian cell lines that would more closely mimic the native environment for CCR5 produce this protein at levels too low for structure determination (137). The only available structure of CCR5 is derived from protein expressed in Sf9 insect cells, incubated with allosteric inhibitor Maraviroc and solubilized in dodecyl-D-maltopyranoside (66). While this structure is useful for visualizing the arrangement of the protein when bound to the drug, this structure assumes a conformation distinct from the unliganded state that is capable of binding chemokines or Env. Therefore, structures of CCR5 in native-like conformations – embedded in a lipid membrane and without ligands - are necessary for more accurate understanding of each protein.
Nanodiscs are a useful tool for protein reconstitution and experimentation. Nanodiscs are a soluble lipid-protein complex that consists of a lipid bilayer surrounded and stabilized by two membrane-scaffolding protein (MSP) constructs (138). Nanodiscs can be made with varying lipid compositions and with different MSP constructs that vary in length (139), and many transmembrane proteins (including trimeric proteins and GPCRs) have been incorporated into nanodiscs and retained function (140-142). Based on this precedent, successful incorporation of CCR5 into the lipid membranes of nanodiscs could increase the functional half-life of each protein and facilitate further biochemical, biophysical, and structural analysis of these proteins.

In this study, we used an efficient doxycycline-regulated mammalian cell expression system to generate CCR5 on a large scale. We observed dimerization of CCR5 and noted that protein overexpression is limited by the ability of the cells to properly post-translationally modify the protein. Reconstituting the solubilized protein into lipid nanodiscs maintained structural integrity and allowed use in biochemical and structural assays. This work is significant as it demonstrates a method for CCR5 overexpression and CCR5 nanodiscs as a potentially useful tool for experimental analysis.

**Materials and Methods**

*Generation of CCR5-expressing cells*

A schema of the CCR5 expression vector is shown in Figure 4.1. In sum, a StrepTagII was added to the 3’ end of the human CCR5 gene. The gene was then cloned into an HIV-1 based lentiviral vector, which contains an upstream tetracycline response element (TRE) and a downstream internal ribosome entry site and the
puromycin (puro) and T2A enhanced green fluorescent protein (EGFP) open reading frames (TRE-CCR5-StrepTagII-IRES.PuroT2A.EGFP).

This vector was packaged in a pseudotyped VSV-G virus and transduced in CHO cells (Invitrogen) that stably express the reverse tet-transactivator protein (tTA). Transduced cells were incubated in 1 μg/mL doxycycline for 1 day and selected in 10 μg/mL puromycin for 5-7 days. Cell clones expressing high EGFP fluorescent cells were isolated using a FACSARia cell sorter (BD Biosciences) and expanded in a serum-free suspension culture medium, CDM4CHO (Thermo Fisher).

The D558 CHO cells expressing CCR5 were transduced with a vector expressing the tyrosylprotein sulfotransferase 1 (TPST-1) gene and selected for high EGFP expression as described above.

**CCR5 expression and purification**

CCR5 in CHO cells was expressed in two ways. First, cells were expanded in a suspension culture in a 14-liter New Brunswick BioFlo 310 fermenter (Eppendorf) to >4 million cells per mL and incubated for 18 hours in 0.2 μg/mL doxycycline. Cells were harvested by centrifugation, snap-frozen in liquid nitrogen, and stored in -80°C until use. Secondly, cells were cultured in static flasks and incubated with doxycycline for 18 hours. Cells were harvested by centrifugation and stored frozen until use.

To purify CCR5 from cell membranes, CCR5-expressing CHO cells were harvested by centrifugation and homogenized in 20% sucrose buffer in the presence of a protease inhibitor cocktail (Roche Complete tablets). Plasma membranes were extracted by ultracentrifugation at 100,000xg and solubilized in 100 mM (NH₄)₂SO₄, 20
mM Tris-HCL (pH 8), 300 mM NaCl, 1% Cymal-5 or Foscholine-12 (Affimetrix). CCR5 was isolated by ultracentrifugating the solution at 200,000xg and affinity-purified with StrepTavidin beads according to the manufacturer’s protocol (Millipore). All buffers were supplemented with 1% Cymal-5.

Flow cytometry analysis
Cells were tested for cell-surface CCR5 expression by labeling with R-Phycoerythrin-conjugated mouse anti-human CD195 (PE-anti-CD195, BD Biosciences), washing with cold 1xPBS, and analyzed with a BD FACSCanto II flow cytometer (BD Biosciences).

Cell-Surface Immunoprecipitation
His-tagged JR-FL gp120 and soluble CD4 (sCD4) were generated in 293F cells and purified from supernatants with Ni-NTA beads (Qiagen). Equal molar amounts of gp120 and sCD4 were incubated at room temperature for 1 hour before use in cell-surface immunoprecipitation. Anti-CCR5 antibody 2D7 was acquired from BD Biosciences.

CHO cells were incubated in the indicated concentration of doxycycline for 15 hrs, washed with PBS, and resuspended in 1XPBS. Complexes of gp120+sCD4 or 2D7 were added to the cells and the reaction was incubated for 2 hours at 37°C. Cells were then washed three times in PBS and lysed in 50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% [vol/vol] Triton X-100, and Complete EDTA-free protease inhibitor cocktail (Roche). Cells were incubated in this buffer for 30 min at 4°C with gentle agitation to enhance lysis. Samples were cleared of unsolublized membranes, organelles, and nucleic acids.
via centrifugation at 14,000xg for 30 min at 4°C. The gp120 glycoproteins in the cleared lysates were then immunoprecipitated with 2 μg C11 antibody/100 μL 50:50 protein A-Sepharose beads for 1 hour at 4 degrees rotating. The 2D7 reaction was incubated with beads alone. Beads were washed three times with 1xPBS, spinning at 30 x g at 4°C. Fifty microliters 2x SDS sample buffer (125 mM Tris, pH 6.8, 10% glycerol, 5.5% beta-mercaptoethanol, 3% sodium dodecyl sulfate) was added to the beads and rotated for 30 min at room temperature. Buffer was added to the beads and incubated at 100 degrees for 10 min.

Silver staining and western blots

Samples in SDS sample buffer were run on a 4-12% Bis-Tris SDS-PAGE and either silver stained or transferred to nitrocellulose membrane for Western blotting. Silver staining was performed using the Pierce Silver Stain Kit (Thermo Fisher) according to the manufacturer’s protocol. Western blotting followed protocols described elsewhere (143). Primary antibodies included anti-CCR5 (Sigma), anti-StrepTagII (Abcam), anti-gp120-HRP (Abcam), and anti-Sulfotyrosine monoclonal (Millipore). Protein-A-HRP (Abcam) served as secondary antibody where necessary.

Production of Membrane Scaffolding Protein MSP1E3D1

Expression plasmids for MSPE3D1 were obtained from the Sligar lab (138). BL21 (DE3) bacteria were transformed with the pET28a kanamycin-resistant vector containing the MSP1E3D1 gene. Colonies were selected on 50 μg/mL kanamycin LB agar plates overnight at 37°C. Colonies were expanded in 1L Terrific Broth with 50 μg/mL
kanamycin, shaking at 250 rpm overnight at 37°C. Protein expression was induced with 1 mM IPTG for 1 hour when OD$_{600}$=0.8-1.0. Cells were harvested by centrifugation, lysed in 80 mL 1X PBS, 1% Triton X-100 and protease inhibitor cocktail, and sonicated for 4 min on ice. Cell lysates were cleared via centrifugation at 17000xg for 30 min at 4°C before protein purification on Ni-NTA beads. MSP1E3D1 was eluted with in 40 mM Tris pH 8, 300 mM NaCl, 500 mM Imidazol, pH 8.0, concentrated and stored at -80°C until use.

Reconstitution of soluble CCR5 in lipid nanodiscs (including size exclusion)

The protocol for nanodisc assembly was adapted from the Sligar lab (144). To reconstitute CCR5 in nanodiscs, we incubated purified soluble CCR5 with MSP1E3D1 and dried brain lipid extract (Avanti Polar Lipids, Inc.) resuspended in 20 mM Tris pH 8, 300 mM NaCl, 4% Cymal-5 in a 1:1:130 molar ratio for 1 hour on ice. The detergent was removed from the reaction by adding SM-2 Biobeads in a ratio of 5 mg beads per 20 μL solution to the reaction and incubating overnight at 4°C in an orbital shaker. Nanodiscs were filtered in an 0.22 micron Ultrafree-MC-GV centrifugal filters and purified from the reaction by running the filtered reaction on a Superose 6 10/300 GL column (GE Healthcare) in 20 mM Tris pH 8, 100 mM NaCl buffer. 1-mL fractions were analyzed for protein content via silver staining and Western blotting. Fractions containing MSP1E3D1 and CCR5 were concentrated and used in binding and immunoprecipitation reactions with 2D7 or anti-strep antibody with Protein A-sepharose beads.

Multiangle Light Scattering
Purified CCR5 and empty nanodiscs were run on a Superose 6 10/300 GL column column in 20 mM Tris pH 8, 100 mM NaCl buffer coupled to a Wyatt DAWN HELEOS II 18 angle light-scattering detector and an Optilab T-rEX differential refractive index detector. Light scattering data were analyzed using the ASTRA 6 software (Wyatt Technology).

**Results**

**Characterization of CCR5 overexpressed in CHO cells**

Human CCR5 tagged with StrepTagII at the C-terminus was transduced and stably expressed in CHO cells (Figure 4.1a). Clones were selected for high EGFP expression and proper morphology and growth kinetics. Clone D558 was selected for use based on high CCR5 expression on the cell surface (Figure 4.1b). CCR5 expression was tested by incubation of D558 in 0.2ug/mL doxycycline for 18 hours in a fermenter, isolating cell membranes, and solubilizing and purifying CCR5 with StrepTavidin Beads. All steps of the CCR5 solubilization and purification were performed with Cymal-5 or Foscholine-12, as previous research has indicated that each detergent maintains the conformational epitope recognized by the 2D7 antibody and gp120 or RANTES binding (136, 137). CCR5 is visible via immunoblotting with an anti-CCR5 antibody, showing two doublet bands representing the CCR5 monomer at ~30 kDa and dimer at ~60 kDa.

An alternative culture method was tested in which cells were grown in static flasks at 33 and 37°C (Figure 4.2). The presence of CCR5 in the solubilized membranes or in the pellets cleared by ultracentrifugation was tested by silver staining or Western
blotting (Figure 4.2a). The optimal growth condition was determined for the incubation of cells at 37°C in 0.5 μg/mL Doxycycline. The process of solubilizing CCR5 from membranes was efficient but incomplete, as there was little CCR5 left in the membrane. Additionally, the monomer doublet band is the dominant species in all samples.

The CCR5 generated in D558 cells was tested for the maintenance of the 2D7 conformational epitope by cell-surface immunoprecipitation (Figure 4.2b). D558 cells were incubated with 2D7 and lysed, and CCR5-2D7 complexes were pulled down with
Protein-A beads. Monomer and dimer doublet bands were pulled down, indicating that CCR5 expressed in CHO cells is in the proper conformation and that 2D7 does not discern between the species forming the doublets.
**Figure 4.2: Dox induction of CHO-R5(strep) cells.** (A) CHO-R5(strep) cells were incubated with indicated concentrations of doxycycline (ng/mL) at 33° or 37°C for 15 hours, and then proteins were extracted from purified membranes. The protein lysates were analyzed by SDS-PAGE and silver stained or immunoblotted with an anti-strep antibody (WB). (B) 2D7 immunoprecipitation of CCR5 from the surface of D558 cells incubated in 0.5 μg/mL Dox at 37° degrees.

**TPST-1 cotransfection and stable expression**

A doublet band is present as a monomer and a dimer in all samples from clone D558 when run on SDS-PAGE. It was hypothesized that this doublet may be due to cleavage of the protein or to incomplete maturation. Tyrosine sulfation of the N-terminus...
of CCR5 is a required modification for protein maturity and contributes to gp120 and MIP-1α binding (40, 137). Precursor and mature CCR5 have been reported to run on SDS-PAGE as a doublet similar to what is seen in our experiments (137).

To determine if this doublet band is due to incomplete tyrosine sulfation, D558 was treated with sodium chlorate during doxycycline incubation (Figure 4.3). Previous
research suggested that chlorate treatment of CCR5-expressing cells would block N-terminal modifications like tyrosine sulfation (40). Chlorate treatment of D558 did not alter migration of CCR5 in SDS-PAGE and was toxic to cells at concentrations >50 mM.

To further implicate tyrosine sulfation as the cause of the doublet band, we also transduced the cells with a plasmid expressing tyrosylprotein sulfotransferase 1 (TPST-1) to form the cell line D749. Clone D749 was cultured in a static flask and incubated with increasing concentrations of doxycycline for induction of CCR5 expression (Figure 4.4). Immunoblotting of cell membrane preparations for the strep tag of CCR5 showed that D749 expresses primarily the larger of the doublet bands (Figure 4.4a). No noticeable increase in tyrosine sulfation was detected by immunoblotting with an anti-Tyrosine Sulfation antibody (Figure 4.4b).

When labeled with PE-conjugated 2D7 and analyzed by flow cytometry, we noted that D749 generated fewer cells expressing CCR5 on the cell surface than D558 cells (Figure 4.5). Since D749 mirrors D558 expressing CCR5 at low doxycycline concentrations (Figure 4.4a) and reduced cell growth delay in D749 cells (observational data), perhaps reduced CCR5 expression overall results in CHO cells that are less overwhelmed by CCR5 protein production and allow more complete CCR5 maturation.

D558 and D749 bind JRFL gp120 via cell surface immunoprecipitation (Figure 4.4C). Therefore, these clones produce CCR5 that is functional for nanodisc reconstitution.
**CCR5-nanodisc assembly**

Previous protocols for nanodisc production have been published (138, 142) and will serve as a template for our approach. The membrane will be stabilized by the membrane scaffold protein E3D1 (MSP1E3D1). The 32k-Da MSP1E3D1 protein contains a 6xHis-tag and an additional 66 amino acids relative to the original MSP1.
protein to increase the diameter of the generated nanodiscs. MSP1E3D1 was purified from *E. coli* DE3 cells that were transformed with pMSP1E3D1 grown in Terrific Broth overnight at 37°C overnight, induced for 1 hour with IPTG. Following cell lysis, MSP1E3D1 was affinity-purified using a Ni-NTA column.

To mimic the native membrane concentration for CCR5, nanodiscs were generated using brain lipid extracts, as 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and cholesterol are highly enriched in HIV-1 viral membranes (145). Nanodiscs were generated as follows: lipids stored in chloroform were dried and resuspended in Cymal-5, incubated with MSP1E3D1 for 1 hour at 4 degrees, and

Figure 4.5: FACS analysis of D558 and D749. Cells were labeled with PE-conjugated 2D7 (PE-2D7) and analyzed by FACS. The two populations observed in SSA-A v. FSC-A were gated to observe differences in PE labeling.
incubated overnight with hydrophobic SM-2 beads. The nanodiscs were then purified by size-exclusion chromatography to remove free lipids, MSP and Env/CCR5. Fractions from size exclusion-chromatography were analyzed by silver stain and Western blot (Figure 4.6a). We observed CCR5 eluting in the same fractions as MSP1E3D1, indicating the formation of CCR5-nanodiscs, while solubilized CCR5 alone does not visualize on gel filtration or SDS-PAGE.
Cryo-TEM can be used to derive images of CCR5 nanodiscs. To do this, samples of CCR5 incorporated in nanodiscs were applied on C-Flat holey carbon grids and then flash plunged into liquid ethane using a Vitrobot, and then transferred to liquid nitrogen. Micrographs were collected on a Tecnai F20 TEM (FEI) with a field-emission gun at 80kV on a CCD camera (Figure 4.6b). We observed densities suggesting that the nanodiscs orient perpendicular to the grids. This may be influenced by the negative charge of the grid attracting the MSP1E3D1 belt around the lipid bilayer or to the thickness of the ice on the grid.

To confirm the presence of CCR5 in nanodiscs by TEM, empty nanodiscs and CCR5 nanodics were bound to grids and bound with 2D7, mouse anti-rabbit antibody, and 5 nm gold-conjugated Protein-A and then negative stained with uranyl acetate (Figure 4.7). Gold-conjugated Protein A should allow identification of CCR5-2D7 complexes on grids. Images of empty nanodiscs show no discernible gold particles. Images of CCR5 nanodiscs showed gold particles in proximity to particles that are approximately 20 nm in length. Improved sample preparation and staining are required for better visualization of CCR5 nanodiscs.

**Validate biochemical function of reconstituted protein**

Solubilizing membrane proteins and reconstitution in nanodiscs could potentially result in some disruption of the protein conformation. Therefore, the maintenance of protein function after reconstitution was investigated.

We immunoprecipitated CCR5 nanodiscs and solubilized CCR5 with the 2D7 antibody conjugated to Protein A beads (Figure 4.8a). CCR5 nanodiscs were eluted
from 2D7 primarily in the presence of SDS sample buffer and detergent, while purified CCR5 was eluted from boiling beads. Higher-order CCR5 structures were seen in both solubilized CCR5 and CCR5 nanodisc samples over time, but CCR5 in nanodiscs was primarily monomeric. Empty nanodiscs and nanodiscs with CCR5 were stable for up to 1 week. Additionally, gp120 was pulled down from immunoprecipitations of StepII-Tag-labeled CCR5 and CCR5 nanodiscs with anti-streptavidin antibody conjugated to protein A beads (Figure 4.8b).

Further, nanodiscs were analyzed via multiple angle light scattering to determine molecular weight and radius (Figure 4.9). No mass increase was associated with the reconstitution of CCR5 into nanodiscs. This may be due to the fixed size of the nanodisc imposed by MSP1E3D1 or to the replacement of the lipids that are incorporated into empty nanodiscs by CCR5. CCR5 nanodiscs incubated with the 2D7 antibody
demonstrated increased mass and radius (Rz) compared to CCR5-nanodiscs alone, supporting the formation of CCR5 nanodisc and 2D7 complexes.
Figure 4.8: 2D7/gp120 immunoprecipitation. (A) Purified CCR5, CCR5 in nanodiscs (R5Nd), and empty nanodiscs (empty Nd) were incubated with 2D7 bound to protein A beads (lane 1), washed (lane 2), and eluted with SDS buffer (lane 3) and then boiled (lane 4). Experiments repeated on day 1 (red), 3 (orange), and 7 (purple). Fractions run on gel electrophoresis and silver stained or western blotted with a 1:5000 anti-streptavidin antibody.

(B) Purified R5, R5Nd, and empty Nd were incubated with 2D7 (green) or gp120, sCD4 and anti-streptavidin antibody (blue) bound to protein A beads. Reaction solution (lane 1), wash fraction (lane 2), SDS elution (lane 3), and boiled elution (lane 4) were run on gel electrophoresis and western blotted with a 1:5000 anti-streptavidin or anti-gp120-HRP antibody.
Figure 4.9: Multiple Angle Light Scattering of Nanodiscs. (A) Nanodiscs (Nd) and CCR5 nanodiscs (NdR5), with or without 2D7 were run on a Superdex 200 3.2/30 in 20 mM Tris pH7.5 150 mM NaCl. Alignment adjusted equally to all graphs according to void volume. UV (green), refractive index (blue), light scattering (red). (B) Hydrodynamic properties of nanodiscs. Mw = molecular weight, Rz = radius, Mw/Mn = polydispersity (where Mw/Mn=1 is complete homogeneity).

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<tr>
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<td>250</td>
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<td>1.004</td>
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<td>271</td>
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<td>1.005</td>
</tr>
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</table>
Discussion

Human CCR5 is a difficult protein to isolate for use in biochemical assays, as it requires a particular lipid environment in the cellular membrane and mammalian cell-specific post-translational modifications to maintain structural integrity. CCR5 expressed in *E. coli* generated a high protein yield, but this expression system generated protein that lacks proper post-translational modifications, which enhances binding of 2D7 and its natural ligands (146). CCR5 expression in a baculovirus/insect cell system generated high protein yield that did maintain structural integrity and proper protein modification, indicating that overexpression of CCR5 was achievable. In this study, we were able to overexpress high-quality recombinant human CCR5 in a tightly regulated TetOn system and to reconstitute the protein in soluble lipid bilayer nanodiscs.

Heterogeneity remains a challenge to overexpressing human CCR5 in CHO cells, as indicated by the doublet bands in both the monomer and dimer forms of CCR5 observed by Western blot. It remains unclear how the two species differ, and it is unknown if this is due to tyrosine-sulfation. The doublet band was observed particularly at high levels of doxycycline-mediated induction even in cells co-expressing TPST-1. A possible reason for this phenomenon could be that overexpression overwhelms the cellular post-translational modification pathway. Still, CCR5 in nanodiscs retained gp120 and 2D7 binding capacities and demonstrated near homogeneity as measured by multiangle light scattering.

Future studies will be required to determine if CCR5-nanodiscs can be used to elucidate the structure of the chemokine receptor. Generation of a CCR5 structure will be interpreted within the context of previously published structures. Similarities between
the structures of CCR5 in nanodiscs and in CCR5 lipid cubic phases could indicate that the lipid membrane only modestly affects protein structure. Observed differences could reflect effects of the lipid membrane environment, or be the result of technical differences in the purification and membrane reconstitution procedures. The effect of the lipid membrane may be especially important when interpreting results from a CCR5 structure, as the majority of the protein is embedded in the membrane. Further biochemical studies of the protein within the context of a lipid membrane would complement such structural analyses.

Additional studies will be needed to validate the functional state of CCR5 in nanodiscs. We anticipate that the CCR5 proteins incorporated in nanodiscs will maintain binding capacities, as indicated by the preliminary biochemical assays described above. Even further, we anticipate that nanodiscs with CCR5 will inhibit viral infectivity by prematurely triggering viral Env. Therefore, CCR5 nanodiscs can also be incorporated into the biochemical or structural studies of HIV-1 Env to understand the conformational changes that lead to and follow CCR5 binding.
Chapter 5

Conclusion
Section 5.1 Overview

This chapter is a summary of the above chapters and the results therein, the perspectives on the implications of this work, and future directions.

Section 5.2 Summary of Results, Perspectives, and Future Directions

This work has attempted to explore the role of CCR5 in human immunodeficiency virus type 1 (HIV-1) infection and replication. The governing questions ask: How essential is CCR5 in infection? What mechanisms will the virus employ to overcome scarce availability of CCR5 on the target cell? Is there a tool that can help researchers use CCR5 for biochemical analysis? These questions are important in the context of HIV-1 basic research and vaccine design. First, the only case of a functional cure of a person living with HIV-1 received a CCR5Δ32/Δ32 hematopoietic stem cell transplant, indicating that cure is possible. Attempts to cure HIV-1 infections in a manner less invasive must therefore understand the role CCR5 plays in infection beyond binding of the virion and interrogate the adaptive capacity of HIV-1 to overcome chemical or immune blocks to CCR5-mediated entry. Second, structural analysis of native, unliganded Env has guided the designs of antibodies for anti-HIV vaccines. HIV-1 Env is metastable and its binding to receptors CD4 and CCR5 trigger the formation of heterogeneous conformations that makes it difficult to isolate Env by crystallography or cryo-electron microscopy along the entry pathway. The additional insights gleaned from a CCR5-bound Env and a resultant broadening of the antigens used in a vaccine could be what is needed to improve vaccine efficacy.
This work has attempted to generate a coreceptor independent HIV-1 by adapting the virus to infect cells with diminishing levels of CCR5 expression on target cells. We assumed that molecular changes in Env would identify residues essential for CCR5 binding and/or downstream conformational changes that permit membrane fusion. Even if a coreceptor independent HIV-1 was not achieved, the levels of CCR5 expression on target cells necessary for infection could be estimated. Here, we isolated HIV-1 NL4.3(JR-FL) that could infect cells at ~1300 CCR5 molecules per cell. This virus acquired a S115N change in the gp120 subunit and E662K change in the gp41 subunit of Env that enhanced the exposure of epitopes in the CD4 binding site and variable loops 2 and 3 (V2 and V3, respectively). The virus also acquired an R564H change in gp41 that conferred the low CCR5 usage phenotype. The mechanism of this phenotype could not be clarified using available tools. Most interestingly, the adapted virus demonstrated reduced cell-free infection but enhanced cell-to-cell transmission. Lastly, human CCR5 overexpressed in mammalian cells could be reconstituted in a lipid bilayer nanodisc and retain anti-CCR5 2D7 and gp120 binding.

This work adds to the literature that HIV-1 Env has a number of ways to maintain an interaction with CCR5 and that CCR5-mediated entry involves a number of mechanisms relying on conformational transitions in gp120 and gp41. The questions that remain unresolved are the exact sequence of events after CCR5 binding and before membrane fusion, what the conformation of Env is at this point, and whether there are small molecules that can target these intermediate states. Further, what is the relationship between CCR5 binding and immune evasion? Finally, this work identifies cell-to-cell transmission as an important dynamic in infection and calls for validation of
its role in pathogenesis (ie, replication of HIV in lymph nodes or the CNS). Development of soluble CCR5 nanodiscs for use in biochemical and structural analysis can assist in this endeavor.

Questions about antigenicity and pathogenesis could be addressed in the context of a natural infection in a competent host, such as non-human primates, or by observing clinical outcomes of patients infected with viruses with the same low CCR5 usage phenotype. While the concerns about the biosafety of generating and using a virus that uses less CCR5 are reasonable, the antibody immune response can neutralize CD4-independent viruses and HIV-1 that uses low levels of CCR5 also increase the neutralization sensitivity of the virus (see Chapter 3).

The information gained from these adaptation experiments have improved our understanding of the Env protein, indicated the mutually exclusive relationship between viral entry and immune evasion, and identified new regions to target therapeutics. Because of this, this work has implications for understanding viral evolution and viral fusion in the context of cell-to-cell transmission. Most significantly, this work encourages exploration of these dynamics in other viruses for the purposes of expanding our knowledge about enveloped viruses, viral infections, and therapies to treat and cure future patients.
Appendices

Appendix 1 Formation of a gp120-sCD4-sCCR5 complex

(A) C-terminally His-tagged JR-FL gp120 was generated and purified from 293F cells. Thrombin tagged soluble 4-domain CD4 was generated in 293F cells and purified by Ni-NTA column. CCR5 expression in D558 cells was induced via incubation in 500 ug/mL doxycycline for 18 hours. CCR5 was purified from the cell membranes and solubilized 100 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8), 300 mM NaCl, 1% Cymal-5. Soluble CCR5 was incubated with gp120 and sCD4 overnight at 4 degrees Celsius. Soluble CCR5 and Complexes with or without gluteraldehyde (+GA) treatment were purified on Superdex 200 10/300 (left). Fractions were Western blotted with anti-StrepTagII (right). (B) Western blot of complexes not treated with GA were Western blotted with anti-StrepTagII or anti-His antibodies.


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