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Insights into the Role of Chemokines and Chemokine Receptors During HIV-1 Pathogenesis

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Abstract

Sexual transmission of HIV-1 is often established by one genetic variant, the transmitted/founder (T/F) virus. T/F HIV-1 may have specific phenotypic properties that are selected for during transmission. To date, the most consistent phenotypic property associated with T/F viruses is use of the chemokine receptor CCR5 as a coreceptor for entry. Small molecule CCR5 antagonists, such as Maraviroc (MVC), inhibit HIV-1 entry by functioning as allosteric inhibitors. These molecules bind within the transmembrane helices of CCR5, inducing a conformational change that prevents the HIV-CCR5 interaction. As with most drugs, HIV-1 has developed strategies to overcome this inhibition. Some viruses develop mutations in the envelope (Env) glycoprotein that enable the use of antagonist-bound CCR5. In Chapter Two, we evaluate 87 CCR5-using viruses to address differences between T/F viruses and viruses isolated from chronically infected individuals (chronic controls-CC) in their ability to mediate entry via varying amounts of CCR5 in the presence of MVC. We demonstrate that CC viruses exhibit partial resistance (PR) to MVC more frequently than T/F viruses, suggesting that T/F and CC HIV-1 differentially utilize CCR5 in a manner that may be biologically important in the context of transmission. Following the discovery of the chemokine receptors CXCR4 and CCR5 as cofactors for HIV-1 entry, it was revealed that their cognate chemokine ligands could inhibit HIV-1 infection in vitro. Multiple cell types have been implicated in secreting chemokines that function to modulate HIV-1 infection. Recently the platelet-derived chemokine PF4 was shown to inhibit HIV-1. However, despite plasma and local concentrations of PF4 being within the range used in these studies, HIV-1 is still able to propagate in vivo. In Chapter Four, we sought to understand the mechanism of action of PF4 and determine it's in vivo relevance. I confirmed and extended previous studies showing that PF4 inhibits infection by HIV-1 and other viruses. However, the inhibitory capacity of PF4 is limited to a defined concentration range, after which inhibition wanes and viral infection is ultimately enhanced at higher chemokine concentrations that are commonly found in vivo. Thus, rather than being a potential anti-viral agent as previously suggested, PF4 may contribute to the hematologic abnormalities commonly seen in HIV-infected individuals by enhancing virus infection in the bone marrow.

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INSIGHTS INTO THE ROLE OF CHEMOKINES AND CHEMOKINE RECEPTORS DURING HIV-1 PATHOGENESIS

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INSIGHTS INTO THE ROLE OF CHEMOKINES AND CHEMOKINE RECEPTORS DURING HIV-1 PATHOGENESIS

DEDICATION

This dissertation is dedicated to my second nuclear family back home in Cameroon—Aunty Anjie, Uncle Paddy, Nyenty, Ayuk, Kimberly, and Anita (Mama). My interest in studying the biology of infectious diseases, particularly HIV-1, was driven by experiencing the impact of such diseases within my family, both during my childhood in Africa and as an adult in The United States.

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ABSTRACT

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Robert W. Doms

Sexual transmission of HIV-1 is often established by one genetic variant, the transmitted/founder (T/F) virus. T/F HIV-1 may have specific phenotypic properties that are selected for during transmission. To date, the most consistent phenotypic property associated with T/F viruses is use of the chemokine receptor CCR5 as a coreceptor for entry. Small molecule CCR5 antagonists, such as Maraviroc (MVC), inhibit HIV-1 entry by functioning as allosteric inhibitors. These molecules bind within the transmembrane helices of CCR5, inducing a conformational change that prevents the HIV-CCR5 interaction. As with most drugs, HIV-1 has developed strategies to overcome this inhibition. Some viruses develop mutations in the envelope (Env) glycoprotein that enable the use of antagonist-bound CCR5. In **Chapter Two**, we evaluate 87 CCR5-using viruses to address differences between T/F viruses and viruses isolated from chronically infected individuals (chronic controls-CC) in their ability to mediate entry via varying amounts of CCR5 in the presence of MVC. We demonstrate that CC viruses exhibit partial resistance (PR) to MVC more frequently than T/F viruses, suggesting that T/F and CC HIV-1 differentially utilize CCR5 in a manner that may be biologically important in the context of transmission. Following the

discovery of the chemokine receptors CXCR4 and CCR5 as cofactors for HIV-1 entry, it was revealed that their cognate chemokine ligands could inhibit HIV-1 infection *in vitro*. Multiple cell types have been implicated in secreting chemokines that function to modulate HIV-1 infection. Recently the platelet-derived chemokine PF4 was shown to inhibit HIV-1. However, despite plasma and local concentrations of PF4 being within the range used in these studies, HIV-1 is still able to propagate *in vivo*. In **Chapter Four**, we sought to understand the mechanism of action of PF4 and determine it's *in vivo* relevance. I confirmed and extended previous studies showing that PF4 inhibits infection by HIV-1 and other viruses. However, the inhibitory capacity of PF4 is limited to a defined concentration range, after which inhibition wanes and viral infection is ultimately enhanced at higher chemokine concentrations that are commonly found *in vivo*. Thus, rather than being a potential anti-viral agent as previously suggested, PF4 may contribute to the hematologic abnormalities commonly seen in HIV-infected individuals by enhancing virus infection in the bone marrow.

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CHAPTER 1

Introduction to HIV-1 entry and transmission

HIV-1 ENTRY

Human immunodeficiency virus type I (HIV-1) infection poses a significant global disease burden, with approximately 34 million people currently infected, and roughly 2.5 million new cases annually (292). HIV-1 infection leads to Acquired Immunodeficiency Syndrome (AIDS), which has caused mortality of an estimated 36 million people since the start of the global pandemic in the mid 1980s, with approximately 1.6 million AIDS-related deaths annually (292). AIDS is a disease characterized by the gradual decline of circulating $CD4^+$ T cells over time. Once a threshold of approximately 200 x 10^9 $CD4^+$ T cells/liter is passed, the individual is said to be immune compromised and highly susceptible to opportunistic infections by otherwise non-life-threatening pathogens. Generally, HIV-1 transmission rates are low, ranging from 0.1% to 10% per coital act (33, 101, 196, 212). However, once an individual becomes infected with HIV-1, a primary or acute viral infection results within a few weeks, and is characterized by high viral replication and massive loss of gut associated lymphoid tissue (GALT) $CD4^+$ T cells. Understanding the early events of HIV-1 infection is important as they provide insight into the later course of disease. Thus, it is most appropriate to study the very earliest interactions between the virus and the host as the starting point of studying HIV-1 pathogenesis. HIV-1 entry into target

cells—primarily $CD4^+$ T cells—is mediated by the type I integral membrane viral glycoprotein Env. The Env precursor (gp160) is proteolytically processed within the cell, generating the surface unit (gp120) and the transmembrane component (gp41), which remain associated via non-covalent interactions. Env comprises gp120 and gp41, and exists as a trimer of heterodimers incorporated into the viral membrane (296).

HIV-1 entry into cells involves four distinct stages: attachment to the host cell, binding of gp120 to the primary receptor (CD4), binding of gp120 to the coreceptor (CCR5 or CXCR4), and gp41-mediated fusion of the viral and host membranes (Fig 1). The initial, and rate-limiting, step of infection *in vitro* involves relatively nonspecific interactions between Env, or other host-derived proteins within the viral membrane, and cellular attachment factors such as the negatively-charged glycosaminoglycan (GAG) heparan sulfate proteoglycan (245). More targeted interactions have also been described between Env and the C-type lectin dendritic cell–specific intercellular adhesion molecular 3-grabbing non-integrin (DC-SIGN) (95). DC-SIGN, and other C-type lectins (148, 278, 279), expressed on dendritic cells can boost HIV-1 infection by binding to high-mannose sugars on Env (114) and enhancing infection efficiency *in vitro* by transferring bound virions to surrounding $CD4^+$ T cells (a process known as *trans*-infection). Additionally, monomeric HIV-1 Env has been shown to specifically interact with the gut homing integrin $\alpha4\beta7$ (14, 57), which is approximately three times larger than CD4, making it a prominent attachment factor for efficient viral capture to increase viral attachment and cell-cell spread.

Notably, the discovery of α4β7 as an attachment factor is interesting because the integrin is upregulated on activated CCR5-expressing $CD4^+$ T cells within the GALT, a site highly relevant to HIV-1 pathogenesis. Whether α4β7 binds trimeric Env remains to be investigated. Viral attachment to the cell brings the virus and cell in close proximity to each other, enhancing the efficiency of infection (194). While significant, these early, low-affinity interactions are not essential for virus entry—as is the case for the viral receptor CD4.

The primary receptor for HIV-1 is CD4 (168, 173), a glycoprotein and member of the immunoglobulin superfamily found on the surface of T helper cells, monocytes, macrophages, and dendritic cells. HIV-1 Env interacts with CD4 (145) to initiate a cascade of conformational changes within gp120 required for successful entry, and with very few exceptions CD4 binding is a prerequisite for HIV-1 entry. HIV-1 gp120 contains five conserved domains (C1–C5) and five genetically variable loops (V1–V5). The variable loops are situated at the surface of gp120 and are involved in immune evasion. V3 is particularly important for coreceptor binding (109, 266). CD4 binding to gp120 induces major structural rearrangements in gp120 by inducing the formation of the bridging sheet (formed by the rearrangement of two pairs of β-sheets from the inner and outer domains of gp120 into a four-stranded β-sheet structure), rotating each gp120 monomer, and the extending of the variable loops (V1/V2 and V3). These conformational changes draw the viral and cell membranes into closer proximity, as well as create and expose the coreceptor-binding site.

Coreceptor engagement follows CD4 binding. In humans, the most important coreceptors are the seven-transmembrane G-protein-coupled receptors (GPCRs) CCR5 and CXCR4. GPCRs are integral membrane proteins with an extracellular N-terminal segment, three extracellular loops (ECL) formed between transmembrane regions, and an intracellular DRY motif that is critical for G-protein signaling components (Fig 2). CCR5 and CXCR4 are chemokine receptors, where binding of the cognate chemokine ligands to the chemokines receptors results in a G-protein-mediated cascade of intracellular signaling events, which regulate trafficking and the effector response of leukocytes. Surface expression of various chemokine receptors is specific for certain types of leukocytes. This expression pattern of chemokine receptors on target cells is the basis for coreceptor tropism of HIV-1, with some viruses exclusively using CCR5 (R5-tropic), some engaging only CXCR4 (X4-tropic), and others taking advantage of both (R5X4/dual-tropic) for entry. CCR5 expression is mainly restricted to memory $CD4^+$ T cells, while CXCR4 expression is more widespread and predominates on naïve $CD4^+$ T cells (30). Despite this expression pattern, most infections are initiated by R5-tropic viruses, while disease progression to AIDS is often associated with the emergence of X4-tropic or dual-tropic variants (64, 247, 267). Several lines of evidence (3, 23, 76, 84, 266) suggest that both the tyrosine-sulfated N-terminal segment and the second extracellular loop (ECL2) are involved in gp120 binding, specifically through interactions with the bridging sheet and the crown of the V3 loop (which shares striking homology with the beta2-beta3 loop in chemokines—the natural ligand of chemokine receptors

(46)). As such, the amino acids in the gp120 coreceptor-binding site are highly conserved between HIV-1, HIV-2, and SIV (223, 224), and could serve as potential targets for the development of broadly-active therapeutic agents.

Binding of gp120 to the coreceptor triggers further conformational changes in Env that enable gp41-mediated fusion of the viral and cellular membranes. The current model of gp41-mediated fusion is based on studies of HIV-1 crystal structures/imaging (52, 290), fusion inhibitors (138, 161, 293), entry kinetic studies (94, 134, 219), and similarities between gp41 and other well-characterized type I membrane fusion proteins including the influenza virus glycoprotein, hemagglutinin (HA) (52, 290). In this model, following the formation of Env-receptor-coreceptor complex, the transmembrane gp41 fusion peptide inserts into the host membrane. The fusion peptide is an N-terminal hydrophobic portion of gp41 primarily involved in anchoring the viral and cell membranes. The fusion peptide of each gp41 in the trimer refolds into a thermodynamically stable structure termed the 6-helix bundle (6HB). The 6HB is formed when an N-terminal helical region (HR1) and a C-terminal helical region (HR2) from each gp41 subunit come together (52, 290). The energy used to form the 6HB juxtaposes the viral and host membranes, driving the formation of a fusion pore (77, 174). Although the mechanisms of fusion for retroviruses have been extensively studied, the location—plasma membrane or endosome—of the fusion pore formation remains controversial (280). Reports showing that the majority of retroviruses do not require low pH to activate the fusion process (62) suggest that HIV-1 likely fuses with the target cell at the plasma membrane, though it is

important to note that pH-independence does not in an of itself imply spatial information. However, multiple lines of evidence suggest that endocytic entry of HIV-1 can lead to infection (177), and may be a preferred route of entry into certain cell types (175). Further studies are needed to elucidate the relevance of endocytic uptake of HIV-1 *in vivo*.

INHIBITING HIV-1 ENTRY

Targeting CD4

Each interaction in the multistep HIV-1 entry pathway can be targeted for drug development. Efforts to block HIV-1 at this stage of infection have led to a class of interventions termed HIV-1 entry inhibitors. Initial attempts to develop specific inhibitors of HIV-1 entry focused on blocking viral binding to the cell. These attempts led to the generation of recombinant soluble CD4 (sCD4) molecules, which lack the transmembrane and cytoplasmic domains of CD4, but retain the two outer-most domains (D1 and D2). These constructs maintain the ability to bind gp120, and thus can act as molecular decoys during infection. Although sCD4 showed promising *in vitro* activity against laboratory-adapted strains of HIV-1 (90, 119, 258, 273), activity in clinical trials did not follow suit (69, 246). Additionally, producing large enough quantities of the protein for clinical use may be challenging. Furthermore, studies demonstrated that HIV-1 primary isolates are less sensitive to neutralization by sCD4 than lab-adapted strains (69, 246, 277). Subsequent studies with PRO 542, a tetravalent CD4-Ig fusion protein comprising D1 and D2 of human CD4 fused to the heavy and light chain constant regions of human IgG2,κ, yielded more encouraging results (7, 120). Moderate

reductions in plasma HIV-1 RNA levels were observed in a phase I trial of PRO 542 in patients with advanced HIV-1 disease. However, disadvantages of this therapeutic platform are that the drug would have to be delivered intravenously, would require a cold chain (which is not practical for treating individuals in resource-limiting settings), and would be expensive to develop.

Conserved structures/domains in gp120, such as the CD4-binding site and coreceptor-binding domain, serve as attractive targets for antibodies or small-molecule inhibitors. Unsurprisingly, small molecule inhibitors that block the gp120-CD4 interaction show greater promise. One such inhibitor (BMS-378806) (159, 285) exhibits potent antiviral activity *in vitro* against HIV-1 subtype B (independent of coreceptor tropism), but is inactive against HIV-2 and SIV (159). BMS-378806 binds within the CD4 binding pocket of gp120, preventing engagement with cellular CD4 receptors. The antiviral activity *in vivo* is demonstrated by a phase I study with the related compound, BMS-488043, which resulted in 1-$\log_{10}$-reduction in plasma HIV-1 RNA in study subjects (107). However, relatively high doses were required (1,800 mg), driving the virus in 17% of subjects to easily acquire resistance. Moreover, baseline resistance to BMS-488043 was detected in another 17% of subjects (306). The compound is no longer in clinical development.

Small molecules targeting CCR5

Multiple methods have been employed to generate inhibitors targeting the gp120-CCR5 interaction. Targeting CCR5 has been an attractive therapeutic angle since the discovery of a naturally occurring mutation in *ccr5* (*ccr5Δ32*) that

truncates CCR5, preventing cell surface expression of the chemokine receptor. Individuals homozygous for this mutation are less susceptible to infection by R5-tropic HIV-1, although they remain sensitive to infection by X4-tropic viruses (117, 160, 244), but still these individuals are rarely infected (27, 189, 268). Drug development efforts to block CCR5 have led to the generation of small molecule antagonists, monoclonal antibodies (mAbs), and analogues of natural CCR5 ligands. The small molecule CCR5 antagonists have been named with the suffix "-viroc", denoting "viral receptor occupancy". These molecules possess no agonist properties and do not impact surface expression of CCR5, yet exhibit potent inhibition of HIV-1 replication *in vitro* against lab-adapted and primary isolates across all subtypes of HIV-1. Three of these inhibitors—aplaviroc (APL), maraviroc (MVC), and vicriviroc (VVC)—have advanced to at least phase II clinical trials and will be discussed here.

Aplaviroc (formerly, compound 873140) is an allosteric noncompetitive inhibitor of CCR5, exhibiting potent antiviral activity during short-term monotherapy studies. Administering 600 mg APL twice daily produced up to a 1.6-$\log_{10}$-reduction in plasma HIV-1 RNA levels during 10 days of treatment (147). Mechanistic studies showed that nanomolar concentrations of APL block the activity of the CCR5 ligand, MIP-1α, but not signaling by another CCR5 ligand—RANTES (288). Although initially promising as an antiviral agent, follow-up studies reported APL-associated hepatotoxicity (drug-induced hepatitis) in 5 subjects during phase IIb and III clinical trials (188). While no fatalities resulted

from the hepatotoxicity, and pathology resolved with drug discontinuance, APL development is not being pursued further due to this side effect.

Maraviroc (formerly, UK-427,857) is also a noncompetitive allosteric inhibitor of CCR5, with potent antiviral activity both *in vitro* and *in vivo*. Like APL, MVC does not induce signaling and calcium mobilization upon binding to CCR5, nor does it impact CCR5 surface expression. However, unlike APL, MVC is a functional CCR5 antagonist that blocks binding of, and signaling by, CCR5 ligands including MIP-1α and RANTES at nanomolar concentrations (80). In a 10-day monotherapy phase IIa clinical trial conducted in 63 subjects infected with R5-tropic HIV-1, 600 mg of MVC daily resulted in ≥1.6 log_{10}-reduction of plasma HIV-1 (86).

The *in vivo* efficacy of MVC was confirmed in two double-blind randomized, placebo-controlled phase III clinical trials—MVC versus Optimized Therapy in Viremic Antiretroviral Treatment-Experienced Patients (MOTIVATE 1 and 2) (85, 103). MOTIVATE 1 was conducted in the United States and Canada, and MOTIVATE 2 was conducted in Australia, Europe, and the United States. All 1049 study participants had been taking one or more drugs from at least three antiretroviral classes and showed evidence of resistance to drugs from these antiretroviral classes, had plasma HIV-1 RNA levels >5,000 copies/mL, median CD4 cells count of 169cells/mm^3, and were infected exclusively with R5-tropic virus as assessed by the Trofile assay (164). Participants were randomized to receive one of two dosing regimens of MVC (300 mg given once or twice daily) or placebo. At 48 weeks, in both studies, the change in HIV-1 RNA from baseline

was greater with MVC than with placebo; -1.66 and -1.82 log_{10} copies/mL with the once-daily and twice-daily regimens, respectively, versus -0.80 with placebo in MOTIVATE 1, and -1.72 and -1.87 log_{10} copies/mL, respectively, versus -0.76 with placebo in MOTIVATE 2. Additionally, in both studies more than twice as many MVC recipients had plasma HIV-1 RNA levels below 50 copies/mL compared to placebo recipients (42-47% in both MVC groups versus 16% in the placebo group in MOTIVATE 1; 45% versus 18% in MOTIVATE 2). Furthermore, increases in CD4 cell counts ranged from 110-130 cells/mm^3 in the MVC arms as compared to 50-70 cells/mm^3 in the placebo arms.

Repeated Trofile testing showed that when virologic failure occurred, it was associated with emergence of X4-tropic virus in 57% of subjects (85). Although all subjects had R5-tropic virus at the failure screening time point, 8% were found to have dual (X4/R5)-tropic or mixed (X4-tropic and R5-tropic) (D/M) virus at baseline (day 0). These variants were undetected before MVC treatment because they were present at levels below the limit of detection of the assay. Subjects infected with D/M-tropic virus at baseline had a lower rate of virologic response, shorter time to virologic failure, and smaller CD4 increases as compared to those exclusively infected with R5-tropic virus. Owing to the results of the MOTIVATE trials, the makers of MVC received approval by the FDA for treatment-experienced patients with poor control of multidrug resistant, R5-tropic HIV-1.

While the approval of MVC targets the treatment of patients with R5-tropic virus, its potential in patients with D/M-tropic virus has also been evaluated (230).

A total of 186 study participants infected with D/M-tropic virus, with median CD4 cell counts of 42 cells/mm^3 and HIV-1 viral load of >5000 copies/mL within each cohort (300 mg MVC once-daily, twice-daily, or placebo), were enrolled in a randomized double-blind, placebo-controlled phase IIb clinical trial A4001029. This study found no significant virologic benefit of MVC as compared to placebo over 24 weeks. These results and those of the MOTIVATE trials suggest that although the presence or outgrowth of X4-tropic virus dampened the response to MVC, it was not associated with rapid CD4 decline or disease progression.

Vicriviroc, (formerly, SCH417690 or SCH-D) is a third class of allosteric noncompetitive CCR5 inhibitor. VVC is an orally bioavailable small molecule CCR5 antagonist that, like MVC, blocks signaling by the C-C chemokines at nanomolar concentrations (265). Early *in vivo* studies demonstrated that VVC produced seizures in animals at high doses, but no VVC-associated adverse effects in the CNS have been reported in human clinical trials. A 14-day monotherapy trial demonstrated a reduction of plasma HIV-1 RNA by approximately 1.0-1.5 $\log_{10}$ copies/mL (248). However, a phase IIb study of VVC (plus dual nucleoside reverse-transcriptase inhibitors—NRTIs) in treatment-naïve HIV-1-infected subjects was halted due to increased rates of virologic failure in the VVC arms compared to the control dual NRTI arm (149). Despite these setbacks, a placebo-controlled phase IIb study conducted in antiretroviral-experienced participants demonstrated potent suppression of HIV-1 by VVC (administered at 5, 10 or 15 mg daily) in combination with a protease inhibitor (297). Mean changes in plasma HIV-1 RNA levels at 24 weeks ranged from 1.5-

1.9 $\log_{10}$ copies/mL in the VVC-treated participants as compared to 0.29 $\log_{10}$ copies/mL in the placebo group. The same trend was observed 48 weeks post treatment. However, development of VVC was not further pursued due to lack of convincing efficacy gains when it was added to background therapy comprising two antiretroviral drugs in two randomized phase III clinical trials (48).

Targeting CXCR4

As with CCR5, the development of small molecule inhibitors of CXCR4 has been an attractive therapeutic option (262). Initial animal toxicity studies with two small molecule inhibitors (AMD3100 and AMD11070) (78) provided enough evidence of safety to proceed with human studies (110). Preliminary human studies with AMD3100 showed selective blockade of CXCR4 and inhibition of X4-tropic HIV-1 and HIV-2, but development of this compound as an anti-HIV-1 drug was discontinued because it lacked bioavailability and caused substantial peripheral leukocytosis—mobilization/release of progenitor hematopoietic stem cells from the bone marrow into the blood (91, 110). This side effect turned out to be a benefit, though not for HIV-1, as AMD3100 (now known as plerixafor) has since been approved for use during autologous transplantation in patients with non-Hodgkin's lymphoma and multiple myeloma (43, 75). Phase I and II studies with another CXCR4 inhibitor AMD11070 showed a reduction in the X4-tropic HIV-1 population in participants infected with D/M virus (180). However, further development of this drug has been halted due to abnormal liver histology in long-term animal studies. The long-term safety of targeting CXCR4 remains in question.

Targeting fusion

Membrane fusion mediated by HIV-1 gp41 is yet another viable drug target in the HIV-1 entry pathway. Because the 6HB structure is critical for membrane fusion, peptide inhibitors mimicking the HR1 or HR2 region of the gp41 subunit have been designed to prevent membrane fusion (122). One such peptide, enfuvirtide (T20), is the only FDA-approved HIV-1 fusion inhibitor (147, 151). T20 is a 36-mer synthetic peptide whose sequence matches that of the HR2 region of gp41. Interaction of T20 with HR1 prevents the association of HR1 with HR2, thus inhibiting fusion and blocking virus entry (294). The drug has minimal toxicity, however because it is a peptide, it must be administered by subcutaneous injection. T20 co-administered with other drugs (darunavir, tirapanavir, and maraviroc) in clinical trials lead to significant improvements in response rates to those compounds (58, 112). Unfortunately, as with most entry inhibitors, viral resistance to T20 emerges rapidly; predominantly in regimens where viral inhibition is incomplete or due to naturally occurring polymorphisms in the gp41 region of genetically diverse forms of HIV-1 (47, 208). This underscores the need for combination therapy in the management of HIV-1 infection (124, 282).

THE VIRAL BOTTLENECK DURING HIV-1 SEXUAL TRANSMISSION

The majority of new HIV-1 infections occur in the developing world, with women being disproportionally affected compared to men. Of these new infections, approximately 80% result from heterosexual intercourse (65, 228,

298). Sexual transmission of HIV-1 is an inefficient process, with the incidence of transmission between discordant couples being estimated to be between 0.1%-10% transmission rate per coital act (33, 101, 196, 212). Sexual transmission of HIV-1 is characterized by a genetic bottleneck in which one or few viral variants from the diverse viral swarm in the donor cross the mucosal epithelium in the recipient and encounter susceptible target cells to seed the infection. This theory of the genetic bottleneck was initially described over 20 years ago, when researchers studying donor-recipient pairs discovered that viral sequences isolated from recipients were usually homogenous and macrophage-tropic, while viral populations of the donors showed heterogeneity both in sequence and cell tropism (299, 300, 304, 307). Other studies suggested that multiple viral variants may initially cross the physical mucosal barrier, however most are extinguished due to intrinsic differences in reproductive rates (131, 152, 154). As a result, successful transmission generally results from the expansion and propagation of a single genetic variant, termed the transmitted/founder (T/F) virus (Fig 3) (131). T/F viruses may have specific properties that are selected for during transmission, and identifying such traits may inform and facilitate the development of pre- and post-exposure therapies targeting these early viruses.

KNOWN PROPERTIES OF TRANSMITTED HIV-1

The first report of genotypic differences between viruses isolated from donor-recipient pairs came from studies of vertical transmission of three mother-infant pairs (300). Infant viral sequences of the V3-V5 region of Env were less diverse than those from their respective mother. Additionally, a potential N-linked

glycosylation site (PNLG) within V3 was conserved in each mother's sequences, but lost in her corresponding infant's viral sequences. Furthermore, fewer PNLGs have been reported in vertically transmitted subtype A and C Envs isolated from mother-infant pairs (229, 301, 303). These data suggest that viruses with fully glycosylated Envs are perhaps selected against as they traverse the placenta into the infant host, or may acquire a fitness cost that hampers their replication in the new host (303).

While these early studies in mother-to-child transmission (MTCT) pairs were invaluable as MTCT accounted for approximately 25% of global transmission during that time (1991) (35), by 1994 MTCT rates had began to dramatically drop (to as low as 4%) owing to the development of highly active therapy preventing vertical transmission (63, 207). This highlighted the importance of studying transmission in pairs that characterized the majority of the pandemic—sexually transmitted pairs. As observed in MTCT pairs, analysis of Envs from acutely infected individuals with subtype C infection also revealed shorter variable loops with fewer PNLGs compared to those from their transmitting partners (73). Other studies reported shorter V1 and V2 regions and fewer PNLGs in acutely infected individuals with subtype A and B virus (56, 235). Moreover, an assessment of thousands of Env sequences to discern patterns in amino acid signatures that are significantly associated either with transmission or with the chronic phase of the infection revealed two statistically associated signatures of acute viruses; the first was located at position 12 in the signal peptide, and the second was a loss of a PNLG in the cytoplasmic tail of gp41

(96). These genotypic signatures are likely not randomly transmitted as work by multiple groups has shown that T/F viruses arise from a minor population of the donor's plasma virus (32, 93, 300). Furthermore, studies have shown that these transmitted minor variants are genetically similar to sequences isolated from earlier time points in the donor, not those circulating at the time of transmission (218), suggesting that the donor's plasma quasispecies at the time of transmission may not accurately predict the T/F virus. These data also support the theory that certain genotypic (or phenotypic) properties are particularly favored during transmission, and are selected even when they account for the minority of sequences present in the donor.

Given the genotypic evidence of the transmission bottleneck, many groups have sought to identify the viral traits/phenotypes that account for transmission fitness by comparing HIV-1 envelope glycoprotein (*env)* sequences from chronically infected individuals with those isolated from their newly-infected partners. Multiples studies reported that viruses that are transmitted between individuals preferentially use CCR5 to mediate entry into target cells (51, 121, 131, 199, 200, 239, 295). This provides some insight into why, as mentioned above, individuals who are homozygous null for *ccr5* rarely become infected, even though they remain susceptible to infection by X4-tropic viruses (27, 189, 268). Additional studies showed that one or few viruses with compact, glycan-restricted Envs that were sensitive to donor neutralizing antibodies, typically established new infections (56, 68, 73, 96, 157, 162). While other groups have not been able to confirm the neutralization sensitivity of T/F Envs (31, 92, 229),

these initial studies served to stimulate research interest in this area. Recent work from Beatrice Hahn and George Shaw has attempted to more precisely identify targetable traits of transmitted viruses using single-genome amplification with replication competent subtype B and C viruses (199). They found that T/F viruses were marginally more infectious and contained approximately twice more Env per virion compared to chronic viruses. T/F viruses were more readily transferred to $CD4^+$T cells by dendritic cells, but replicated with similar kinetics in primary cells as chronic viruses. Lastly, T/F were more resistant to IFN-α treatment compared to chronic viruses.

While the field has made great progress in elucidating properties that are associated with transmitted HIV-1, so far the only consistent phenotype associated with transmitted viruses is the use of CCR5. Approximately one-half of transmissions are thought to occur when the donor is still in the acute stage of infection (209, 289), therefore this property may have easily been missed had the studies discussed above not assessed differences between T/F viruses and viruses isolated from chronically-infected individuals, where X4-tropism is more prevalent (28, 202, 222, 247, 261). This underscores the importance of comparing T/F viruses to chronic viruses to increase the likelihood of detecting subtle differences that significantly impact transmission that may otherwise be overlooked. The genotypic and phenotypic properties outlined here likely act in concert to evade host immune pressure and foster viral diversification, and may serve as attractive therapeutic targets for the development of AIDS interventions.

PROJECT GOAL I: IDENTIFYING TARGETABLE PROPERTIES OF TRANSMITTED VIRUSES

T/F viruses may have specific properties that are selected for during transmission, and identifying such traits may inform and facilitate the development of pre- and post-exposure therapies targeting these early viruses. To date, the most consistent property associated with transmitted viruses is the use of the CCR5 coreceptor. CCR5 is a GPCR that functions as a chemokine receptor to regulate trafficking and the effector response of leukocytes. Ligand (MIP1-α, MIP1-β, and RANTES)-binding induces a conformational change of the protein and activates trimeric G-proteins, which leads to subsequent signaling events that mediate CCR5 effector function. Thus, like other GPCRs, CCR5 is conformationally plastic. We, and others, have used conformation-dependent monoclonal antibodies to confirm antigenically distinct forms of CCR5 on primary cells and cell lines (25, 153). The impact of this conformational heterogeneity on infection varies by virus strain. We know that viruses differ in their ability to interact with CCR5, with some viruses being heavily reliant on the amino terminus (NT) and others on the second extracellular loop (ECL2) for entry (3, 272). Additionally, some viruses are naturally capable of utilizing structurally altered antagonist-bound CCR5 for entry (108, 271).

Although we know that a spectrum of CCR5 engagement by various HIV-1 exists, the implications of these interactions are unknown. We were interested in understanding how various viruses interact with CCR5—particularly, different conformations of CCR5. To this end, I evaluated a panel of 87 R5-tropic viruses

(from MVC treatment-naïve patients) to address differences between T/F and chronic control (CC) HIV-1 in their ability to mediate entry via varying amounts of CCR5 in the presence of MVC. Our lab had previously reported no statistical difference in MVC sensitivity between subtypes B and C T/F and CC viruses on a microglial cell line—NP2/CD4/CCR5 (200, 295).

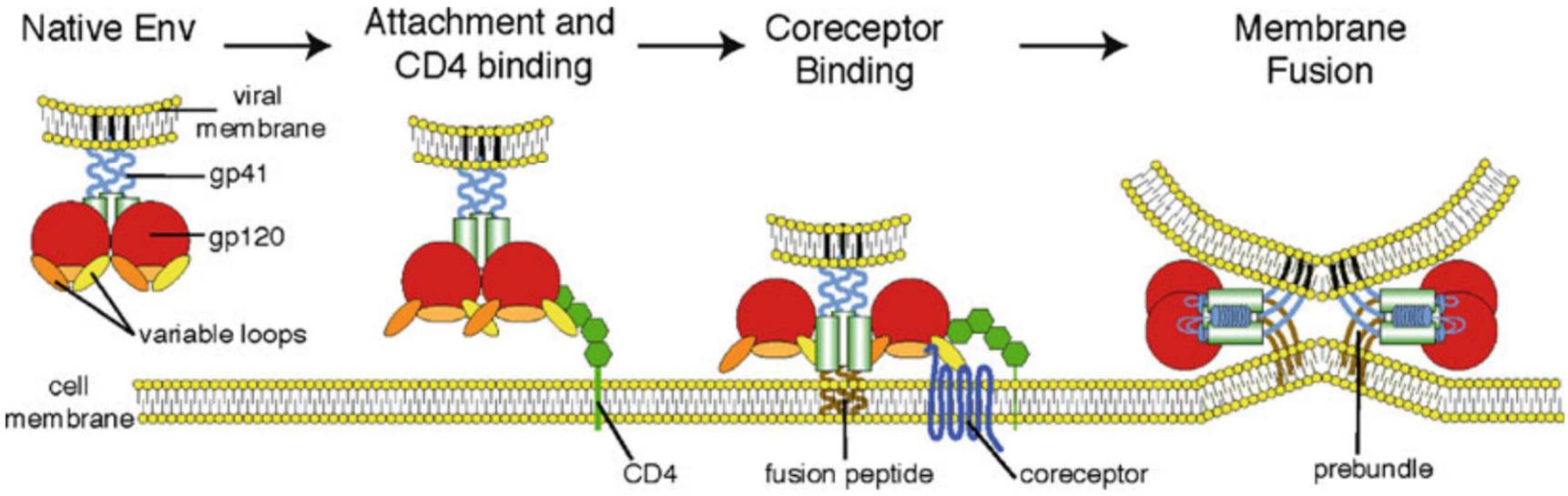
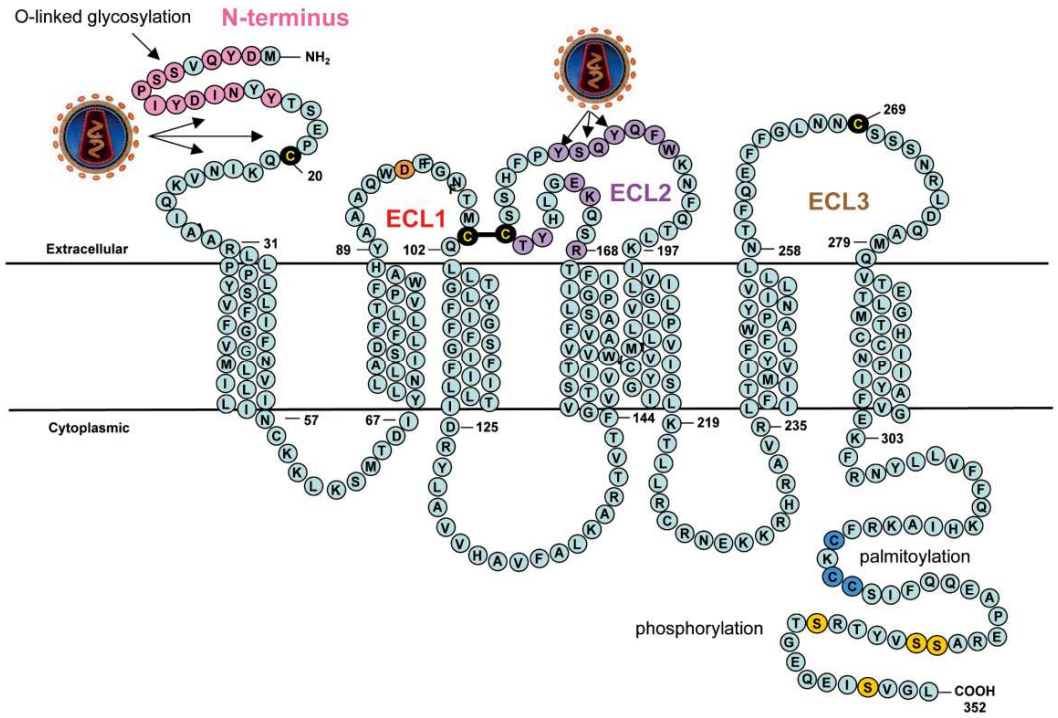


Figure 1: HIV-1 entry process. HIV-1 entry can be divided into three key steps, all mediated by the viral envelop glycoprotein (Env) gp120. First, nonspecific, low-affinity interactions bring the virion into close proximity to the cell surface, allowing gp120 to bind to the receptor CD4. Second, CD4 binding induces a conformational change in Env exposing the coreceptor binding site and facilitating coreceptor (typically, CCR5 or CXCR4) engagement. Coreceptor binding induces another structural change in Env, triggering the formation of the fusion pore and membrane fusion of the viral and host membranes. (Image from Wilen et al. *Adv Exp Med Biol.* 2012; 726: 223-42)

Figure 2: Schematic of CCR5 coreceptor. CCR5 is a seven-transmembrane G-protein-coupled receptor (GPCR) that functions as a



chemokine receptor, and serves as a coreceptor for HIV-1 infection. HIV-1 Env binds the 352 amino acid protein at the amino terminus (N-terminus) and/or the second extracellular loop (ECL2) at the pink and purple colored residues, respectively. Sites of posttranslational modifications are also shown and contribute to changes in CCR5 3D structure. Conformational plasticity is a key feature of GPCRs such as CCR5. (Image adapted from Lopalco et al. *J Transl Med* 2010 **9** (Suppl 1):S4)

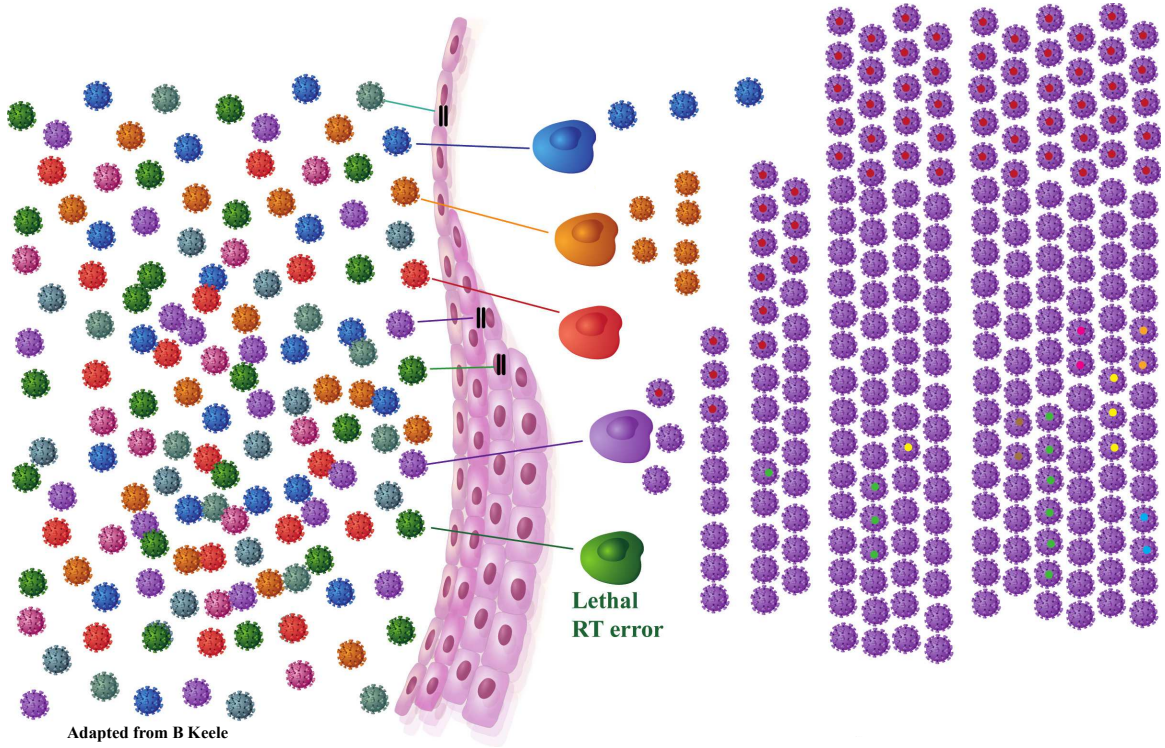


Figure 3: HIV-1 genetic bottleneck. Sexual transmission of HIV-1 is characterized by a genetic bottleneck in which a diverse viral swarm in the donor, represented by the differentially-colored virions on the left, must initially cross the mucosal epithelium in a new host and encounter target cells to infect. Multiple variants may cross this physical barrier, however the majority will die out or be outcompeted. As a result, approximately 80% of the time, successful transmission is carried out by a single genetic variant, termed the transmitted/founder (T/F) virus. (Image adapted from Brandon Keele)

CHAPTER 2

Transmitted/Founder and Chronic HIV-1 Envelope Proteins are Distinguished by Differential Utilization of CCR5

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ABSTRACT

Infection by HIV-1 most often results from the successful transmission and propagation of a single virus variant, termed the transmitted/founder (T/F) virus. Here, we compared the attachment and entry properties of envelope (Env) glycoproteins from T/F and Chronic Control (CC) viruses. Using a panel of 40 T/F and 47 CC Envs, all derived by single genome amplification, we found that 52% of clade B and C CC Envs exhibited partial resistance to the CCR5 antagonist maraviroc (MVC) on cells expressing high levels of CCR5, while only 15% of T/F Envs exhibited this same property. Moreover, subtle differences in the magnitude with which MVC inhibited infection on cells expressing low levels of CCR5, including primary CD4+ T cells, was highly predictive of MVC-resistance when CCR5 expression levels were high. These results are consistent with previous observations showing a greater sensitivity of T/F Envs to MVC inhibition on cells expressing very high levels of CCR5 and indicate that CC Envs are often capable of recognizing MVC-bound CCR5, albeit inefficiently on cells expressing physiologic levels of CCR5. When CCR5 expression levels are high, this phenotype becomes readily detectable. The utilization of drug-bound CCR5 conformations by many CC Envs was seen with other CCR5 antagonists, with replication competent viruses, and did not obviously correlate with other phenotypic traits. The striking ability of clade B and C CC Envs to use MVC-bound CCR5 relative to T/F Envs argues that the more promiscuous use of CCR5 by these Env proteins is selected against at the level of virus transmission and is selected for during chronic infection.

INTRODUCTION

Physical and innate immune barriers serve to make mucosal transmission of HIV-1 a relatively inefficient process. As a result, establishment of virus infection in a naïve host most often results from the transmission and subsequent propagation of a single virus strain, termed a transmitted/founder (T/F) virus (19, 73, 131, 158, 198, 210, 299, 300, 304, 307). Virologic traits that might enable a virus to overcome one or more of these barriers could be selected for during transmission, and identification of such traits should lead to a greater understanding of the earliest events in HIV-1 infection and could suggest new prevention strategies.

Single genome amplification has enabled the inference of a large number of T/F *envs* from multiple virus clades (1, 105, 131, 190, 238, 239). This has made it possible to seek genotypic and phenotypic differences between T/F Env proteins and those derived from chronically infected individuals (CC Envs). Several phenotypic characteristics are clearly associated with transmission: T/F Envs virtually always use CCR5 rather than CXCR4 or other non-canonical coreceptors (24, 51, 121, 131) and generally infect T cells but not macrophages (131, 190, 239, 295) as a result of requiring relatively high levels of CD4 to mediate virus entry (98, 171, 182, 203, 204, 242). Other phenotypic and genotypic traits that have been linked to transmission are less well defined: Envs isolated from acute infection have sometimes been reported to be more neutralization sensitive (295) have on average fewer putative N-linked glycosylation sites (73, 96), and have shorter variable loops (56, 73, 234, 235)

compared to Envs isolated from chronically infected individuals.

Recently, Swanstrom and colleagues reported an additional phenotypic difference between clade C T/F and chronic Env proteins (127). They found that CC Envs were more likely than T/F Envs to exhibit partial resistance to the CCR5 antagonist maraviroc (MVC) on 293T cells expressing high levels of CCR5, but not on 293T cells expressing lower levels of CCR5. This finding suggests that CC Envs are often capable of infecting cells by using the MVC-bound conformation of CCR5 or that they are able to utilize a form of CCR5 that is unable to bind MVC. However, work by us and others has not revealed differences in MVC sensitivity between T/F and CC Envs on an NP2 cell line (200, 219, 272, 295).

To reconcile these findings, we examined a previously described panel of clade B and clade C Env proteins from T/F and chronic viruses (200, 295) for their ability to infect cell lines and primary human CD4 + T cells in the presence of saturating concentrations of MVC. On 293T cells expressing high levels of CCR5, we confirmed that clade C CC Envs were much more likely than T/F Envs to mediate infection in the presence of MVC as shown by Swanstrom and colleagues with a different panel of clade C Env proteins (127). We found this property to be shared by clade B Envs and extended these observations further to include cell lines expressing 5- to 10-fold lower levels of CCR5 and on primary human CD4 + T cells. Subtle differences in the efficiency with which MVC inhibited infection of 87 different T/F and chronic viruses on cells expressing low levels of CCR5 was highly predictive of more overt MVC-resistance when CCR5 expression levels were high. This property was also seen with other CCR5

antagonists, with replication competent infectious molecular clones (IMCs), and did not correlate with other phenotypic properties. Since the efficiency of virus entry in the presence of MVC increased with CCR5 expression levels, we conclude that many CC Envs can utilize at least some of the drug-bound conformations of CCR5, albeit inefficiently. Nonetheless, the differential ability of clade B and C chronic and T/F Envs to use MVC-bound CCR5 argues that the more promiscuous use of CCR5 conformations by many chronic viral Env proteins is selected against at the level of virus transmission by a mechanism(s) that has not yet been elucidated by *in vitro* assays, but is selected for during chronic infection. Differences in the populations of CD4+CCR5+ cells that are targeted by HIV in acute versus chronic infection may reveal differences in how T/F and CC Envs mediate virus infection and could suggest new prevention strategies.

MATERIALS AND METHODS

Description of Envs and infectious molecular clones (IMCs). The derivations of all the Env clones and IMCs used in this study have been described previously (131, 200, 239, 295). Briefly, the sequences of all Envs and IMCs were inferred from single genome sequencing of acute or chronically infected donors. The Envs or IMCs were then amplified from the first-round PCR product or gene synthesized and then sub-cloned into pCDNA3.1+ directional TOPO or pCRXL TOPO vector (Invitrogen). Clade B CC Envs were randomly selected amongst sequences from the chronic swarm that were predicted to be functional, while clade C CC Envs were generated by determining the most-recent common ancestor of discrete clonally-expanded populations, evident as minor populations in phylogenetic trees of chronic sequences (200). A summary of the geographic origin and infection status for Envs and IMCs is shown in Table S1.

Cell culture. 293T17, 293T-derived Affinofile, NP2/CD4/CCR5, NP2/CD4/CXCR4, and U87/CD4 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin/streptomycin. Affinofile cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin per ml, 50 μg/ml blasticidin per ml, and 200 μg/ml G418 per ml.

Pseudovirus production and normalization. HIV-1 Env pseudoviruses were produced by calcium phosphate co-transfection of 6 μg of pcDNA3.1$^+$ containing the desired *env* clone with 10 μg of HIV-1 core (pNL43-**Δ**Env-vpr$^+$-luc$^+$

or pNL43-**Δ**Env-vpr$^+$-eGFP) into 293T17 cells. At 72 h post transfection, the pseudovirus-containing supernatant was harvested and filtered through a 0.45-μm filter, aliquoted, and stored at -80°C. For primary CD4$^+$ T cell infections, pseudovirus was concentrated approximately 100-fold by ultracentrifugation at 113,000 x g for 2 h at 4°C through a 20% sucrose cushion. Pelleted pseudovirus was then resuspended in phosphate-buffered saline (PBS). All luciferase-encoding pseudoviral stocks were serially diluted on NP2/CD4/CCR5 cells to define the linear range of the assay. Relative light units (RLUs) of all viruses used were well within the 5-log linear range of the assay.

Virus inhibition assays. The HEK293T-based CD4/CCR5 dual-inducible cell line (Affinofile) was employed to assess sensitivity of pseudovirions and replication competent viruses to CCR5 antagonists at different levels of CCR5 surface expressions. CD4 expression was induced with minocycline and CCR5 expression was induced with ponasterone A (pon A) as described previously(126). Briefly, 650 cells were plated in each well of a 96-well plate and then 48 h later they were induced with a final concentration of 10 ng/ml minocycline to maximally express CD4 and either 2 μM or 0.031 μM pon A to express high and low CCR5, respectively. 18 h post-induction, CD4 and CCR5 expression levels were determined by quantitative flow cytometry (BD QuantiBrite) as described below. Induced Affinofile cells, NP2/CD4/CCR5 or U87/CD4 cells transiently transfected with pcDNA3.1$^+$ encoding CCR5 using Lipofectamine 2000 (Invitrogen) were used for CCR5 antagonist inhibition studies. Cells were preincubated for 30min at 37°C with either 2μM of drug,

previously shown to be saturating (57), or three-fold serial dilutions from 6μM to 0.1μM of the CCR5 antagonists maraviroc (MVC), aplaviroc (APL), CMPD-167, TAK-779, vicriviroc (VVC), or media alone prior to infection with the indicated luciferase reporter pseudovirus. After addition of pseudovirus, plates were spinoculated at 450 x g for 2 h at 4°C and then incubated at 37°C. We also performed experiments without spinoculation and obtained similar results: spinoculation increased infection by approximately 2-fold, but did not impact the sensitivity of viruses to maraviroc. Cells were lysed with Brite-Glo (Promega) at 72 h post-infection and relative light units (RLUs) assessed on a Luminoskan Ascent luminometer. All inhibition assays were done in triplicate in each of at least two independent experiments using a MVC-sensitive and MVC-partially resistant R5-tropic Env as controls.

Primary human CD4$^+$ T cell infections. Primary human CD4$^+$ T cells, purified by negative selection, were obtained from the Human Immunology Core of the University of Pennsylvania's Center for AIDS Research (CFAR). Cells were infected as described previously (295). Briefly, 1.5 x 10^6 cells per condition were stimulated with plate-bound anti-CD3 (clone OKT3; eBiosciences) and anti-CD28 (clone 28.2; BD Biosciences) and 20 U/ml recombinant interleukin-2 (IL-2; Aldesleukin) in Rosewell Park Memorial Institude (RPMI) medium containing 10% FBS. Three days post-stimulation cells were transferred to 96-well V-bottom plates and incubated for 30 min with 6μM MVC or a no drug control. Approximately 5μg p24 of concentrated HIV-green fluorescent protein (GFP) pseudovirus was used to infect cells in triplicate in a total volume of 50μl at a cell

concentration of 1 x 10^7 cells/ml. Plates were then spinoculated at 1,200 x g for 2 h at 4°C. Cells were resuspended at 1 x 10^6 cells/ml. Three days post-infection, cells were stained with live/dead aqua (1µl) (Invitrogen), anti-CD4 Alexa Fluor 700 (2µl) (Invitrogen), and anti-CD3 Qdot 655 (0.5µl) (Invitrogen) and examined by flow cytometry and the fraction of cells that were GFP-positive determined. HIV-infected cells were defined as live/dead CD3+CD4-GFP+ as cell surface CD4 is down-regulated upon infection.

Flow cytometry. Cell staining was perferomed at room temperature in 50µl FACS wash buffer (PBS, 2.5% FBS, 2 mM EDTA). For $CD4^+$ T cells, cells were first washed in PBS, then live/ dead aqua (Invitrogen) was added and incubated for 10 min. Next, anti-CCR7 IgM (1µl) in FACS buffer was added and incubated for 30 min. Cells were then washed in FACS buffer before staining with anti-CD4–Qdot 655 (0.5µl) (Invitrogen), anti-CD45RO–phycoerythrin (PE)-Texas Red (3µl) (Beckman Coulter), and anti-CCR5 (2D7)–PE (5µl) (BD Biosciences) for 30 min. Cells were then washed in FACS buffer and resuspended in 1% paraformaldehyde (PFA). Samples were run on an LSRII (BD) instrument and analyzed with FlowJo 10.0 (Treestar). Cells were gated as follows: singlets (FSC-A by FSC-H), then live cells (SSC-A by live/dead), then lymphocytes (SSC-A by FSC-A), then CCR5+ cells (SSC-A by 2D7-PE).

Quantitative FACS. Quantitative FACS was performed to convert mean fluorescence intensity (MFI) into antibody-binding sites (ABS) by using a standardized microbeads kit (BD Biosciences) according to the manufacturer's

instructions. The MFI of the isotype control for each experiment was converted to ABS and subtracted from the ABS value obtained with the experimental sample.

Statistical and correlation analyses. Infection values obtained with T/F and chronic Env pseudovirions were compared using Mann-Whitney tests, Fisher's exact, or t-tests, and correlations were assessed using Spearman tests. P-values of less than 0.05 were considered significant. Data were analyzed with Prism 5.0 software.

Ethics statement. All human cells used in this study were from normal healthy donors who provided written informed consent after approval by the University of Pennsylvania's Institutional Review Board.

RESULTS

Partial resistance to MVC on Affinofile cells with high CCR5 surface expression. Swanstrom and colleagues observed that chronic clade C Envs mediate low levels of infection on Affinofile cells expressing high levels of CCR5 in the presence of MVC more frequently than T/F Envs. To assess if an independent panel of 87 clade B and C Envs (200, 295) would recapitulate this finding, we also utilized the 293T-derived Affinofile cell system. Among these pseudoviruses were 24 clade B and 16 clade C T/F Envs, as well as 28 clade B and 19 clade C CC Envs, all derived by single-genome amplification and described in earlier reports (131, 200, 295). The Affinofile cell line makes it possible to independently modulate surface expression of CD4 and CCR5 by applying different concentrations of the transcriptional activators minocycline and pon A, respectively (126).We examined the sensitivity of HIV-1 Env pseudoviruses to MVC on Affinofile cells expressing relatively low (low CCR5 Affinofile cells) or high levels of CCR5 (high CCR5 Affinofile cells), while maximally inducing CD4 levels. PonA treatment increased the overall expression of CCR5 on Affinofile cells from 2,723 (uninduced) to 23,470 (high induction) antibody binding sites (ABS) per cell (Fig. 1A). To confirm that 6μM MVC was saturating at the highest level of CCR5 expression, we assessed the difference in residual infection between the two highest concentrations of MVC employed and found that there was no increase in inhibition of infection from 2μM to 6μM MVC,

indicating that 2μM was saturating and that further increases in drug did not have any additional inhibitory effect (P = 0.70 by paired t-test) (Fig. 1B).

Affinofile cells expressing low or high levels of CCR5 were infected by each of the 87 pseudoviruses in the presence or absence of increasing concentrations (to a maximum of 6μM) of MVC, after which we calculated the maximal percent inhibition (MPI). We imposed an arbitrary MPI cutoff of 95%, where MPI values >95% were considered MVC sensitive, while samples with MPIs less than or equal to 95% were assumed to be partially resistant to MVC. We found that infection by all 87 pseudoviruses was inhibited by >95% at saturating concentrations of MVC on low CCR5 Affinofile and NP2/CD4/CCR5 cells, consistent with our previous results (200, 295). On high CCR5 Affinofile cells, 57 of 87 (66%) of the pseudoviruses were inhibited by MVC by >95% (representative pseudovirus is shown in Fig. 1C, top panel), while 30 of 87 (34%) of the pseudoviruses exhibited partial resistance defined by a MPI ≤ 95% in the presence of 6μM MVC, with some pseudoviruses exhibiting considerable MVC resistance (MPI values as low as 60%) (Fig. 1C, middle panel). JRFL, which is MVC sensitive, was included as a control in all experiments (Fig. 1C, bottom panel). When CCR5 levels were high, the amount of MVC needed to achieve maximal inhibition increased for all viruses as expected. These results confirmed a central finding of Swanstrom and colleagues (127), in that a considerable number of viruses do indeed exhibit a reduced MPI when MVC is used to inhibit infection on high CCR5 Affinofile cells.

Partial MVC resistance is enriched in chronic HIV-1, but is not clade or antagonist specific. There was a striking difference in the frequency with which T/F and CC Envs exhibited partial MVC resistance (15% [6 of 40] versus 52% [24 of 47]; $P < 0.0001$ by Fisher's exact test) (Fig. 2A). This partial resistance was not clade-specific as 50% (14 of 28) of clade B and 53% (10 of 19) of clade C CC Envs exhibited partial resistance to MVC, whereas only 17% (4 of 24) of clade B and 13% (2 of 16) of clade C T/F Envs exhibited this property under conditions of high CCR5 expression (clade B $P = 0.02$, clade C $P = 0.02$ by Fisher's exact test) (Fig. 2B). Not only was the distribution of MVC-sensitive and resistant Envs similar for clade B and C pseudoviruses, but these distributions were similar to those reported by Swanstrom and colleagues with their panel of clade C Envs (127). Thus, partial resistance to MVC on cells expressing high levels of CCR5 is reproducible, and is not clade specific.

To assess if MVC-resistance predicted resistance to other CCR5 antagonists, we evaluated the sensitivity of four partially MVC-resistant pseudoviruses to four additional small molecule CCR5 inhibitors: aplaviroc, CMPD-167, TAK-779, and VVC. Similar to the results with MVC, all four pseudoviruses tested were inhibited by >95% by all CCR5 antagonists on Affinofile cells expressing low levels of the coreceptor. However, at high CCR5 surface expression, all four pseudoviruses were partially resistant to the other CCR5 antagonists, similar to the results obtained with MVC (representative results shown in Figure 2C). Furthermore, the residual infection seen at high levels of CCR5 on Affinofile cells in the presence of CCR5 antagonists was not

due to the use of low endogenous levels of CXCR4 present on 293T cells, as the addition of the CXCR4 inhibitor AMD3100 did not affect the MPI of partially resistant pseudoviruses (data not shown). Finally, we tested four IMCs whose Env proteins had been tested as part of the pseudovirus panel, including two T/F IMCs that were sensitive to MVC on high CCR5 Affinofile cells and two chronic IMCs that exhibited partial MVC resistance. The results with the replication competent viruses mirrored the results obtained with the respective pseudoviruses (data not shown), indicating that the partial resistance phenotype observed under conditions of high CCR5 is not an artifact of the pseudotyping system. Taken together, these results suggest that clade B and C CC Envs exhibit partial resistance to CCR5 antagonists at high levels of CCR5 much more frequently than T/F Envs.

MVC sensitivity on cells expressing low CCR5 levels predicts MVC resistance on cells expressing high levels of CCR5. We previously reported that T/F and chronic Env pseudovirus infection of NP2/CD4/CCR5 cells expressing CD4 and CCR5 were equally sensitive to MVC, exhibiting similar IC_{50} values. In addition, all T/F and CC Envs were inhibited by saturating concentrations of MVC by >95% (200, 295). However, dose-response curves with different slopes can appear similar on traditional semi-log plots, masking differences in maximal inhibition (243, 252). Therefore, we performed new infection assays using the entire panel of viruses on NP2/CD4/CCR5 cells, which stably express intermediate levels of CCR5 (Fig. 1A), and compared the results to those obtained on low CCR5 and high CCR5 Affinofile cells by plotting the

residual infection for each virus on a log-scale (Fig. 3). While MVC inhibited all pseudoviruses by >95% on both low CCR5 Affinofile and NP2/CD4/CCR5 cells, there was considerable variability in residual infection values for different T/F and chronic Env pseudotypes. On NP2/CD4/CCR5 cells there was a significant difference in median residual infection between T/F and CC Envs (T/F median=0.06%, chronic median=0.15%; P = 0.01 by Mann-Whitney test). The magnitude of this difference was more pronounced on high CCR5 Affinofile cells (T/F median=0.54%, chronic median=4.8%; P = 0.0003 by Mann-Whitney test) but was not apparent on low CCR5 Affinofile cells (T/F median=0.18%, chronic median=0.14%; P = 0.94 by Mann-Whitney test). When we re-examined the NP2/CD4/CCR5 infection data from our previous studies (200, 295), the same correlations were observed.

Are the low and variable levels of infection on low CCR5 Affinofile and NP2/CD4/CCR5 cells in the presence of MVC predictive of more efficient infection in the presence of MVC when CCR5 levels are high? To determine this, we plotted residual infection on low CCR5 Affinofile (Fig. 4A) or NP2/CD4/CCR5 cells (Fig. 4B) in the presence of saturating MVC against that of each virus on high CCR5 Affinofile cells in the presence of saturating MVC. We found a moderate correlation between these values when comparing low CCR5 Affinofile and high CCR5 Affinofile cells (Spearman correlation coefficient= 0.22; P = 0.04) (Fig. 4A) and a more significant correlation when comparing NP2/CD4/CCR5 to high CCR5 Affinofile cells (Spearman correlation coefficient= 0.65; P < 0.0001) (Fig. 4B). These results argue that an appreciable number of CC Envs can

utilize MVC-bound CCR5 but that this ability is less obvious on cells expressing low to moderate levels of CCR5, unless results are plotted on a log scale. When this is done, small differences in residual virus infection are not only evident, but highly predictive of substantive infection when CCR5 expression levels are high. To further confirm this finding, we examined a subset (4 T/F and 8 CC Envs) of our pseudoviruses on U87/CD4 cells in which CCR5 was transiently expressed, resulting in high levels of CCR5 (Fig. 1A). These experiments recapitulated our findings on high CCR5 Affinofile cells: viruses that exhibited MPIs of <95% on high CCR5 Affinofile cells likewise exhibited reduced MPIs on transiently transfected U87/CD4 cells, while viruses that remained highly sensitive to MVC under these conditions were likewise efficiently inhibited on CCR5-expressing U87/CD4 cells (data not shown).

Utilization of CCR5 in the presence of MVC is evident on primary human CD4+ T cells. To assess whether this phenotype is recapitulated under more physiologically relevant conditions, we infected primary human CD4+ T cells with GFP-expressing Env pseudoviruses in the presence or absence of saturating concentrations of MVC. To maximize the sensitivity of this primary cell assay, we selected three T/F pseudoviruses that were efficiently inhibited by MVC on high CCR5 Affinofile cells and three chronic pseudoviruses that exhibited significant resistance to MVC under these conditions. Peripheral blood CD4+ T cells from two uninfected donors, in each of two independent experiments, were purified by negative selection and stimulated with plate-bound anti-CD3/anti-CD28 and IL-2 for three days. Cells were incubated in the

presence or absence of saturating levels of MVC (6μM) 30 min prior to infection. Three days post-infection, cells were analyzed by flow cytometry and infected cells were determined by gating on live CD3+CD4-GFP+ events. We found that the three chronic pseudoviruses exhibited significant residual infection compared to T/Fs on CD4+ lymphocytes in the presence of saturating MVC (P = 0.04 by t-test) (Fig. 5). Further, these differences reflected those previously observed for these viruses on high CCR5 Affinofile cells and on NP2/CD4/CCR5 cells (Fig. 5). The differences were most pronounced on high CCR5 Affinofile cells (which express the highest levels of CCR5), less pronounced on NP2/CD4/CCR5 cells (intermediate CCR5 levels), and the least pronounced (but still observable) on primary CD4+ T cells (low CCR5 levels). Thus, these results are again consistent with the hypothesis that many CC Envs can utilize MVC-bound CCR5, and that high levels of CCR5 magnify this property, revealing differences in how T/F and CC Envs engage this coreceptor.

Ability to use MVC-bound CCR5 is not related to overall level of infectivity or other viral phenotypes. The ability of some HIV-1 strains to recognize MVC-bound CCR5 has been linked to differences in how the Env protein engages its chemokine coreceptor (214, 215, 291). As a result, it is possible that the striking difference in the frequencies with which T/F and chronic viruses mediate infection in the presence of MVC, albeit inefficiently at physiologic levels of CCR5 expression, is a surrogate measure for some other phenotypic property. In two previous studies, we tested these clade B and C pseudoviruses in a variety of phenotypic assays, comparing T/F to CC Envs

(200, 295). With the exception of some clade-specific neutralization sensitivity, no significant genotypic or phenotypic differences were identified. In light of our current findings, we re-analyzed these data, comparing Envs that were partially resistant to MVC on high CCR5 Affinofile cells to 18 previously determined Env properties (Table 1). None of the phenotypic characteristics previously measured for these panels correlated strongly with incomplete inhibition by MVC, including CD4 use efficiency, neutralization by monoclonal antibodies (b12, VRC01, PG9, PG16) or by pooled sera from patients infected with either clade B or C HIV-1 strains (clade B/C HIV Ig), or primary CD4+ T cell subset tropism. Except for V5 length, we found no strong genetic correlates of incomplete MVC inhibition, including V1-V4 individual and total variable loop lengths and potential N-linked glycosylation sites. We also asked whether the ability to utilize CCR5 in the presence of MVC was simply a property of highly functional Env proteins or highly infectious Env pseudoviruses. To address this, we plotted pseudoviral infectivity (RLU) in the absence of MVC against residual infection at saturating concentrations of MVC. There was no correlation between overall infectivity and the partial resistance phenotype (Spearman correlation coefficient = 0.02 P = 0.84) (Fig. 6). Therefore, residual infection was not a surrogate measure for increased infectivity.

DISCUSSION

In addition to using CCR5 and being T-cell tropic, several other phenotypic and genotypic traits have been associated with T/F Env proteins relative to those from chronic viruses (56, 73, 131, 200, 234, 235, 238, 239, 295). Among these, the differential utilization of CCR5 first observed by Swanstrom and colleagues, confirmed and extended here, is arguably the most robust. Both clade B and C T/F Envs are far more likely to be inhibited in entry by MVC on cells expressing high levels of CCR5 than are CC Envs. The ability of many CC Envs to mediate infection in the presence of MVC under conditions where CCR5 expression levels are high indicates that these Envs can utilize one or more conformations of CCR5 better than most T/F Envs. This suggests that there is selective pressure against the more promiscuous CCR5 utilization phenotype exhibited by many CC Envs at the level of HIV transmission and selection for this trait during chronic infection. Questions that remain to be addressed are the mechanism that underlie this phenotype, whether genetic signatures associated with differential use of CCR5 can be identified, and whether this phenotype observed on a cell line expressing levels of CCR5 that greatly exceed those seen on primary cells is a correlate for another property that would be more meaningful at the level of mucosal transmission of HIV-1.

MVC is a CCR5 antagonist that potently inhibits infection by virtually all R5 strains of HIV-1 *in vitro* (80), blocks vaginal transmission of SHIV-162P3 in a rhesus macaque model (281), and significantly diminishes virus loads in HIV-1 infected individuals (86). Clearly, MVC is an effective inhibitor of HIV infection

under most experimental and clinical conditions. Thus, the finding by Swanstrom and colleagues that approximately one-half of CC Envs are inhibited less than 95% by MVC on 293T cells expressing high levels of CD4 and CCR5 was surprising. Here, we confirmed this finding using a large panel of Clade C Envs, and found that Clade B Envs also exhibited this property to a similar magnitude.

What is the mechanism that underlies the differential effects of MVC on T/F versus chronic HIV-1 Envs? CCR5 antagonists are allosteric inhibitors – upon binding to CCR5, they induce a conformation that is not recognized by most HIV-1 strains (26, 82, 130, 133, 250, 276, 288). Thus, the ability of many chronic HIV-1 Envs to mediate some degree of infection in the presence of saturating concentrations of MVC could mean that under these conditions a fraction of CCR5 molecules assume a conformation that is unable to bind MVC but that can be utilized by many chronic Env proteins. If so, then CCR5 may exhibit enhanced conformational heterogeneity when expression levels are high relative to when expression levels are lower, with one or more of these conformations not binding MVC or doing so only poorly. CCR5 and seven-transmembrane domain receptors in general can assume different conformations as a result of binding ligands or G proteins (18, 50, 84, 186, 187), and CCR5 has been shown to exhibit antigenic heterogeneity (11, 21, 22, 153). It is also possible that at high levels of CCR5 expression posttranslational modifications such as sulfation of the N-terminal domain or coupling to G proteins could become saturated, resulting in conformational heterogeneity. Thus, our results could be explained by a model in which one such conformation fails to bind MVC or does so only poorly, with this

conformation being recognized by a large subset of CC Envs but by only a small fraction of T/F Env proteins. When coupled with the fact that HIV-1 Env interactions with CCR5 are variable as judged by the differential effects that some mutations in CCR5 have on infection by diverse HIV-1 strains (146, 272), it is certainly plausible that CCR5 conformation could be influenced by expression levels in a manner that preferentially allows infection by chronic but not T/F viruses in the presence of MVC.

A second possible mechanism to account for the ability of some Envs to mediate infection in the presence of MVC when CCR5 levels are high is that these Envs can utilize the drug-bound conformation of CCR5 very inefficiently, such that under physiological levels of CCR5 expression infection appears fully suppressed by MVC and by other CCR5 antagonists. Our data as well as work by Gorry and colleagues (225) clearly favors this interpretation. Typically, virus inhibition curves are plotted on a linear scale. However, if inhibition is plotted on a log-scale and a highly reproducible and quantitative assay is used, different antiretroviral agents can be revealed to exhibit considerable variability in their abilities to maximally inhibit HIV-1 (243, 252). When we examined the ability of MVC to inhibit HIV-1 infection on NP2/CD4/CCR5 cells in this way, it became apparent that while some viruses were inhibited by 99.9%, others were inhibited by 99.0% or somewhat less. We found that these small, residual levels of infection were highly predictive of the ability of a virus to mediate appreciable levels of infection when both CCR5 and MVC levels are high. When a subset of viruses were tested on primary human CD4+ T cells in this way, the same results

were obtained: viruses that mediated obvious levels of infection on CCR5-high cells in the presence of MVC were inhibited less efficiently than viruses that remained highly sensitive to MVC regardless of CCR5 expression levels. Roche et al. have also found that some Envs that appear fully sensitive to MVC on cells expressing low to moderate levels of CCR5 exhibit some degree of resistance when CCR5 levels are high (225). Thus, we conclude that using a cell line that expresses high levels of CCR5 simply amplifies a phenotype that is already present on cells expressing lower levels of CCR5 that can be easily over-looked (200, 295).

Given that some R5 HIV-1 strains can mediate readily detectable levels of infection in the presence of high levels of both CCR5 and MVC, can this information be used to predict clinical outcomes when MVC is used as part of an antiviral regimen? The most common mechanism that underlies clinical failure associated with MVC treatment is expansion of pre-existing viral variants that utilize CXCR4 to mediate infection (102, 104, 147, 149, 178, 276). Less commonly, mutations in the Env protein enable it to use MVC-bound CCR5, sometimes with impressive efficiency (272, 291). The question is whether these clinical failures are associated with preexisting viruses that are not completely inhibited by MVC, with this property being clearly evident only when using cells that express high CCR5 levels. In fact, Roche et al. found that a viral Env predisposed to acquire high-level resistance to CCR5 antagonists exhibited a low level ability to use MVC-bound CCR5 on cell lines expressing high levels of this coreceptor (225). Whether this will be a general property of viral Envs from

patients who fail MVC via this pathway is not known, but could be examined by testing the ability of Envs from such individuals to infect Affinofile CCR5-high cells in the presence of MVC.

The most important question resulting from this work is why the ability to use MVC-bound CCR5 is associated so clearly with chronic but not T/F Envs. What results in the selection of this phenotype during the course of natural HIV-1 infection in the absence of MVC therapy, and what selects against this phenotype at the level of mucosal transmission? Clearly, the reason cannot have anything to do with MVC per se as none of the patients from whom we derived Envs had been treated with MVC. Thus, the striking difference between chronic and T/F Envs seen by Swanstrom and colleagues and in our study is likely a surrogate for some other viral property. In our previous studies, we compared the phenotypic properties of panels of T/F and geographically-matched CC Envs (200, 295). We reanalyzed these data, comparing Envs that were almost totally inhibited by MVC on CCR5-high cells to those that were not regardless of whether they were chronic or T/F Envs (Table 1). These analyses failed to identify any cross-clade phenotypic differences between these two groups, including the ability to infect different CD4+ T cell subsets, use alternative coreceptors, neutralization sensitivity, dependence upon CD4 levels, and the ability to be captured by DCs and transmitted to T cells. In addition, there were no obvious genetic signatures between the two groups save for a correlation with V5 loop length, with longer V5 loops being correlated with reduced MPI on high CCR5 Affinofile cells. While Swanstrom and colleagues found that slightly higher

glycosylation was associated with the MVC-resistant phenotype (127), this correlation was not evident in our panel of combined clade B and C Envs. Given the plasticity with which Envs can interact with their coreceptors and the multiple Env determinants that can influence CCR5 interactions (137, 142, 143, 146, 170, 260, 263, 265, 272), this is perhaps not surprising: much larger numbers of Env clones and sequences may be needed to identify genetic characteristics that are consistently linked with the ability to utilize MVC-bound CCR5. The more promiscuous utilization of CCR5 conformations by CC Envs may reflect selection pressures in chronic infection where neutralizing antibodies are abundant and certain populations of CD4+CCR5+ cells have been diminished or otherwise altered by viral cytopathicity. Earlier studies have shown that R5 viruses can become increasingly resistant to entry inhibitors over time, consistent with alterations in CCR5 use (100, 129, 221). More promiscuous utilization of CCR5 could expand viral tropism under these conditions, in contrast to *de novo* infection of naïve hosts where virus infection of a more homogeneous population of CD4+CCR5+ cells could be favored. Further characterization of the CD4+CCR5+ cells that are targeted during acute infection may reveal differences in the abilities of T/F and chronic Env proteins to mediate virus entry as well as identify new approaches to prevention.

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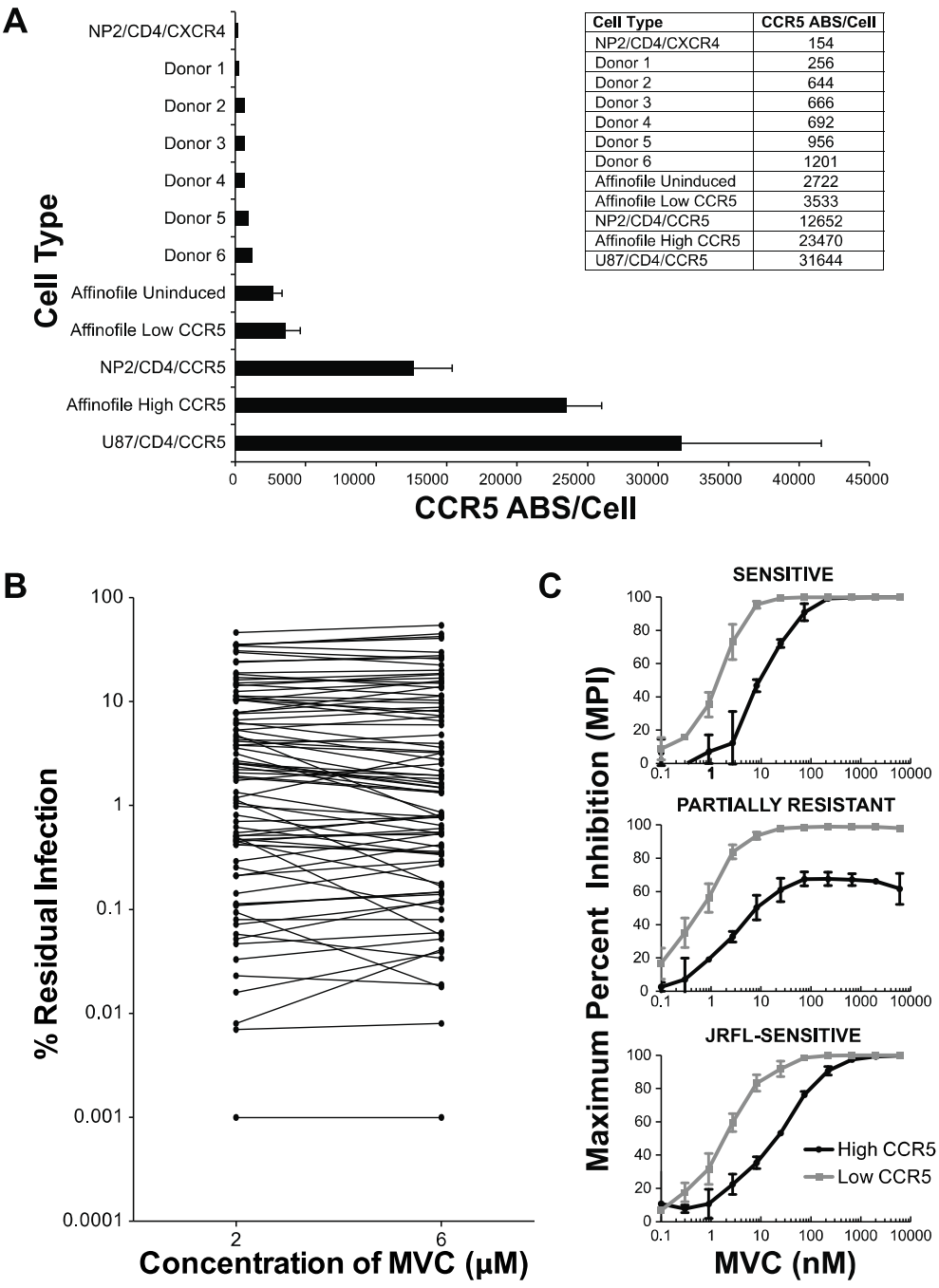
FIGURES



Cell Type	CCR5 ABS/Cell
NP2/CD4/CXCR4	154
Donor 1	256
Donor 2	644
Donor 3	666
Donor 4	692
Donor 5	956
Donor 6	1201
Affinofile Uninduced	2722
Affinofile Low CCR5	3533
NP2/CD4/CCR5	12652
Affinofile High CCR5	23470
U87/CD4/CCR5	31644

Figure 1. Partial resistance to saturating amounts of maraviroc on Affinofile cells expressing high CCR5. (A) Quantitative assessment of cell surface expression of CCR5 was determined by staining cells with PE-labeled CCR5

mAb 2D7 followed by quantitative flow cytometry, making it possible to calculate the number of 2D7 binding sites per cell. (B) MVC saturation was confirmed by measuring residual infection between the two highest concentrations used. No differences in virus inhibition were observed between 2μM and 6μM MVC (*P* = 0.70). (C) MVC sensitive pseudoviruses dispayed a MPI >95% on both low (grey) and high (black) CCR5 293-Affinofile cells. Two phenotypes were observed. 57 pseudoviruses and the JRFL control virus displayed a MPI >95% regardless of CCR5 expression levels (1st and 3rd panels). However, 30 pseudoviruses were efficiently inhibited by MVC on cells expressing low CCR5, but exhibited MPI values of ≤95% on high CCR5 Affinofile (middle panel). All experiments were done in at least triplicate in each of at least three independent experiments. Error bars represent standard deviations.

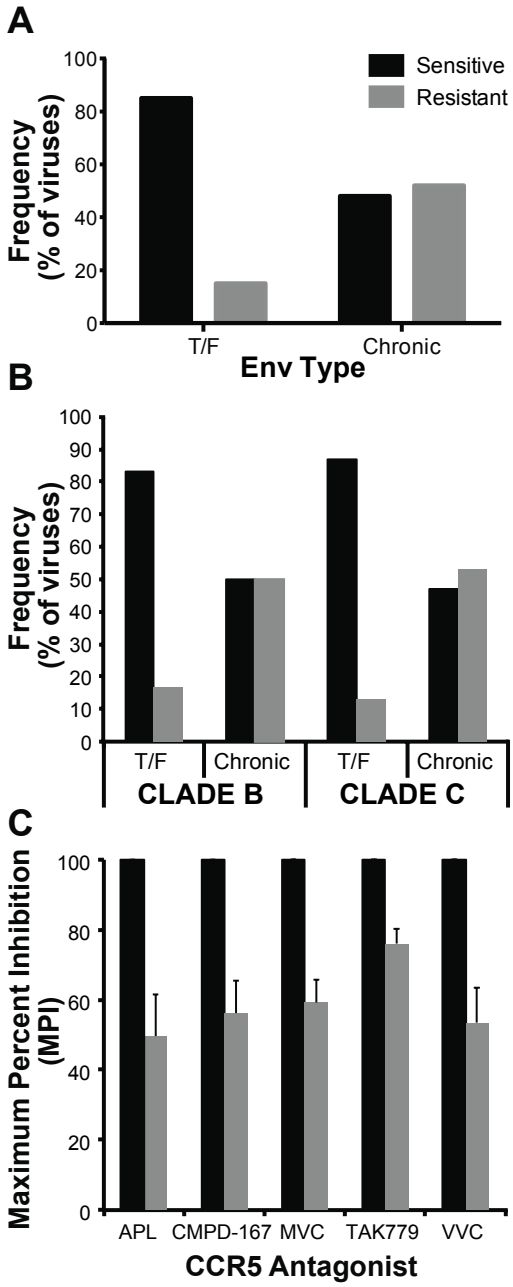


Figure 2. Partial resistance is enriched in chronic HIV-1 Envs compared to T/F Envs, irrespective of clade and CCR5 antagonist. (A) 87 pseudoviruses were tested on high CCR5 Affinofile cells. There was a higher frequency of partial reisistance to MVC in CC Envs compared to T/F Envs ($P < 0.0001$). (B)

Data was segregated by clade (52 clade B and 35 clade C) and independently confirmed an enrichment of the partially resistant phenotype in CC Envs (Clade B P = 0.02; clade C P = 0.02). (C) Representative resistant (704010330.G5h) and sensitive (700010040.C9.4520) pseudoviruses were tested for sensitivity to other CCR5 antagonists on high CCR5 Affinofile cells. Cells were pretreated with varying concentrations of MVC, aplaviroc, CMPD-167, TAK779, and VVC prior to infection. MPI values of MVC-resistant (grey) and sensitive (black) pseudovirus are shown. Data were analyzed by Fisher's exact test. All infections were done in at least triplicate in each of at least three independent experiments. Error bars represent standard deviations.

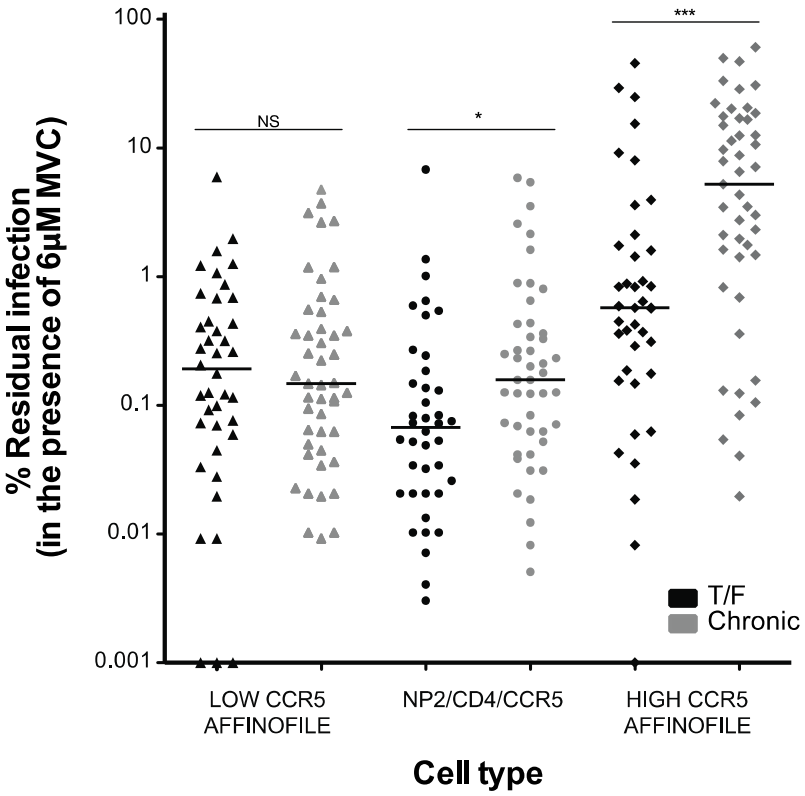


Figure 3. Residual infection on cells expressing different levels of CCR5. All pseudoviruses were tested on low CCR5 Affinofile cells, NP2/CD4/CCR5 cells, and high CCR5 Affinofile cells and the residual infection in the presence of saturating MVC was plotted for all cell types evaluated. There was a significant difference between T/F (black) and chronic (grey) residual infection in both NP2/CD4/CCR5 and high CCR5 Affinofile cells (NP2/CD4/CCR5 P = 0.01, high CCR5 Affinofiles P = 0.0003), but not on low CCR5 Affinofile cells (P = 0.94). Data were analyzed by Mann-Whitney test. All experiments were done in at least triplicate in each of at least two independent experiments.

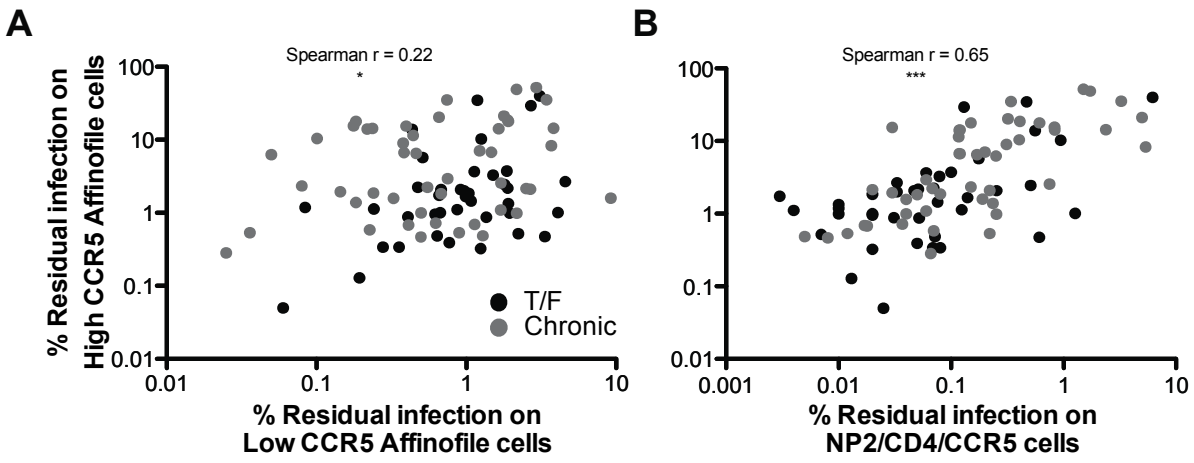


Figure 4. Residual infection on cells expressing low levels of CCR5 is predictive of MVC resistance on cells expressing high CCR5. (A) The residual infection on low CCR5 Affinofile cells was plotted against that on high CCR5 Affinofiles in the presence of saturating MVC. Residual infection on low CCR5 Affinofiles was predictive of partial MVC resistance on high CCR5 Affinofile cells (Spearman correlation coefficient = 0.22; *P* = 0.04). (B) The residual infection on high CCR5 Affinofile cells was also plotted against that on NP2/CD4/CCR5 in the presence of saturating MVC. Similarly, residual infection on NP2/CD4/CCR5 was predictive of residual infection on high CCR5 Affinofile cells (Spearman correlation coefficient = 0.65; $P < 0.0001$).

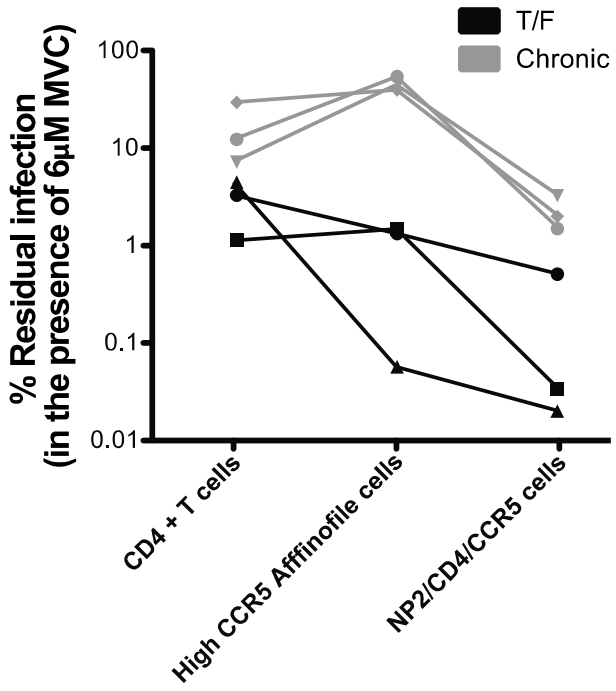


Figure 5. CC Envs exhibit greater residual infection compared to T/F Envs on primary human CD4 + T cells. Levels of residual infection for three T/F (black) and three chronic (grey) Envs on CD4 + T cells were assessed in the presence of saturating MVC. Higher levels of residual infection were mediated by CC Envs in the presence of MVC compared to T/F Envs (chronic median = 1.1% versus T/F median = 0.51%; *P* = 0.10) . Residual infection for the three T/F and CC Envs were compared between CD4 + T cells, High CCR5 Affinofile cells, and NP2/CD4/CCR5 cells . In all three cell types, CC Envs displayed higher residual infection compared to T/F Envs. All primary cell infections were done with at least two donors in triplicate in at least two independent experiments.

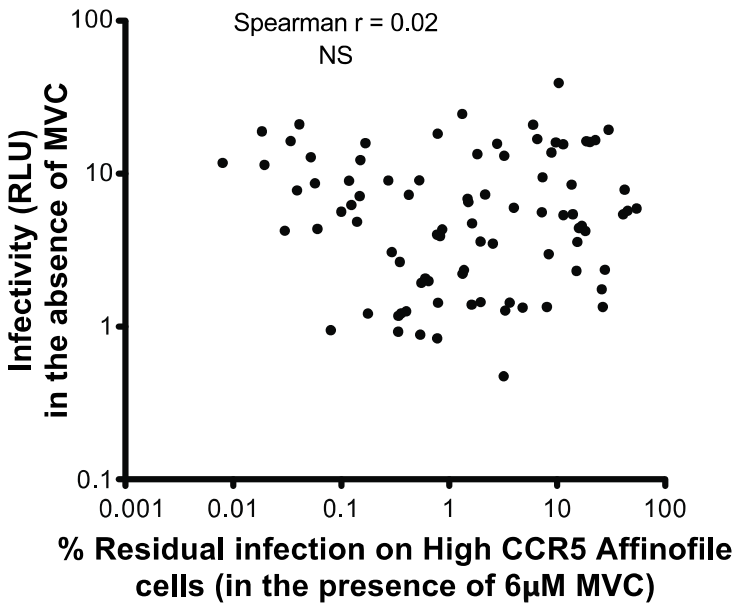


Figure 6. Infectivity is not associated with partial resistance phenotype. Pseudoviral infectivity in the absence of MVC was plotted against residual infection in the presence of saturating MVC to determine whether partial reistance correlated with infectivity. There was no correlation (Spearman correlation coefficient = 0.02; *P* = 0.84).

TABLE 1 Genotypic and phenotypic correlates of partial resistance.

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Env Type	Clade	Env clone designation	Coreceptor tropism	Geographic location	Risk factor[a]	Gender	Viral load (copies/ml)	Fiebig stage/ min time since infection	Reference	Accession number
T/F	B	REJO.D12.1972	R5	Alabama	HSX	M	722,349	V	2	EU576707
	B	THRO.F4.2026	R5	Alabama	MSM	M	5,413,140	V	2	EU577077
	B	1018-10.A5.1732	R5	South Carolina	SPD	M	270,000	III	2	EU575091
	B	WITO.B10.2062	R5	Alabama	HSX	M	325,064	II	2	EU577388
	B	RHPA.A19.2000	R5	Alabama	HSX	F	1,458,354	V	2	EU576734
	B	SUMAd5.82.1713	R5	Alabama	MSM	M	939,260	II	2	EU577061
	B	700010040.C9.4520	R5	North Carolina	MSM	M	298,026	V	2	EU576418
	B	700010058.A4.4375	R5	North Carolina	unknown	M	394,649	III	2	EU576440
	B	1053-07.B15.1648	R5	South Carolina	SPD	M	1,400,000	III	2	EU575201
	B	9010-09.A1.4924	R5	South Carolina	SPD	F	146,954	II	2	EU575771
	B	9015-07.A1.4729	R5	South Carolina	SPD	M	500,000	II	2	EU575795
	B	TT35P.11H8.2874	R5	Trinidad	HSX	M	1,849,301	II	2	EU577329
	B	9021-14.B2-4571	R5	California	SPD	M	143,379	II	2	EU575924
	B	1006-11.C3.1601	R5	North Carolina	SPD	M	1,600,000	III	2	EU575025
	B	1056-10.TA11.1826	R5	South Carolina	SPD	M	140,000	II	2	EU575305
	B	SC05.8C11.2344	R5	Trinidad	HSX	M	9,980,952	II	2	EU576774
	B	SC20.8A8.2437	R5	Trinidad	HSX	M	2,789,313	IV	2	EU576838
	B	9032-08.A1.4685	R5	Alabama	SPD	M	40,815	III	2	EU576114
	B	PRB956-04.B22.4267	R5	Virginia	SPD	n/a	600,000	II	2	EU576603
	B	PRB959-02.A7.4345	R5	South Carolina	SPD	n/a	>2,000,000	II	2	EU576693
	B	034v1 (IDU)	R5	Montreal	IDU	M	75,000,000	III	9	GU562170
	B	034v2 (IDU)	R5	Montreal	IDU	M	75,000,000	III	9	GU562198
	B	AD17.1 env	R5	New York	MSM	M	47,600,000	II	10	n/a
	C	706010018. 2E3	R5	S. Africa	HSX	F	93,700	VI	11	FJ444047
	C	20258279-V2_3A5	R5	S. Africa	SPD	F	281,838	IV	31	HQ595763
	C	2833264_3G11	R5	S. Africa	SPD	M	234,423	I/II	31	HQ595757
	C	21197826-V1_3A1	R5	S. Africa	SPD	F	343,923	I/II	31	HQ595753
	C	21283649_3E8	R5	S. Africa	SPD	M	3,180	I/II	31	HQ595756
	C	20927783_3E2	R5	S. Africa	SPD	F	1,886	I/II	31	HQ595750
	C	1245045_3C7	R5	S. Africa	SPD	M	234,068	I/II	31	HQ595742
	C	20258279-V4_3D10	R5	S. Africa	SPD	F	281,838	IV	31	HQ595764

Env Type	Clade	Env clone designation	Coreceptor tropism	Geographic location	Risk factor[a]	Gender	Viral load (copies/ml)	Fiebig stage/ min time since infection	Reference	Accession number
T/F	C	ZM247Fv1.Rev-	R5	Zambia	HSX	F	10,823,500	II	12, 13, 18	n/a
	C	ZM249M-B10	R5	Zambia	HSX	M	2,000,000	IV	12, 13, 18	EU166862
	C	704809221.1B3	R5	S. Africa	HSX	M	750,000	I/II	11	FJ444116
	C	703010054.2A2	R5	Malawi	HSX	M	13,936	V	11	FJ443808
	C	703010217.B6	R5	Malawi	HSX	F	102,602	V/VI	11	FJ443589
	C	ZM247Fv2.fs	R5	Zambia	HSX	F	10,823,500	II	12, 13, 18	n/a
	C	704010042. 2E5	R5	S. Africa	HSX	M	181,000	IV	11	FJ443745
	C	705010185.tf	R5	S. Africa	HSX	F	14,800	I/II	in prep[b]	n/a
Chronic	B	HEMA.A4.2125	R5	Alabama	MSM	M	49,755	1yr 10mo	2	EU578133
	B	SHKE.A7.2118	R5	Alabama	MSM	M	544,000	1yr 2mo	2	EU578458
	B	HEMA.A23.2143	R5	Alabama	MSM	M	49,755	1yr 10mo	2	EU578132
	B	SHKE.A26.4112	R5	Alabama	MSM	M	544,000	1yr 2mo	2	EU578453
	B	WICU.B4.2973	R5	Alabama	MSM	M	8,424	5yr 11mo	2	EU578642
	B	OLLA.A14.1923	R5	Alabama	HSX	F	382,000	2yr 1mo	2	EU578231
	B	SAMI.A8.1863	R5	Alabama	MSM	M	116,000	3yr 11mo	2	EU578272
	B	SHKE.A4.2116	R5	Alabama	MSM	M	544,000	1yr 2mo	2	EU578456
	B	SMRE.A13.4127	R5	Alabama	HSX	F	135,858	1yr 4mo	2	EU578471
	B	TALA.A2.1780	R5	Alabama	MSM	M	228,200	6yr 11mo	2	EU578494
	B	WICU.C1.2992	R5	Alabama	MSM	M	8,424	5yr 11mo	2	EU578657
	B	YOMI.F2.4137	R5	Alabama	MSM	M	14,178	6yr 1mo	2	EU578683
	B	SC05.A10.2362	R5	Trinidad	HSX	M	19,514	5yr 5mo	2	EU578358
	B	SC05.8H2.3243	R5	Trinidad	HSX	M	19,514	5yr 5mo	2	EU576786
	B	SC05.8A11.2363	R5	Trinidad	HSX	M	19,514	5yr 5mo	2	EU578359
	B	1632-ta9	R5	Washington	MSM	M	97,800	2yr 5mo	26	HQ216892
	B	1632-a17	R5	Washington	MSM	M	97,800	2yr 5mo	26	HQ216864
	B	1632-a6	R5	Washington	MSM	M	97,800	2yr 5mo	26	HQ216883
	B	1632-ta1	R5	Washington	MSM	M	97,800	2yr 5mo	26	HQ216887
	B	1632-a7	R5	Washington	MSM	M	97,800	2yr 5mo	26	HQ216884
	B	1632-a23	R5	Washington	MSM	M	97,800	2yr 5mo	26	HQ216869
	B	1588-ta7	R5	Washington	MSM/ IDU	M	99,600	7yr 2mo	26	HQ216783
	B	1470-d27	R5	Washington	MSM/ IDU	M	492,200	4yr 3mo	26	HQ216683
	B	1451-d17	R5	Washington	MSM	M	532,000	20yr 3mo	26	HQ216655
	B	1451-c16	R5	Washington	MSM	M	532,000	20yr 3mo	26	HQ216638
	B	1451-d1	R5	Washington	MSM	M	532,000	20yr 3mo	26	HQ216651

Env Type	Clade	Env clone designation	Coreceptor tropism	Geographic location	Risk factor[a]	Gender	Viral load (copies/ml)	Fiebig stage/ min time since infection	Reference	Accession number
Chronic	B	1599-b11	R5	Washington	IDU	M,F	112,000	6yr 7mo	26	HQ216802
	B	1444-a21	R5	Washington	MSM	M	86,300	7yr	26	HQ216583
	C	704010330.G5h	R5	S. Africa	HSX	M	46,100	n/a	31	JQ777128
	C	704010207.D11	R5	S. Africa	HSX	F	15,400	n/a	31	JQ777073
	C	702010141.E80	R5	Malawi	HSX	F	151,282	n/a	31	JQ779320
	C	702010432.E16	R5	Malawi	HSX	M	40,570	n/a	31	JQ779232
	C	703010167.E15	R5	Malawi	HSX	F	73,505	n/a	31	JQ779889
	C	ZM414.1	R5	Zambia	HSX	F	213,600	n/a	68	GU329415
	C	707010457	R5	Tanzania	HSX	F	234,671	n/a	31	KC156220 [c]
	C	ZM414.20	R5	Zambia	HSX	F	213,600	n/a	68	GU329426
	C	705010534.E35	R5	S. Africa	HSX	F	63,300	n/a	31	JQ779192
	C	704010499.H1	R5	S. Africa	HSX	F	15,200	n/a	31	JQ777164
	C	704010461.A7h	R5	S. Africa	HSX	F	22,900	n/a	31	JQ777137
	C	704010028.F6	R5	S. Africa	HSX	F	9,220	n/a	31	JQ777039
	C	703010269.E30	R5	Malawi	HSX	F	30,434	n/a	31	JQ777184
	C	704010273. E5	R5	S. Africa	HSX	F	25,700	n/a	31	JQ777098
	C	3902.bmLG14	R5	Malawi	HSX	F	19,900	n/a	Kirchher JL et al.[c]	HM070661
	C	4403.A18	R5	Malawi	HSX	F	100,892	n/a	Kirchher JL et al.[c]	HM070677
	C	4403.D1	R5	Malawi	HSX	F	100,892	n/a	Kirchher JL et al.[c]	HM070689
	C	4403.bmR.B6	R5	Malawi	HSX	F	100,892	n/a	Kirchher JL et al.[c]	HM070754

[a] HSX= heterosexual exposure; MSM=men who have sex with men; IDU=intravenous-drug user.

[b] Kappes JC et al. manuscript in preparation.

[c] IMC Accession number.

[d] Kirchherr JL et al.2011. Identification of amino acid substitutions associated with neutralization phenotype in the human immunodeficiency virus type-1 subtype C gp120. Virology **409**:163–174.

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CHAPTER 3

Chemokines and HIV-1: Viral inhibition and enhancement

CHEMOKINES: THE BASICS

Chemokines are a group of low-molecular weight (7 to 12 kDa) secreted proteins that primarily function in leukocyte development, maturation, and homing (192). They are also central players in many physiopathological processes such as allergies, angiogenesis, inflammation, infectious and autoimmune diseases, tumor growth and hematopoiesis. Chemokines induce chemotaxis in a variety of cells (monocytes, neutrophils, lymphocytes, eosinophils, fibroblasts, and keratinocytes) by interacting with chemokine receptors, which are members of the seven-transmembrane GPCR superfamily and glycosaminoglycans (GAGs) to promote migration and impart directionality to cell movement (237). GPCRs couple chemokine binding outside the cell to activation of intracellular signaling cascades that lead to cell motility. GAGs are complex carbohydrates attached to protein cores on the cell surface (proteoglycans) or free flowing in the extracellular space. In addition to numerous functions, GAGs immobilize chemokines to provide a chemotactic gradient to direct migrating cells to sites of injury or inflammation (213). To date, more than 50 chemokines and 20 chemokines receptors have been described (Fig 1) (34, 165, 191, 302). Multiple chemokines can bind and signal through the same receptor, providing redundancy in chemokine/receptor function.

As a group, chemokines exhibit between 20-70% amino acid homology (115, 191) and are characterized by the presence of three to four conserved cysteine residues. They can be divided into four subfamilies (CXC, CC, C, or CX3C), based on the positioning of the amino terminal cysteine residues (17). Structurally, CC-chemokines are defined by the tandem arrangement of cysteine residues in the amino terminus, while CXC-subfamily cysteines are separated by a single amino acid. The majority of described chemokines belong to these two subfamilies. The third subfamily (C) members contain a single cysteine residue in the conserved position. The fourth subfamily (CX3C) has two cysteines separated by three variable amino acids in the amino terminus. This subtle structural difference informs the quaternary structure of the chemokine and has implications not only for receptor recognition, but also oligomer formation (123, 237). It is well known that chemokines oligomerize in a variety of homo- and hetero-oligomeric forms at high concentrations both in solution and in physiological settings to carry out their effector functions (16, 184, 185, 284). However, studies have reported differences in the quaternary structures of CC- and CXC-chemokines and suggested that these differences may be important for how chemokine subfamilies are recognized by, and signal through, their cognate receptors and GAGs (123, 195, 216, 220, 286). Therefore the dynamic equilibrium of chemokine oligomeric states is critical for function, allowing specific interactions with both GPCRs and GAGs.

CHEMOKINES AND HIV-1 PATHOGENESIS

Important discoveries in the last two decades have defined a close relationship among chemokines, their receptors, and HIV-1 infection (6, 55, 59, 60, 71, 79, 81, 88). For several years, it was known that $CD8^+$ T cells secrete soluble factors that suppress HIV-1 replication in $CD4^+$ T cells (38, 283). Supporting evidence for the presence of a noncytolytic suppressive product of $CD8^+$ T cells came from clinical reports that correlated disease progression with the absence or presence of $CD8^+$ T cell activity (99, 167). Furthermore, it was shown that a high level of CD8+ T cell suppressor activity was associated with long-term survivors of HIV-1 infection (45). Lastly, the noncytolytic activity of $CD8^+$ T cells controlled disease pathology in nonhuman primates (49, 83, 211). The identity of these soluble factors remained elusive until Cocchi et al. demonstrated that the β-chemokines MIP-1α (macrophage inflammatory protein 1α), MIP-1β (macrophage inflammatory protein 1β) and RANTES (regulated on activation, normal T expressed and secreted) were responsible for a significant fraction of the noncytolytic $CD8^+$ T cell suppressive effect (59). These chemokines, in addition to others belonging to the α-chemokine family (ie: stromal cell-derived factor 1—SDF-1), suppress viral replication of R5- and X4-tropic HIV-1 strains *in vitro* most commonly by competing with Env for binding to the cognate chemokine receptor (29, 193). Receptor downregulation in response to chemokine binding can also suppress viral replication by decreasing the density of surface coreceptors (8, 166, 254). *In vivo* studies to corroborate these

in vitro findings are limited by the inherent difficulties in measuring circulating chemokine concentrations. However, further research by Cocchi et al. demonstrated an inverse relationship between levels of β-chemokines *in vivo* and the outcome of HIV-1 infection, where higher concentrations of MIP-1α, MIP-1β, and RANTES from $CD8^+$ T cells correlated with asymptomatic HIV-1 infection (61). Additionally, nonhuman primate studies have shown that immunization with simian immunodeficiency virus (SIV) grown in human $CD4^+$ T cells induces unusually high production of RANTES, MIP-1α, and MIP-1β, which protects macaques from subsequent SIV challenge (287).

Paradoxically, chemokines have also been described to enhance HIV-1 infection, at least *in vitro*. RANTES can modulate virus infection in a variety of ways that are dependent upon its concentration. At low concentrations, RANTES inhibits HIV-1 infection by blocking its interaction with the viral coreceptor CCR5 (59, 274). At high and likely supra-physiological concentrations, RANTES forms oligomers that bind to the viral Env protein as well as cell surface GAGs, enhancing virus attachment and infection (132, 135, 274). RANTES can also modulate HIV-1 infection by transducing signals via CCR5 that over time render cells more permissive for viral replication (97, 274). In addition to RANTES, previous work has demonstrated that the α-chemokine SDF-1 enhances R5-tropic, but not X4-tropic, HIV-1 and vesicular stomatitis virus (VSV) infection *in vitro* (169). Specifically, SDF-1 was reported to enhance infection via signaling-mediated increase of Tat transactivation of the HIV-1 long terminal repeat.

Additional studies have highlighted novel ways in which chemokines enhance HIV-1 infection both *in vitro* and *in vivo*. In 2007, Saleh et al. identified a novel mechanism of HIV-1 latent infection of resting $CD4^+$ T cells, in which the CCR7 ligands, CCL19 and CCL21, were found to significantly increase the permissiveness of resting $CD4^+$ T cells to HIV-1 infection (241). Interestingly, this enhancement was attributed to chemokine-mediated increases in viral DNA nuclear import and integration, but not to productive HIV-1 replication. The same group further showed that the mechanism of the CCL19-CCR7 interaction is similar to that of the HIV-1 gp120-CXCR4 interaction in triggering cofilin activation and actin dynamics, which drastically enhance viral nuclear import and integration (44, 302). These results are consistent with *in vivo* data showing that enhanced levels of CCL19 and CCL21 in HIV-1-infected patients correlate with viral load, disease progression and response to HAART (70).

Canonically, chemokine suppression of HIV-1 infection involves competitive inhibition of viral entry by binding to the cognate chemokine coreceptors. These more recent results suggest that HIV-1 infection could also be affected by chemokines interacting with multiple receptors such as CCR7 (and others not discussed in this section) that may synergize with or antagonize HIV-1-mediated coreceptor signaling pathways. This widens therapeutic opportunities and our understanding of viral pathogenesis, as multiple surface receptors and intracellular signaling molecules might now serve as attractive targets for therapy.

PLATELET FACTOR 4 (PF4) AND HIV-1 INFECTION

Although MIP-1α, MIP-1β, and RANTES were initially shown to contribute to the HIV-1 suppressive effect of $CD8^+$ T cells, other cells types have been implicated in the production of anti-HIV-1 chemokines. Several studies have shown that monocytes, macrophages, and natural killer cells are important sources of CC- and CXC-chemokines that antagonize HIV-1 entry and replication *in vitro* (38, 42, 59, 259, 283). More recently, activated platelets have been shown to possess anti-HIV-1 properties (259). Platelets are specialized blood cells that primarily function to promote coagulation at sites of vascular injury by adhering to subendothelial matrix proteins. During vascular injury, activated platelets release a number of mediators from their α-granules, including connective tissue-activating peptide III (CTAP-III/CXCL7), RANTES, and platelet factor 4 (PF4/CXCL4) (36, 118).

PF4 is a cationic α-chemokine that functions primarily to promote coagulation by moderating the effects of heparin-like molecules (20, 257), and can be found in nanomolar and micromolar concentrations within plasma and serum, respectively (37, 54, 89, 128). PF4 has been shown to be chemotactic for immune cells by acting through interactions with a splice variant of the GPCR CXCR3B and an integral chondroitin sulfate proteoglycan expressed on the surface of cells (140, 150, 181). Additionally, there is evidence that PF4 is involved in the differentiation of monocytes to macrophages. Recently, PF4 has been described to possess potent and broadly-active antiviral activity against HIV-1 *in vitro* (15, 259).

The antiviral role of PF4 during HIV-1 infection is not unanticipated, as several previous studies have linked platelet malfunction and HIV-1 infection—with thrombocytopenia (platelet count below 150,000cells/mm^3) and thrombosis (clotting) being frequent complications during infection (66, 201, 251). These hemostatic disorders affect roughly 10-50% of HIV-1-infected individuals (67, 236). Individuals with platelet dysfunctions that increase the risk of thrombosis are likely to receive heparin treatment (87). PF4 binds with high affinity (4-30nM) to heparin and heparin-like molecules (163, 264), leading to the development of ultra large complexes (ULCs), which are highly immunogenic (217). Antibodies against these ULCs bind to PF4 on platelets and monocytes, activating these cells, resulting in both thrombocytopenia and thrombosis in a condition known as heparin-induced thrombocytopenia and thrombosis (HITT) (9). Unsurprisingly, when the incidence of HITT in HIV-1-infected patients treated with unfractionated heparin (UFH) therapy was compared with that in an UFH-treated uninfected control group, HIV-1 infection correlated with increased incidence of HITT (270).

PROJECT GOAL II: ELUCIDATING THE MECHANISM AND PHYSIOLOGICAL RELEVANCE OF PF4 ACTIVITY DURING HIV-1 INFECTION

PF4 has been shown to directly inhibit HIV-1 infection *in vitro* at concentrations less than 0.5μM (15, 259). However, despite plasma and local tissue concentrations of PF4 ranging from 0.25nM to 10μM (37, 54, 89, 128), HIV-1 is able to successfully replicate and escape the inhibitory effects of PF4 *in vivo*. The mechanism of this discordance remains unclear. In this study, we sought to understand the mechanism of action of PF4 and whether it's *in vitro* activity was biologically meaningful in the context of human infection; in an effort to clarify whether PF4 is a suitable therapeutic candidate for HIV-1 infection.

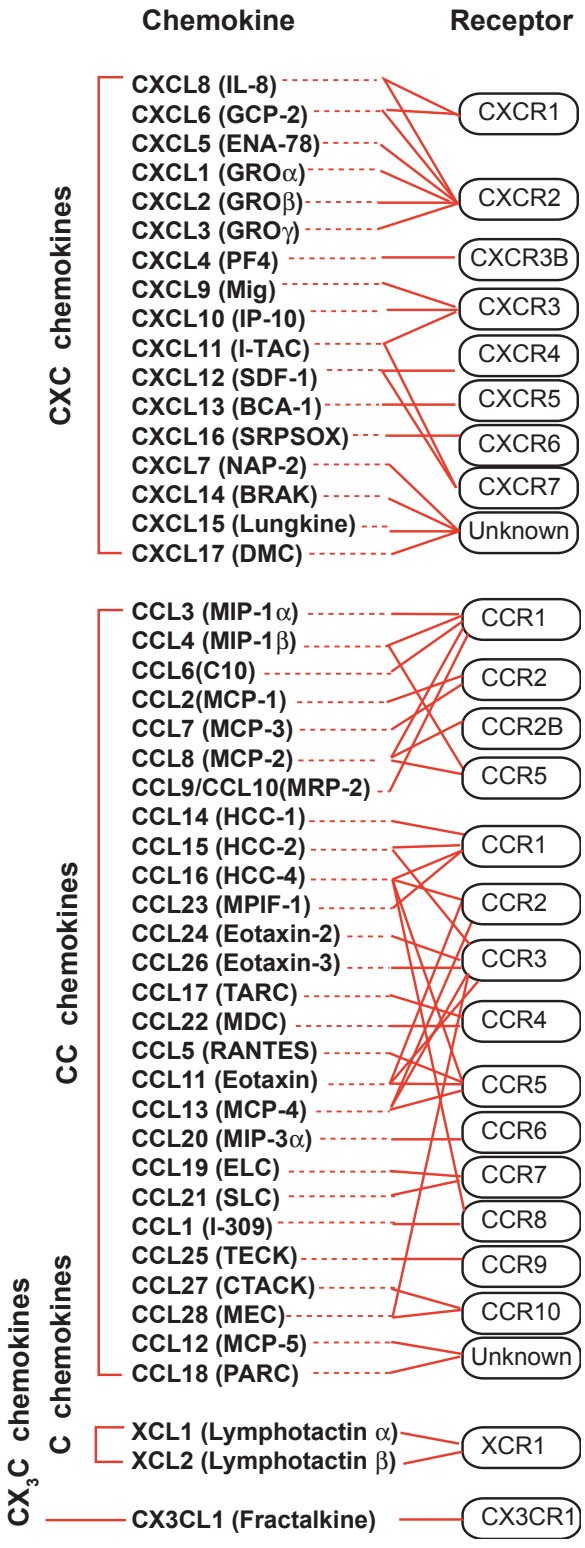

Figure 1: Known human chemokines and chemokine receptors. More than 50 chemokines and 20 chemokine receptors have been identified to date. The difference in numbers, coupled with the fact that multiple chemokines can bind to, and signal through, the same chemokine receptor suggests a redundancy in chemokine/receptor function. (Image adapted from Wu et al *Retrovirology* 2010; 7:86)

CHAPTER 4

Platelet-Factor 4 (PF4) inhibits and enhances HIV-1 infection in a concentration-dependent manner by modulating viral attachment

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ABSTRACT

Platelet-factor 4 (PF4) has been recently shown to inhibit infection by a broad range of HIV-1 isolates *in vitro*. We found that the inhibitory effects of PF4 are limited to a defined concentration range where PF4 exists largely in a monomeric state. Under these conditions, PF4 bound the HIV-1 envelope protein and inhibited HIV-1 attachment to the cell surface. However, as concentrations increased to the point where PF4 exists largely in tetrameric or higher-order forms, viral infection *in vitro* was enhanced. Enhancement could be inhibited by mutations in PF4 that shift the oligomeric equilibrium towards the monomeric state, or by using soluble glycosaminoglycans (GAGs) to which tetrameric PF4 avidly binds. We conclude that at physiologically relevant concentrations, oligomeric PF4 enhances infection by HIV-1 by interacting with the viral envelope protein as well as cell surface GAGs, enhancing virus attachment to the cell surface. This effect was not specific to HIV-1, as enhancement was seen with some but not all other viruses tested. The biphasic effects of PF4 on HIV-1 infection suggest that native PF4 will not be a useful antiviral agent, and that PF4 could contribute to the hematologic abnormalities commonly seen in HIV-infected individuals by enhancing virus infection in the bone marrow.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) entry into target cells results from sequential interactions between the HIV-1 envelope glycoprotein (Env) with the cellular receptor CD4 and a coreceptor, either CCR5 or CXCR4 (6, 55, 71, 79, 81, 88). The efficiency of this process can be regulated *in vivo* by cytokines and chemokines that bind to the viral coreceptors or that influence coreceptor expression levels (38, 42, 59, 74). A variety of cell types secrete cytokines or chemokines that can modulate HIV infection, including activated platelets that have been shown to possess anti HIV-1 properties *in vitro* (38, 42, 59, 259, 283).

During vascular injury, activated platelets release a number of mediators from their α-granules, including connective tissue-activating peptide III (CTAP-III/CXCL7), RANTES (CCL5), and Platelet-factor 4 (PF4/CXCL4) (36, 118). PF4 is a cationic α-chemokine that functions primarily to promote coagulation by moderating the effects of heparin-like molecules (20, 257). PF4 is present in nanomolar and micromolar concentrations within plasma (0.5 to 3 nM) and serum (0.4 to 1.9 μM), respectively (37, 54, 89, 128). PF4 has been shown to be chemotactic for immune cells by acting through interactions with a splice variant of the G-protein coupled receptor CXCR3B and cell surface proteoglycans (140, 150, 181). Recently, PF4 has been described to possess potent and broadly-active antiviral activity against HIV-1 *in vitro* at concentrations less than 0.5 μM

(15, 259), though the mechanism and *in vivo* relevance of these results are uncertain.

In this study, we find that the previously reported inhibitory effects of PF4 are limited to a narrow concentration range where PF4 exists predominantly as a monomer (172). Under these conditions, PF4 binds directly to Env and inhibits virus infection by preventing its attachment to the cell surface. At physiologic concentrations, where PF4 exists largely as a tetramer, it enhanced infection several-fold above untreated controls. This biphasic activity of PF4 was not restricted to the HIV-1 Env, as we observed similar results with HIV-1 pseudoviruses bearing the glycoproteins of murine leukemia virus (HIV-1_{MLV}), simian immunodeficiency viruses (HIV-$1_{SIVmac316}$ and HIV-$1_{SIVsmmE660}$) and vesicular stomatitis virus (HIV-1_{VSV-G}). However, PF4 did not antagonize nor enhance the entry of pseudoviruses bearing the glycoprotein of influenza (HIV-1_{H5N1}). We further demonstrated that PF4 carries out its dual activity during viral infection by modulating viral attachment to the cell. Lastly, we provide evidence that oligomeric PF4 directly interacts with cellular glycosaminoglycans (GAGs) as well as the HIV-1 envelope glycoprotein gp120, perhaps serving as a bridge between cell surface GAGs and the viral envelope glycoprotein, thus enhancing virus attachment and infection at high PF4 concentrations. This could play a role *in vivo*, as PF4 produced in the bone marrow could impact virus infection of stem cells and other progenitors, contributing to the hematologic abnormalities commonly associated with HIV/AIDS.

METHODS

Cell Culture. 293T17 and HeLa-derived JC53 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) fetal bovine serum (FBS)—D10F media. Multinuclear activation of galactosidase indicator cells stably expressing human CD4 and CCR5 (MAGI-CCR5) were obtained from the National Institutes of Health-AIDS Research and Reference Reagent Program and maintained in DMEM supplemented with 10% FBS and 1mg/ml puromycin. SupT1-CCR5 and Jurkat-CCR5 immortalized cell lines were maintained in Roswell Park Memorial Institute (RPMI) medium containing 10% FBS (R10F media). Primary human $CD4^+$ T cells were obtained from the Human Immunology Core of the University of Pennsylvania's Center for AIDS Research.

Virus production and normalization. HIV-1 Env pseudoviruses were produced by calcium phosphate co-transfection of 6 μg of pcDNA3.1$^+$ containing the desired env clone with 10 μg of HIV-1 core (pNL43-ΔEnv-vpr$^+$ -luc$^+$ or pNL43-ΔEnv-vpr$^+$ -eGFP) into 293T17 cells. At 72 hr post-transfection, the pseudovirus-containing supernatant was harvested and filtered through a 0.45-μm-pore-size filter and stored at -80°C. Influenza (H5N1) pseudoviruses were produced by calcium phosphate co-transfection of 400 ng of pCMV8/R containing H5 (VRC 7705) and 100 ng of pCMV8/R containing N1 (VRC 7708) with 10 μg of HIV-1 core (pNL43-ΔEnv-vpr$^+$ -luc$^+$) into 293T17 cells. HEPES buffer (1:100; Invitrogen) was added to the media to maintain basic pH and minimize acid-induced HA triggering. At 48 hr post-transfection, the pseudovirus-containing supernatant was harvested as described for HIV-1 Env pseudoviruses. GFP pseudoviruses

were concentrated approximately 100-fold by ultracentrifugation at 113,000 x g for 2 hr at 4°C through a 20% sucrose cushion. Pelleted pseudovirus was then resuspended in phosphate-buffered saline (PBS) and stored at -80°C.

Virus inhibition assay. MAGI-CCR5 cells were treated with varying concentrations of PF4 or media only prior to infection with the indicated luciferase reporter pseudovirus or full-length infectious molecular clone. After addition of pseudovirus, plates were spinoculated at 1,200 x g for 2 hr at 4°C and then incubated at 37°C. Viral inoculum was replaced with fresh complete media (supplemented with PF4 for replication competent infections) after 4 hr. Inhibition assays were also conducted with the SupT1-CCR5 and Jurkat-CCR5 cell lines as described above. For single-cycle infections using luciferase-encoding pseudovirus, cells were lysed with Brite-Glo (Promega) at 72 hr postinfection, and relative light units (RLUs) were measured. For spreading infections of replication competent infectious virus, HIV-1 replication was assessed by measuring p24 Gag protein in cell-free culture supernatants taken between days 3 and 9 postinfection using a commercial enzyme immunoassay (AlphaLISA; Perkin-Elmer). All inhibition assays were done in at least duplicate in each of at least three independent experiments.

Viral attachment assay. Human $CD4^+$ T cells (10^6 cells per condition) were stimulated for 3-5 days with anti-CD3/anti-CD28 beads (Invitrogen) and 20 U/ml recombinant interleukin-2 (IL-2; Aldesleukin; Prometheus Laboratories) in R10F media. Cells were pretreated with 200 µl of 200 nM or 4 µM $PF4^{WT}$ with or without 10 µg/ml soluble heparan sulfate proteoglycan (HSP), 4 µM $PF4^{K50E}$, 15

μM maraviroc (MVC), 15 μM plerixafor (AMD3100), 40 μg/ml DEAE-dextran, or serum-free PBS at room temperature for 30 mins. Cells were subsequently exposed to 200 μl of undiluted HIV-1_{R3A} (2.3 μg p24/ml) and incubated at 37°C for 4 hr in the absence or presence of each drug treatment. To determine background signal level, 2 x 10^6 untreated cells were infected and incubated at 4°C for 4 hr. After incubation, cells were washed twice in PBS to remove unbound virus. Cells were then split into two aliquots: one aliquot was treated with 50 μl of prewarmed 0.05% trypsin-EDTA at 37°C for 10 mins, followed by trypsin inactivation with 5 ml cold R10F media. The other aliquot was left trypsin-untreated in R10F media. Both trypsin-treated and -untreated cells were washed three times with cold PBS, then the cell pellets lysed with 100 μl of 0.5% (wt/vol) Triton X-100. Cell-associated p24 was measured using the p24 AlphaLISA (Perkin-Elmer). The final p24 concentration was calculated by subtracting the concentration of the trypsin-treated cells incubated at 4°C from the p24 signal measured in each test sample.

Primary human $CD4^+$ T cell infections. Primary human $CD4^+$ T cells (10^6 cells per condition) were stimulated with anti-CD3/anti-CD28 magnetic beads (Invitrogen) and 20 U/ml recombinant IL-2 in R10F media. Three days post-stimulation, cells were transferred to 24-well plates and incubated for 30 min with no PF4, 200 nM PF4, or 4 μM PF4. Viral input was normalized by reverse transcriptase (RT) activity as determined by a colorimetric assay (Roche). Approximately 3 ng RT of replication competent HIV-1_{CH077} was used to infect cells in duplicate in a total volume of 250 μl. Plates were incubated at 37°C, and

media was replaced every 48 hr with fresh IL-2 containing R10F with PF4. HIV-1 replication was assessed by measuring the p24 Gag protein in cell-free culture supernatants collected 6 days postinfection. Assays were done in duplicate with each of at least three independent donors.

Generation and purification of PF4 in S2 cells. cDNA encoding human PF4 was cloned into the plasmid pMT/BiP/V5-His A (Invitrogen) for expression. PF4 expression was induced by adding copper sulfate (0.5mM) to S2 cells. The induced S2 cells were then incubated in serum-free medium Insect-Xpress (Lonza) for 3 to 5 days; supernatants were collected and the media filtered through a 0.22-μm filter. $PF4^{WT}$ was purified from the media on a heparin HiTrap column on an ATKA Prime (GE Healthcare) at 4°C in 10 mM Tris, 1 mM EDTA, pH 8.0 buffer. Media were loaded in buffer containing 0.5 M NaCl, and PF4 was eluted at 1.8 M NaCl using a linear gradient. Fractions containing purified PF4 as detected by silver staining of 12% NuPAGE Bis-Tris gels (Invitrogen) were pooled, concentrated, and the buffer was exchanged into 50 mM HEPES, 0.5 M NaCl, pH ~7.2 using an Amicon Ultra centrifugal filter (3K NMWL; Millipore). Protein was quantified using a bicinchoninic acid assay (Pierce Chemical). $PF4^{K50E}$ and $PF4^{E28R/K50E}$ were purified as $PF4^{WT}$, with the following modifications: the column buffer system used was 50 mM MES, 1 mM EDTA, and pH 6.5. Media were loaded in buffer containing 0.3 M NaCl and the proteins were eluted at 1.3 M NaCl using a linear gradient. Commercially available PF4 isolated from human platelets (Calbiochem) and commercially available

recombinant PF4 (R&D Systems) were tested along with laboratory-generated recombinant PF4.

Enzyme-linked immunosorbent assay (ELISA). Chemokines were immobilized on 96-well plates (Immulon 4HBX; Thermo Scientific) in PBS-/- (Life Technologies) overnight at room temperature. Wells were washed three times with 250 μl PBS containing 0.1% Tween-20 (PBST), followed by blocking with 200 μl of 1% BSA in PBS (BPBS) at room temperature for 1 hr. Wells were then washed three times with PBST. In binding experiments, 50 μl of purified gp120 was added to chemokine-coated or control wells and allowed to react for 30 mins at room temperature. Wells were then washed three times. To detect bound gp120, 50 μl of a gp120-specific polyclonal rabbit serum (1170) created in our laboratory (1:1250 dilution in BPBS) was added to wells and reacted at room temperature for 30 mins. The wells were washed three times before the addition of 50 μl HRP-conjugated secondary goat anti-rabbit antibody (1:2500 dilution in BPBS; Cell Signaling Technology). After 30 mins reaction at room temperature, the wells were washed six times. To visualize color, 100 μl of the tetramethylbenzidine (TMB) substrate solution (R&D Systems) was added to the wells. The OD_{450} was measured using an MRX Revelation microplate reader (Dynex Technologies) immediately after the addition of 50 μl 2 N sulfuric acid stop solution (R&D Systems).

Antibody inhibition assays. PF4 was preincubated in the absence or presence of 2X excess RTO or KKO anti-hPF4 antibodies at room temperature for 25 mins. The antibody-PF4 mixture was then added to MAGI-CCR5 cells prior

to infection with the indicated luciferase reporter pseudovirus. After addition of pseudovirus, plates were spinoculated at 1,200 x g for 2 hr at 4°C and then incubated at 37°C. Infection inoculum was replaced with fresh complete media after 2 hr. Cells were then lysed with Brite-Glo (Promega) at 72 hr postinfection, and RLUs were measured.

Statistical analysis. Infection values obtained with or without PF4 treatment were compared using *t* tests. P values of less than 0.05 were considered significant. Data were analyzed with Prism 5.0 software.

Ethics statement. These studies were approved by the University of Pennsylvania's Institutional Review Board. All human cells used in this study were from normal healthy donors who provided written informed consent.

RESULTS

PF4 exhibits a biphasic effect on HIV-1 entry. Initial studies evaluating the effect of recombinant human PF4 on HIV-1 infection *in vitro* demonstrated that PF4 possesses broad antiviral activity against multiple HIV-1 genetic clades, irrespective of coreceptor tropism (15, 259). We utilized an independent panel of previously described (200, 295)14 clade B and 9 clade C HIV-1 Envs (Table 1) to further explore the antiviral properties of this chemokine. Of the 23 Envs tested, 18 were CCR5 (R5)-tropic, one was CXCR4 (X4)-tropic, and four were R5/X4 (dual)-tropic, and 12 of the 23 Envs were derived from transmitted/founder (T/F) viruses (96, 121, 131, 136, 200, 238, 239). Multinuclear activation of galactosidase indicator cells stably expressing human CD4 and CCR5 (MAGI-R5 cells) (53) were pretreated with increasing concentrations of recombinant PF4 for 30 minutes, and subsequently infected with HIV-1 Env pseudoviruses. PF4 inhibited infection by all HIV-1 Env pseudoviruses tested, with maximal inhibition occurring at approximately 200 nM added protein (mean % inhibition = 85 $\pm$ 6; $p < 0.0001$). T/F Envs were inhibited as efficiently as Envs derived from other viruses. However, when PF4 was added at concentrations above 200 nM, infection was inhibited less efficiently and was enhanced above (mean % infection = 174 $\pm$ 67; $p < 0.0001$) that of untreated controls at the highest concentration of PF4 tested (Figs 1A and 1B).

For our studies, we tested commercially available PF4 isolated from human platelets, commercially available recombinant PF4, as well as laboratory-produced recombinant PF4. We found that both recombinant forms of PF4

impacted HIV-1 infection identically over a broad concentration range, while the concentration needed for native PF4 to maximally inhibit (24 nM) and then enhance (200 nM) HIV-1 infection was approximately one-log less, perhaps reflecting inefficient refolding of the recombinant proteins. Nonetheless, as all three forms of PF4 exhibited similar biphasic effects on HIV-1 infection, we proceeded with the laboratory-developed recombinant PF4 for subsequent experiments.

The MAGI assay was also performed utilizing replication competent R5-, X4-, and dual-tropic HIV-1 (CH141, HxB, and R3A, respectively). As was observed with the pseudoviruses, 200 nM PF4 maximally inhibited infection of the clade C primary isolate HIV-1_{CH141} (mean % inhibition = 93 $\pm$ 4; P = 0.0007), the clade B primary isolate HIV-1_{R3A} (mean % inhibition = 86, $\pm$ 10; P = 0.0044), and the laboratory-adapted strain HIV-1_{HxB} (mean % inhibition = 90 $\pm$ 9; P = 0.0034). In contrast, at 4 μM, PF4 enhanced infection of all three viruses by 2-3 fold (Fig 1C). A saturating concentration of the membrane fusion inhibitor enfuvirtide (T20) was used as a negative control in these experiments, inhibiting infection of all three viruses by 94-99% of untreated control.

PF4 activity is evident on multiple cell types against a variety of viruses. To evaluate whether the activity of PF4 was specific to the MAGI-R5 cells, $CD4^+$ T cell lines stably expressing CCR5 (SupT1-R5 and Jurkat-R5) and primary human $CD4^+$ T cells were infected with either HIV-1 pseudoviruses or replication competent HIV-1 in the absence or presence of PF4. The biphasic activity of PF4 was observed in all cell types with all viruses tested (Fig 1D).

These results confirm the findings of previous studies (15, 259), that infection by R5- and X4-tropic HIV-1 strains is reduced in the presence of PF4. However, the inhibitory effects of PF4 were limited to a relatively narrow concentration range, above which viral infection *in vitro* was consistently enhanced.

To assess the specificity of anti-viral PF4 activity, we examined the ability of HIV-1 pseudotyped viruses bearing the envelope glycoproteins from $SIV_{sm}E660$, $SIV_{mac}316$, MLV, influenza (H5N1) or VSV to infect MAGI-R5 cells in the absence or presence of increasing concentrations of PF4. Infection by HIV-$1_{SIVmac316}$ and HIV-1_{MLV} was robustly inhibited by PF4 at approximately 200 nM (mean % inhibition = 84 $\pm$ 1; p value < 0.0001 and 95 $\pm$ 2; P = 0.0002, respectively), but significantly enhanced (mean % infection = 265 $\pm$ 71; p < 0.0212 and 286 $\pm$ 65; P = 0.0015, respectively) by 4 μM PF4. Infection by HIV-$1_{VSV\text{-}G}$ and HIV-$1_{SIVsmmE660}$ was inhibited to a lesser degree (mean % inhibition = 78 $\pm$ 5; P = 0.0013 and 70 $\pm$ 20; P = 0.0256, respectively) with inhibition being lost as PF4 concentrations were increased, while HIV-1_{H5N1} infection was unaffected by PF4 (mean % inhibition = 4 $\pm$ 8; P = 0.2483) (Fig 1E). Given that these pseudoviruses share a common HIV-1 core (NL4.3) yet were inhibited to varying degrees by PF4, we conclude that both the inhibitory as well as the viral enhancement effects of PF4 occur at the level of entry and are not restricted to HIV-1.

PF4 modulates viral entry by acting on viral attachment. To explore the mechanism of action by which PF4 modulates HIV-1 infection, we first performed time of addition experiments and found that, as previously reported

(15), PF4 most strongly inhibited HIV-1 when added before or simultaneously with the virus, consistent with it impacting HIV-1 entry (data not shown). To determine if PF4 impacts the first step of virus infection – binding of virions to the cell surface – we examined the ability of low and high concentrations of the chemokine to interfere with viral attachment. For this purpose, primary human $CD4^+$ T cells were exposed to HIV-1_{R3A} for 4 hr at 37°C in the presence or absence of PF4. Cells were then split into two aliquots; one aliquot was treated with trypsin to remove attached viral particles remaining on the cell surface, while the other aliquot was left untreated. Attachment was measured by quantifying total vs. trypsin-resistant cell-associated HIV-1 p24 Gag protein. When added at 200 nM, PF4 inhibited virus infection and also significantly reduced viral attachment to cells by 55% ± 9% compared to the no treatment control (P = 0.0008) (Fig 2). In contrast, the infection-enhancing concentration of PF4 (4 μM) increased viral attachment to primary cells by 61% ± 12% relative to the no treatment control (P = 0.0006). The polycation DEAE-dextran was used as a positive control and enhanced viral attachment 42-fold above the untreated control (P = 0.0004). The coreceptor small molecule antagonists maraviroc (MVC) and AMD-3100, which block viral engagement of CCR5 and CXCR4 respectively, had no significant effect on viral attachment as expected (Fig 2). These data suggest that low levels of PF4 inhibit viral infection by decreasing viral adsorption to cells, while higher concentrations of the chemokine enhance viral attachment and thus increase infection.

PF4 interacts with cellular GAGs. PF4 could potentially impact virus attachment by interacting with cell surface components, with the viral glycoprotein or with both. There is considerable evidence that many chemokines interact with cell surface GAGs which in turn facilitate attachment of many viruses to cells (106, 125, 213). To determine if PF4 may interact with cell surface molecules in a manner that impacts virus attachment, we treated MAGI-R5 cells with an inhibitory concentration of PF4 (200 nM) for 30 minutes, then either washed the cells with PBS (wash) or left unwashed (no wash), and subsequently infected with HIV-1 pseudovirus. We hypothesized that if PF4's mechanism of action entailed interactions with the viral glycoprotein, then pre-binding to the cell surface followed by washing should have little impact on virus infection. If, however, PF4 binds to a cell surface molecule that participates in virus attachment, then washing off pre-bound PF4 should reduce the chemokine's effect on virus infection depending on the affinity of the interaction. Pre-binding PF4 to the cell surface followed by extensive washing decreased subsequent infection by HIV-1_{MLV}, HIV-1_{JRFL}, and HIV-1_{VSV-G} by 45% ± 12% (P = 0.016), 51% ± 7% (P = 0.007), and 24% ± 16% (P = 0.13), respectively (Fig 3A); consistent with PF4 interacting with a cell surface molecule(s) to antagonize viral infection. As expected, pre-binding and then washing PF4 from cells had no effect on HIV-1_{H5N1} infection (mean % infection = 106 ± 9; P = 0.4080) (Fig 3A).

Since it is well established that PF4 binds to negatively charged GAGs such as heparan sulfate proteoglycans (HSP) and chondroitin sulfate proteoglycans (CSP),(141, 163, 205, 231) we performed competition assays

using soluble HSP. MAGI-R5 cells were infected with HIV-1 pseudoviruses in the presence or absence of increasing concentrations of soluble HSP and no, low (200 nM), or high (2 μM) concentrations of PF4. As the concentration of soluble HSP was increased, the ability of the low concentration of PF4 to inhibit infection waned, eventually reaching the same level as no PF4. In addition, high amounts of HSP ablated the capacity of the high concentration of PF4 to enhance infection (HIV-1_{JRFL} shown in Fig 3B). Similar results were observed with soluble CSP (data not shown). The fact that soluble HSP reduced the ability of PF4 to inhibit virus infection and entirely ablated the ability of high concentrations of PF4 to enhance virus infection could be linked to virus binding—at inhibitory concentrations of PF4, the presence of 10 μg/ml soluble HSP slightly increased virus binding (mean % cell-associated p24 200nM vs. 200nM + HSP = 55 ± 9 vs. 70 ± 9; $P = 0.3$), while at enhancing concentrations of PF4 the presence of HSP strongly reduced virus binding to the cell surface (mean % cell-associated p24 = 57 ± 7; $P < 0.0001$) (Fig 3C). These data are consistent with PF4 being able to bind to GAGs, particularly at high PF4 concentrations, with this in turn being linked to enhanced virus binding to the cell surface.

HIV-1 gp120 binds specifically to PF4. The ability of PF4 to bind to GAGs does not, by itself, explain how it can enhance virus infection at high concentrations. Therefore, we hypothesized that at high concentrations, PF4 might function as an electrostatic bridge between virions and cell surface GAGs to modulate attachment. If this is true, not only should PF4 interact with cell surface GAGs, it should also interact with the virus. To test this, we assessed the

ability of purified HIV-1 envelope glycoprotein gp120 to bind to polystyrene-immobilized PF4 by ELISA. Graded amounts of PF4 were immobilized overnight before incubation with varying concentrations of purified HIV-1_{JRFL} gp120 for 30 mins. After washing, bound gp120 was detected using a rabbit polyclonal serum (4). We found that gp120 bound to PF4 in a concentration-dependent manner (Fig 4A). Likewise, gp120 also bound to RANTES in a concentration-dependent manner, consistent with previous work (274). In contrast, gp120 failed to bind to immobilized IL-8 or SDF-1 using the same assay conditions (Fig 4B). From these results, we conclude that the bimolecular interaction observed between gp120 and PF4/RANTES is direct and has some degree of specificity.

PF4 oligomerization state correlates with its effects on virus infection. PF4 in solution exists in a dynamic state; at low concentrations it is largely monomeric, while at high concentrations it forms tetramers (20, 172, 179, 233). Chemical cross-linking was used to confirm this equilibrium using the recombinant PF4 used in our studies (217). Although this equilibrium exists in solution *in vitro*, under physiologic conditions it has been hypothesized that PF4 exists primarily as a tetramer and avidly binds to heparin and GAGs to form the ultra-large antigenic complexes noted in the clinical disorder of heparin-induced thrombocytopenia (HIT) (10, 20, 116, 172, 179, 217). Mutations that decrease the formation of PF4 tetramers have been shown to reduce the formation of these large complexes. Rauova et al. disrupted the ionic interactions between recombinant PF4 dimers by substituting Lys at position 50 with Glu to create $PF4^{K50E}$, which shifts the equilibrium of PF4 to favor dimers and monomers (217).

Additionally, a double mutant was generated in which the Glu^{28} and Lys^{50} in PF4 were replaced with Arg and Glu ($PF4^{E28R/K50E}$) to reinstate the ionic interactions between dimers, significantly restoring the ability to form tetramers at high concentrations (217). Given this concentration-dependent oligomerization of PF4 and its two opposing effects on viral entry, we reasoned that inhibition might be linked to the presence of monomers that bind directly to Env and prevent it from interacting with the cell surface, whereas PF4 tetramers may function to enhance viral infection by forming a bridge between cell surface GAGs and Env, overriding the inhibitory effect and thereby enhancing virus attachment.

To explore the effect of oligomeric state on PF4 activity, MAGI-R5 cells were infected with HIV-1 pseudoviruses in the absence or presence of increasing concentrations of recombinant $PF4^{WT}$, $PF4^{K50E}$, or $PF4^{E28R/K50E}$. For all viruses tested, $PF4^{K50E}$ exhibited an approximately 1-log increase in IC_{50} compared to $PF4^{WT}$, while the double mutant $PF4^{E28R/K50E}$ partially rescued this loss in potency (Fig 5A). As previously observed, HIV-1_{JRFL} and HIV-1_{MLV} infection was enhanced approximately 2-fold of untreated control (mean % infection = 210 ± 51 and 200 ± 32, respectively) at the highest concentration (4 μM) of $PF4^{WT}$ tested. Relative to $PF4^{WT}$, 4 μM of the monomer-favoring $PF4^{K50E}$ did not enhance, but rather inhibited HIV-1_{JRFL} and HIV-1_{MLV} infection (mean % inhibition = 66 ± 11; P = 0.028 and 63% ± 8%; P = 0.008, respectively), while the complementary mutant $PF4^{E28R/K50E}$ restored the enhancing activity (mean % infection = 121 ± 9; p =0.45 and 136% ± 18%; P = 0.16, respectively), although not to the levels of $PF4^{WT}$ (Fig 5A & 5B). Compared to the untreated control, HIV-1_{VSV-G} infection was

inhibited by 4 μM $PF4^{K50E}$ (mean % inhibition = 33 ± 9; *P* = 0.0154), but not significantly impacted by 4 μM $PF4^{WT}$ or $PF4^{E28R/K50E}$ (mean % infection = 97 ± 16; *P* = 0.89 and 104 ± 11; p =0.19, respectively) (Fig 5A & 5B). Additionally, we tested the ability of $PF4^{K50E}$ to modulate viral attachment as we had previously done with low and high concentrations of $PF4^{WT}$. As observed with the low concentration of $PF4^{WT}$, the monomer-favoring $PF4^{K50E}$ significantly decreased viral attachment at 4 μM compared to untreated controls (% cell-associated p24 = 35 ± 6; p < 0.0001) (Fig 5C). From these data, we conclude that the antiviral property of PF4 occurs under conditions where tetramers are not prevalent, while the presence of tetramers is associated with enhancement of viral infection.

Antibodies that recognize distinct oligomeric forms of PF4 were used to further probe the role of oligomeric state on PF4 activity during viral infection. The monoclonal anti-hPF4 antibody RTO selectively binds to PF4 monomers while the HIT-like monoclonal antibody KKO induces the oligomerization of PF4 tetramers and preferentially recognizes large complexes comprised of PF4 tetramers (13, 232). If monomers and dimers are responsible for the inhibitory effect of PF4 on virus infection, then both RTO and KKO should ablate the inhibitory effect of 200 nM PF4, which in fact we observed (Fig 6). However, at 4 μM PF4 where preformed tetramers predominate, the antibodies should differ in their effects—RTO should not impact PF4 activity since it preferentially binds monomers, while KKO may further enhance infectivity by inducing even greater PF4 oligomerization which enhances attachment of virus to the cell surface. We found this to be the case, with KKO further enhancing infectivity of both $HIV\text{-}1_{JRFL}$

and HIV-1_{MLV} pseudotypes in the presence of 4 μM PF4 (Fig 6). These results further support the hypothesis that oligomeric forms of PF4 enhance viral infection.

DISCUSSION

Increased platelet activation, thrombocytopenia, and thrombosis are complications associated with HIV-1 infection (10, 66, 113, 201, 251). Several studies describe an anti-viral role for chemokines released from activated platelets including RANTES and PF4 (15, 59, 259). RANTES can modulate virus infection through diverse mechanisms that are dependent upon its concentration. At low concentrations, RANTES can inhibit HIV infection by blocking its interaction with the viral coreceptor CCR5 (12, 40, 59, 166, 193, 256, 274, 275). At high, and likely supra-physiological concentrations, RANTES forms higher-order oligomers that bind to the viral Env protein as well as cell surface GAGs, enhancing virus attachment and infection, at least *in vitro* (274). RANTES can also modulate virus infection by transducing signals via CCR5 that over time render cells more permissive for virus replication (97). Likewise, we find that PF4 can both inhibit and enhance HIV-1 infection in a concentration-dependent manner, with enhancement being observed at PF4 concentrations likely to be found in proximity to cell surfaces.

Recent studies that examined the impact of PF4 on HIV-1 infection suggested that the *in vitro* inhibitory effects of PF4 could be exploited therapeutically (15, 259). Enhancement of infection by PF4 was not reported in these studies, though concentrations greater than 650 nM were not tested. However, a previous study by Schwartzkopff et al. showed that higher concentrations of PF4 (4 μM) actually enhanced HIV-1 infection in macrophages (249). The availability of recombinant PF4, PF4 mutations that impact its

oligomeric properties, and oligomeric-state specific anti-PF4 antibodies allowed us to more fully explore this chemokine's biphasic effects on HIV-1 infection. We confirmed earlier findings that at low concentrations PF4 inhibits HIV-1 infection by approximately 1-log, and linked this inhibition to the ability of PF4 to bind directly to the gp120 subunit of the viral Env protein and to decrease binding of virions to the cell surface. However, PF4 also inhibited infection by several other viruses that bear little similarity to HIV-1, and inhibited all of the genetically diverse HIV-1 strains that we tested indicting that it has broad anti-viral potency. From this we conclude that while PF4 can engage the viral Env protein, it likely does so in a relatively non-specific manner that may well limit its therapeutic use. Further, the fact that PF4 inhibited infection of all HIV-1 strains we tested *in vitro* suggests that it has not applied sufficient selective pressure *in vivo* to drive the development of widespread resistance.

PF4 exists in dynamic equilibrium in solution, where monomers assemble into tetramers via dimer intermediates in a concentration dependent manner (20, 172, 179, 233). However, it is likely that under physiological conditions PF4 exists predominantly as a tetramer complexed with GAGs (20, 116, 172, 179). The biphasic effect of PF4 on HIV-1 infection can be explained by its tendency to oligomerize at physiologically relevant concentrations and its ability to bind to cell surface GAGs in a manner analogous to RANTES (20, 116). As PF4 concentrations increased, with a concomitant shift toward tetrameric and higher-order complexes, the inhibitory capacity of PF4 waned and viral infection was ultimately enhanced. This finding was observed for all HIV-1 strains tested as

well as virions bearing the SIV and MLV glycoproteins. Collectively, these data suggest that *in vivo*, where PF4 likely exists primarily as a GAG-associated tetramer, the inhibitory effects of the monomeric chemokine are less likely to predominate.

The mechanism by which PF4 enhanced virus infection at high concentrations was again at the level of virus binding. Like most chemokines, oligomeric PF4 binds with high affinity to polyanionic GAGs such as heparin and heparan sulfate proteoglycans (HSP) (264). Since PF4 can also bind to the viral Env protein, we hypothesized that PF4 oligomers can function as an electrostatic bridge between virions and cell surface GAGs. In support of this hypothesis, we found that high concentrations of PF4 enhanced virus binding, and that the addition of soluble heparan and chondroitin sulfate proteoglycans significantly mitigated the enhancing, but not the inhibitory, effects of PF4 by decreasing viral attachment to cells. This is not entirely surprising, as PF4 tetramers have been shown to exhibit higher affinity for GAGs due to their favorable quaternary structure, which exposes a ring of basic amino acids (264, 305). Additionally, by promoting oligomerization, the HIT-like antibody KKO likely induced the formation of ultra large complexes of PF4 tetramers that further potentiated the basal enhancement effect of 4μM PF4 *in vitro.*

While RANTES and PF4 can both inhibit viral infection at low concentrations, the mechanisms are different: RANTES inhibits virus infection in a highly specific manner by interacting with the viral coreceptor while PF4 inhibits HIV-1 infection by binding to the viral Env protein. In contrast, both chemokines

can enhance virus infection at high concentrations by forming higher-ordered complexes and enhancing virus binding to cells through interactions with cell surface GAGs. However, while the concentrations at which RANTES enhances HIV-1 infection *in vitro* are unlikely to be found *in vivo*, it is likely that physiological concentrations of PF4 can enhance virus infection. These findings underscore the importance of examining the full spectrum of relevant concentrations when assessing the impact of chemokines on virus infection given their ability to form oligomers that in turn influence their interactions with cell surface molecules. In addition, it is possible that the enhancing effect of PF4 on virus infection could impact HIV-1 pathogenesis. PF4 is produced by megakaryocytes in the bone marrow leading to intramedullary release (Lambert M *et al.*, submitted), and regulates hematopoietic stem cell cycle activity (39). The presence of PF4 in the bone marrow could enhance HIV-1 infection of stem cells and other progenitors, contributing to the well-documented hematopoietic abnormalities that are common in HIV-infected individuals.

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FIGURES

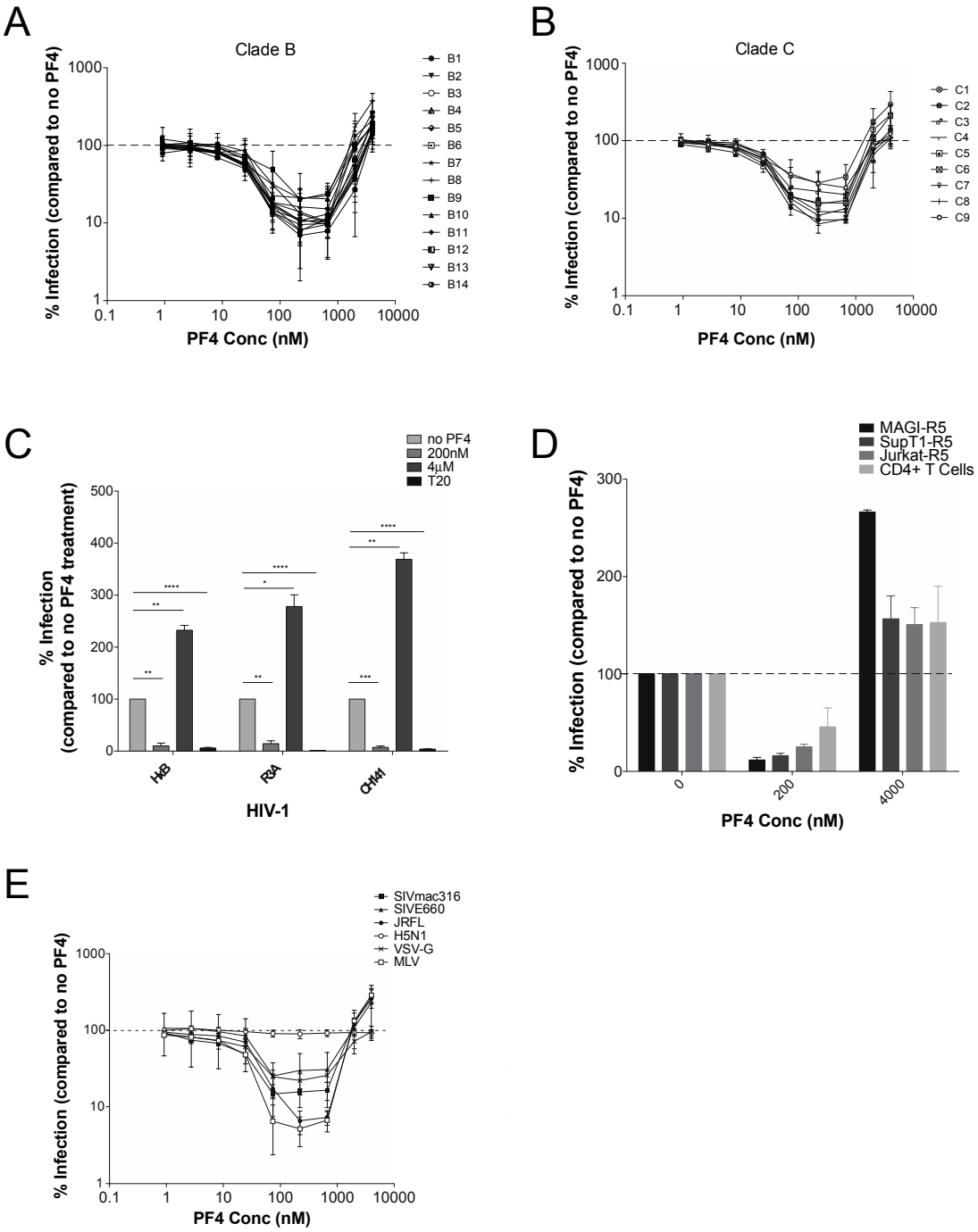

Figure 1: PF4 exhibits biphasic activity on viral entry. (A) 14 clade B and (B) 9 clade C HIV envelope pseudotypes were tested on MAGI-R5 cells in the

absence or presence of increasing concentrations of PF4. PF4 inhibited infected of all Env pseudoviruses tested at concentrations below 200nM. However, at concentrations above 2μM, infection of all viruses was enhanced 2-5 fold by PF4. (C) The dual activity of PF4 was recapitulated with CCR5- and CXCR4-using replication competent HIV-1. (D) PF4 exhibited biphasic activity against a replication competent dual-tropic primary HIV-1 isolate (CH077) on multiple cell lines. (E) The effect of PF4 was not specific to HIV-1 envelope as the dual activity was evident to varying degrees with HIV-1 pseudoviruses bearing the glycoprotein of SIVmac316, SIVE660, VSV, and MLV. HIV-1 pseudotypes bearing the influenza glycoprotein H5N1 infection was unaffected by the presence of PF4. All experiments were done in at least triplicate in each of at least three independent experiments. Error bars represent standard deviations. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$

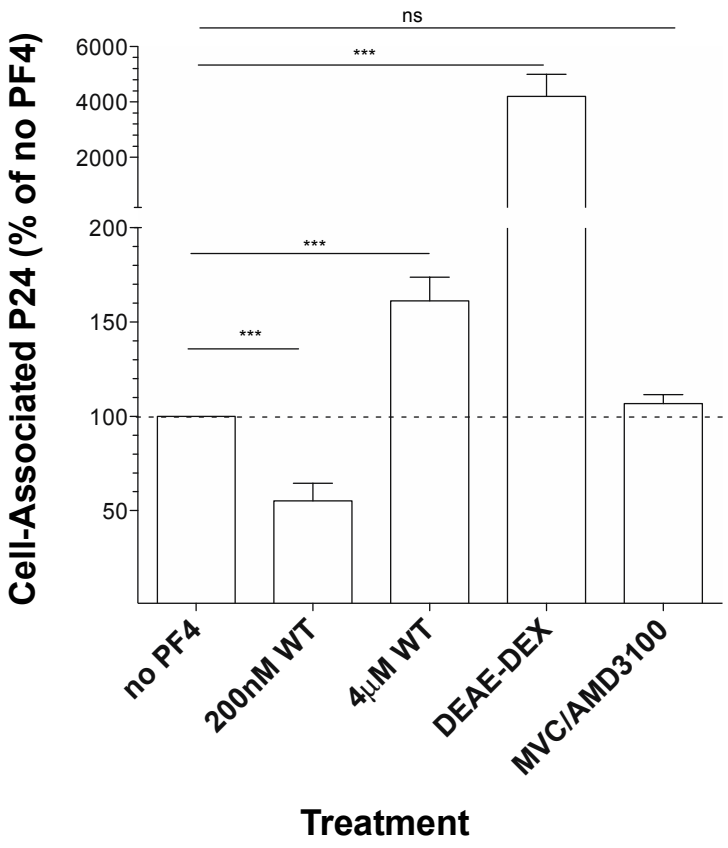

Figure 2: PF4 modulates viral attachment to cells. Replication competent HIV-1 R3A binding to the cell surface was assessed in the absence or presence of PF4. The inhibitory concentration of PF4 (200nM) significantly decreased viral attachment to cells compared to the untreated control (% cell-associated p24 = 55.2 ± 9.4; p = 0.0008). Contrastingly, the enhancing concentration of PF4 (4μM) significantly increased viral binding to cells compared to control (% cell-associated p24 = 161.3 ± 12.5; p = 0.0006). The positive control DEAE-dextran increased viral attachment approximately 42-fold above control (% cell-associated p24 = 4207.0 ± 790.7; p = 0.0004). The small molecule coreceptor antagonists maraviroc (MVC) and AMD3100 served as negative controls and did

not impact viral attachment as they work downstream of early attachment events. All experiments were done in duplicate with three donors in at least three independent experiments. Error bars represent standard error.

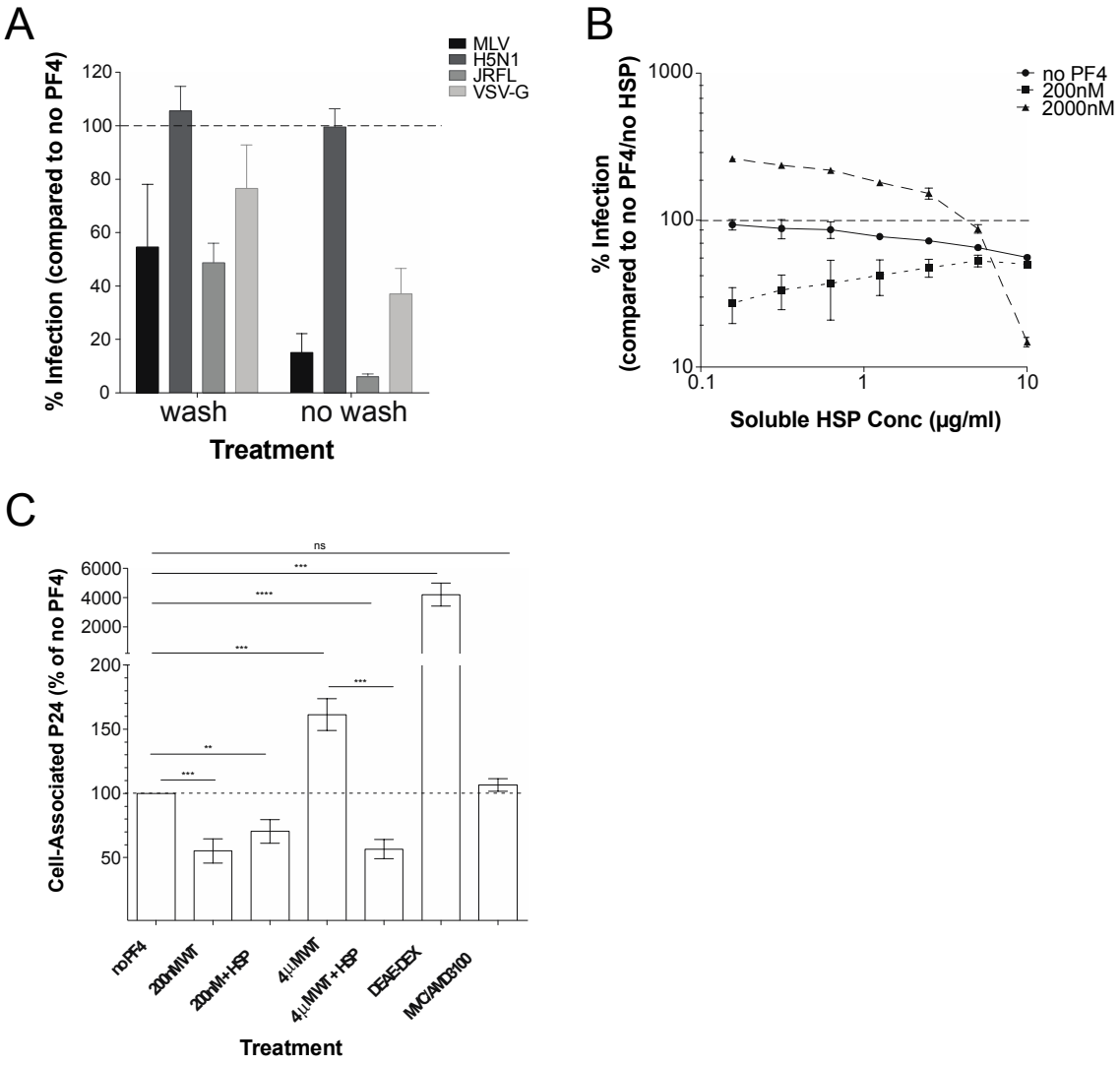

Figure 3: PF4 interacts with cell surface glycosaminoglycans. (A) To address whether PF4 interacts with a cell surface molecule to modulate infection, cells were pretreated with 200nM PF4 and either washed five times with PBS or left unwashed, followed by infection with HIV-1 pseudoviruses bearing the glycoprotein of either MLV, H5N1, JRFL, or VSV. Washing off PF4 before infection reduced entry by approximately 2-fold. Infection was inhibited at least 3-fold when PF4 was left on cells. The negative control HIV-1_{H5N1} infection was

unaffected by either treatment. (B) Effect of cell surface GAGs on PF4 activity. Infection of HIV-1_{JRFL} was measured in the presence of 0nM, 200nM, or 2000nM PF4 with increasing concentrations of soluble HSP. 10μg/ml HSP significantly diminished the effect of 2000nM PF4 on viral infection, impacting infection aprroximately 18-fold. HSP had a 2-fold effect on infection in the presence of low concentrations of PF4 and in the absence of PF4. (C) Addition of soluble heparan sulfate proteoglycan (HSP) to the low concentration of PF4 did not significantly impact its effect on viral binding. However, adding HSP to the high concentration of PF4 significantly decreased cell-associated virus to levels comparable to 200nM PF4. All experiments were done in at least triplicate in each of at least three independent experiments. Error bars represent standard deviations.

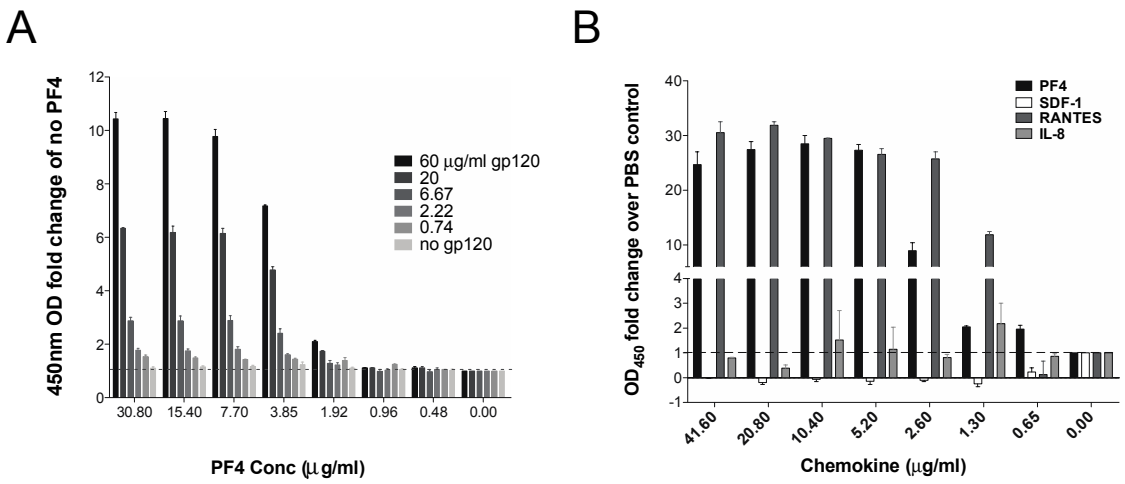

Figure 4: HIV-1 gp120 binds specifically to immobilized PF4. (A) Graded amounts of PF4 were immobilized overnight before incubating with varying concentrations of purified HIV-1 JRFL gp120. JRFL gp120 bound to PF4 in a concentration-dependent manner. (B) To assess selectivity of gp120 binding, we tested the ability of other chemokines of similar size and charge to PF4 to bind gp120. RANTES was used as a positive control and bound JRFL gp120. However, gp120 did not bind IL-8 or SDF-1. All experiments were done in at least triplicate in each of at least three independent experiments. Error bars represent standard deviations.

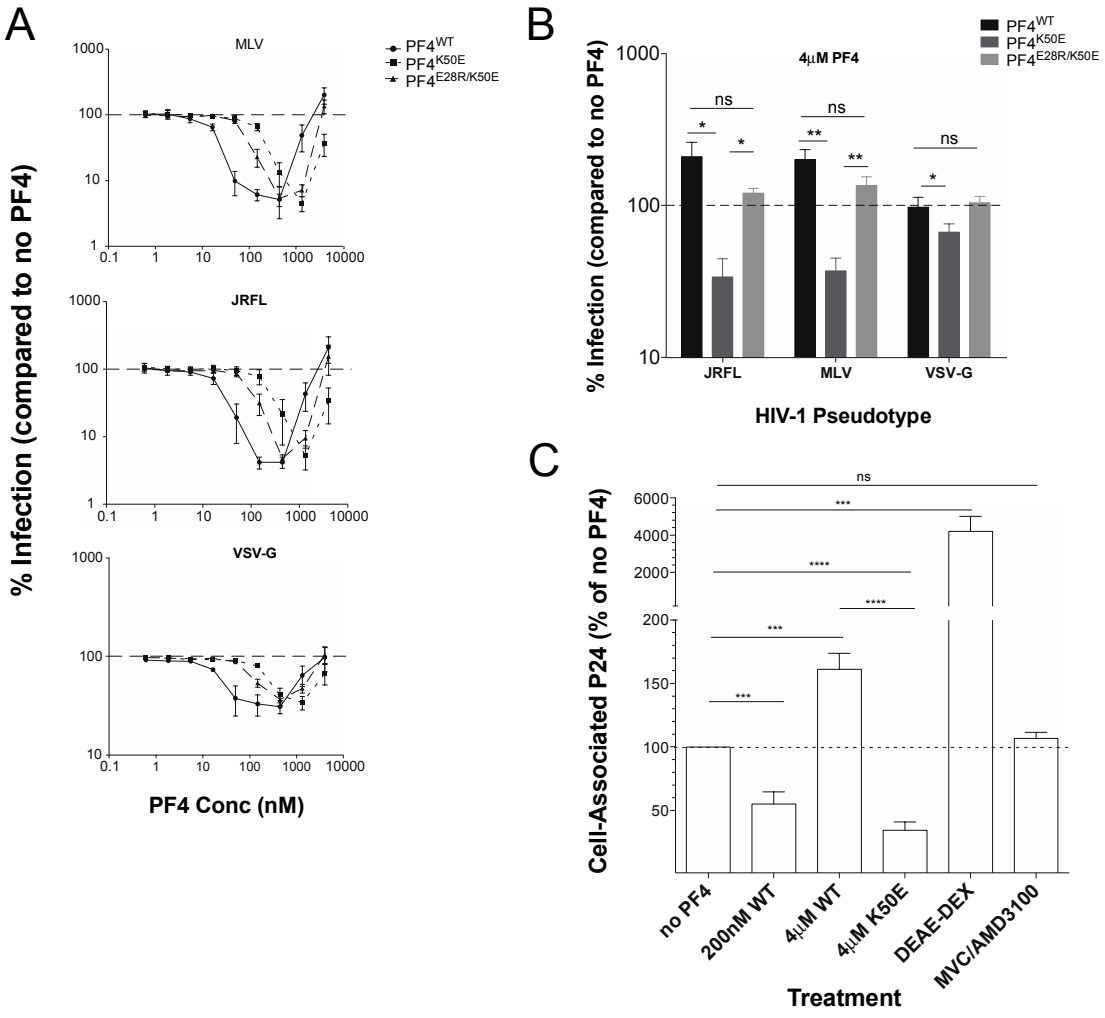

Figure 5: PF4 oligomeric state correlates with its biphasic activity on viral infection. (A) MAGI-R5 cells were infected with HIV-1 pseudoviruses bearing the glycoprotein of MLV (top), JRFL (middle), and VSV (bottom) in the absence or increasing presence of $PF4^{WT}$, $PF4^{K50E}$, or $PF4^{E28R/K50E}$. (B) Virus entry was measured at high (4μM) concentrations of $PF4^{WT}$, $PF4^{K50E}$, or $PF4^{E28R/K50E}$. PF4WT enhanced infection of $HIV\text{-}1_{JRFL}$ and $HIV\text{-}1_{MLV}$ approximately 2-fold. $PF4^{K50E}$ significantly inhibited $HIV\text{-}1_{JRFL}$, $HIV\text{-}1_{MLV}$, and $HIV\text{-}1_{VSV\text{-}G}$ entry.

$PF4^{E28R/K50E}$ restored the enhancement observed with $PF4^{WT}$. $PF4^{WT}$ and $PF4^{E28R/K50E}$ did not significantly impact HIV-1_{VSV-G} entry. (C) High concentrations of the monomer-favoring mutant $PF4^{K50E}$ significantly reduced viral binding to cells. All experiments were done in at least triplicate in each of at least three independent experiments. Error bars represent standard deviations. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$

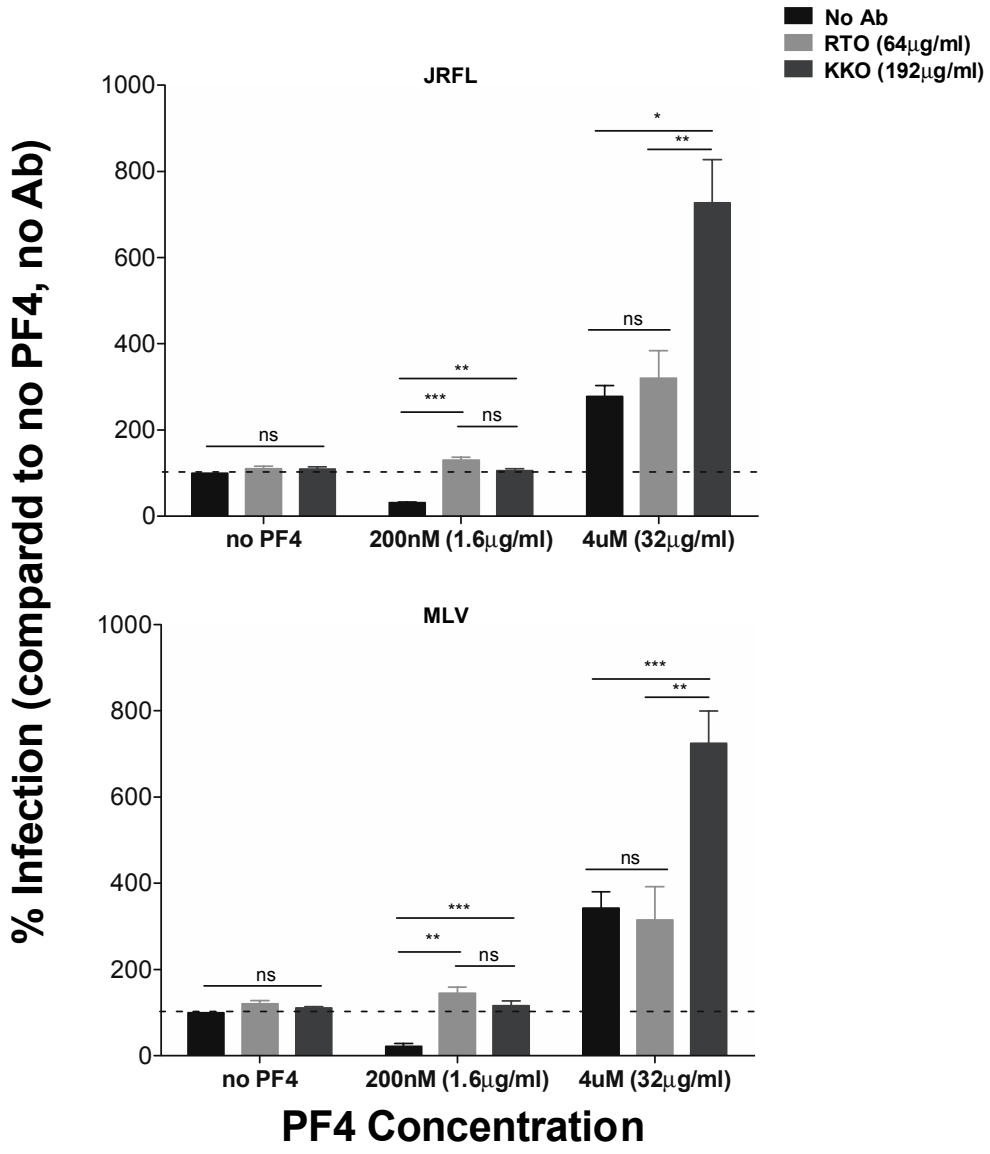

Figure 6: Antibodies recognizing distinct oligomeric forms of PF4 impact chemokine activity during infection. 0, 200nM, or 4μM PF4 was preincubated in the absence or presence of excess RTO or KKO anti-hPF4 antibodies. The mixture was then added to MAGI-R5 cells, followed by HIV-1_{JRFL} (top) or HIV-1_{MLV} (bottom) infection. For both pseudoviruses tested, RTO and KKO did not

significantly impact viral infection in the absence of PF4. However, both antibodies prevented 200nM PF4-mediated viral inhibition. At 4μM PF4, RTO did not impact viral enhancement compared to no antibody control. KKO further enhanced viral infection in the presence of 4μM PF4. All experiments were done in at least triplicate in each of at least three independent experiments. Error bars represent standard deviations. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0001$

TABLE I Summary of T/F and chronic HIV-1 *env* clones

Env Type	Clade	Numerical designation	Env clone designation	Coreceptor tropism	Reference	Accession number
T/F	B	1	REJO.D12.1972	R5	27	EU576707
	B	2	WEAUd15.410.5017	R5/X4	27	EU289202
	B	3	700010058.A4.4375	R5	27	EU576440
	B	4	700010077.SA2.6559	R5/X4	27	EU578999.2
	B	5	TT35P.11H8.2874	R5	27	EU577329
	B	6	1006-11.C3.1601	R5	27	EU575025
	B	7	1056-10.TA11.1826	R5	27	EU575305
	B	8	1058-11.B11.1550	R5/X4	27	EU289187
	C	1	2833264_3G11	R5	32	HQ595757
	C	2	1245045_3C7	R5	32	HQ595742
	C	3	ZM246F.F	R5	28-30	n/a
	C	4	ZM247Fv2.fs	R5	28-30	n/a
Chronic	B	9	CRPE.B28.4072	R5/X4	27	EU578065.1
	B	10	JOTO.TA1.2247	X4	27	EU578181.1
	B	11	OLLA.A14.1923	R5	27	EU578231
	B	12	SAMI.A8.1863	R5	27	EU578272
	B	13	SHKE.A26.4112	R5	27	EU578453
	B	14	1632-a6	R5	31	HQ216883
	C	5	704010330.G5h	R5	32	JQ777128
	C	6	702010141_w12_e80.F	R5	32	JQ779320
	C	7	704010461.A7h	R5	32	JQ777137
	C	8	4403.A18	R5	33	HM070677
	C	9	4403.bmL.B6	R5	33	HM070754

CHAPTER 5

Summary, discussion, and future directions

PROJECT I: T/F AND CC DIFFERENTIALLY UTILIZE CCR5

The concept of selective HIV-1 transmission was initially suggested by studies assessing envelope (*env)* diversity between donor-recipient pairs (transmission pairs) (299, 300, 304, 307). While these studies incited substantial interest and work in this area, they were limited by the fact that they were relatively qualitative in nature—chronically infected individuals were described as harboring genetically diverse viral quasispecies, and acutely-infected individuals could be grouped as having a “homogenous” or “heterogeneous” infection, born of a single donor viral variant or multiple closely-related donor variants, respectively. Thus, while it was apparent that HIV-1 transmission involved a genetic bottleneck, a more quantitative approach to enumerate and define the characteristics of transmitted viruses was required. The development of single-genome amplification (SGA) aimed to precisely identify and quantitatively characterize the transmitted/founder (T/F) virus(es). SGA involves limiting dilution of vRNA/cDNA, such that only a single template is amplified, followed by amplicon sequencing (131). SGA overcomes the limitations of conventional PCR followed by cloning and sequencing of the amplified template by eliminating *Taq* polymerase-induced recombination and ensuring proportional representation of target sequences (197, 238, 253, 255). Using this method, George Shaw and

colleagues were able to unambiguously infer the T/F *env* lineage of 98 out of 102 acutely-infected individuals; each lineage was characterized by a distinctive set of identical or near identical sequences (131). Subsequent cloning and *in vitro* characterization of pseudoviruses bearing each of these Envs, demonstrated that all T/F Envs were functional; capable of mediating CD4- and CCR5-dependent cell entry. This is expected as these Envs successfully initiated infection in a new host. In contrast, it is not uncommon to find nonfunctional Envs in cloned Envs from chronically infected individuals (136, 200, 240). Studying SGA-derived T/F Envs has revealed that the most consistent property associated with transmitted viruses is the use of the CCR5 coreceptor (24, 51, 121, 131).

CCR5 is a seven-transmembrane domain G-protein-coupled receptor (GPCR) that primarily functions as a chemokine receptor to regulate trafficking and the effector response of leukocytes. While CCR5 use is a major contributor to the genetic bottleneck, it is clear that on average one, or a select few, CCR5-using (R5-tropic) variants with specific genetic signatures (see **Chapter 1**) cross the mucosal epithelium to infect a new host. The use of CCR5 to mediate viral entry can be inhibited by the small molecule antagonist maraviroc (MVC). MVC, and other small molecule antagonists discussed in chapter 1, function as allosteric inhibitors, inducing a conformational change of CCR5, which prevents the viral envelope glycoprotein (Env) gp120 from engaging the coreceptor. However, some viruses become resistant to MVC by acquiring mutations that enable them to recognize and bind to the drug-bound conformation of CCR5. Thus, coreceptor conformation is clearly important. We know that, like other

GPCRs (139), CCR5 exists in different conformations, perhaps related to membrane microdomains (186, 187), differences in posttranslational modifications such as sulfation and glycosylation (50, 84), and coupling to G proteins (226, 269). We and others have shown that distinct CCR5 conformations can been detected on multiple cell types through the use of conformation-dependent antibodies (25, 153). Additionally, studies on MVC resistant viruses have shown variability in how viruses can productively engage CCR5—with some being more reliant on the amino terminus of CCR5, while others preferentially engage the second extracellular loop (3, 272). Moreover, some viruses are naturally resistant to CCR5-antagonists like MVC and can mediate entry via antagonist-bound CCR5 (108, 271). Given this background, it is logical to ask whether T/F viruses interact with CCR5 in a manner that is different from what is seen with viruses isolated from chronically infected individuals (chronic controls—CC). Understanding this phenotypic difference in coreceptor usage can provide insight into how Env-CCR5 interactions shape transmission fitness, and also inform the development of tests to better predict whether a patient will benefit from a CCR5-antagonist therapy.

While we know that there exists a continuum of CCR5 engagement by various HIV-1 strains, the implications of these interactions are unknown. We were interested in understanding how various viruses interact with CCR5—particularly, different conformations of CCR5, and whether this interaction was meaningful in the context of transmission. To this end, we evaluated a panel of 87 CCR5-using viruses (from MVC treatment-naïve patients) to address

differences between T/F and CC viruses in their ability to mediate entry via CCR5 in the presence of MVC. Our lab had previously reported no statistical difference in MVC sensitivity between subtypes B and C T/F and CC viruses on a microglial cell line—NP2/CD4/CCR5—stably expressing human CD4 and CCR5 (200, 295).

In project I, however, we described a difference in the magnitude of MVC inhibition of T/F and CC viruses that was readily apparent on cells with high surface levels of CCR5. We can modulate cell surface expression of CCR5 using the 293-derived Affinofile cell line (126). Affinofile cells expressing low or high levels of CCR5 were infected with each of 87 viruses in the absence or presence of increasing concentrations (to saturating levels—6μM) of MVC, after which we calculated the maximal percent inhibition (MPI). The MPI measures residual entry of resistant viruses in the presence of MVC. We imposed an arbitrary MPI cutoff of 95%, where MPI values >95% were considered fully sensitive (FS) to MVC, while samples with MPIs ≤95% were defined as partially resistant (PR) to MVC. All 87 viruses were inhibited by >95% at 6μM MVC on low-CCR5 Affinofiles. However, on high-CCR5 Affinofiles, only 66% of the viruses were inhibited by MVC by >95%, while 34% of the viruses exhibited PR in the presence of 6μM MVC, with some viruses exhibiting extensive MVC resistance. These results were confirmed using additional CCR5 antagonists, replication competent HIV-1, and on primary human $CD4^+$ T cells. When we parsed the viruses in our panel by Env type, we found a difference in the frequency with which T/F and CC viruses exhibited partial MVC resistance (15% and 52%, respectively). From these data, we conclude that at baseline, there is a

significant variability in MVC MPI among different viral isolates and that the magnitude of this variability is in part dependent on CCR5 expression. Furthermore, these results suggest that T/F and CC viruses differentially utilize CCR5 to mediate entry (111).

What does differential utilization of CCR5 signify in the context of transmission? The fact that the PR phenotype segregates with CC Envs suggests that this property is favored by the virus during a chronic infection, but disadvantageous to the virus (and likely selected against) during transmission and acute infection. One hypothesis is that at later stages of infection, when the virus is pressured by the host immune system and readily available target cell numbers precipitously decline, the ability to use multiple conformations of CCR5 could expand the availability of $CD4^+$ $CCR5^+$ target cells. Indeed, Paul Gorry and colleagues have shown that R5-tropic viruses become increasingly resistant to CCR5 inhibitors with time, consistent with changes in CCR5 use (100, 221).

Another hypothesis is that different conformational variants of CCR5 exist on mucosal targets cells that are readily available during acute infection/transmission, compared to $CD4^+$ $CCR5^+$ cells in the blood or other distal tissues later in infection. Employing a number of mAbs specific for different epitopes on CCR5, Berro et al (25) revealed the existence of multiple antigenic forms of CCR5 expressed on the surfaces of U87/CD4/CCR5 cells and $CD4^+$ T cells. The authors further showed that CCR5 antagonist-sensitive and -resistant viruses use the forms of coreceptor differentially for entry in the presence or absence of CCR5 antagonists. These results and our data suggest that

differences in CCR5 conformation may provide additional insights into the coreceptor determinants of the PR phenotype and the significance of this phenotype during HIV-1 transmission/pathogenesis.

Future studies should aim to define the molecular determinants of the PR phenotype and address the impact of differential CCR5 usage on mucosal transmission. First, chimeras can be used to elucidate the viral and coreceptor contribution to the PR phenotype by identifying Env and coreceptor molecular determinants of this phenotype. CCR5 is known to be conformationally dynamic, thus, comparing the conformational forms of CCR5 on target cells from mucosal tissue to those on blood-derived target cells using monoclonal antibodies will reveal whether there exists differences in CCR5 antigenic forms in distinct compartments. Next, it is important to investigate the role of this phenotypic difference during mucosal transmission *in vivo*. The bone marrow-liver-thymus (BLT) humanized mouse model may be a suitable system for these studies because it has been shown to be the only mouse model that leads to the generation of a human mucosal immune system. Additionally, the model has been shown to faithfully repopulate the mouse female reproductive tract with the appropriate human immune cells (DCs, Macrophages, and T cells) (72). Moreover, these mice exhibit high numbers of human immune cells in the blood, as well as organs and tissues (176). Together these experiments will define the mechanisms and implications of variable CCR5 usage during mucosal transmission, which may provide new insights into HIV-1 therapy.

PROJECT II: PF4 INHIBITS AND ENHANCES HIV-1 INFECTION

Recent studies have described an anti-viral role for platelets (259). These specialized anucleated blood cells release a number of chemokines to promote coagulation. One such platelet-derived chemokine is platelet factor-4 (PF4), which was recently described to posses potent and broad anti-HIV-1 activity (15, 259). However, despite *in vivo* plasma and local concentrations of PF4 being within the range used in these studies (36), HIV-1 is still able to establish productive infection. In this study, we sought to understand the mechanism of action of PF4 and whether it's *in vitro* activity was biologically meaningful in the context of human infection.

Utilizing a distinct panel of primary HIV-1 isolates and other viruses, we showed that the inhibitory effects of PF4 are not restricted to HIV-1 and constrained to a narrow concentration window. As the concentration of PF4 increased, the inhibitory capacity of the chemokine waned, and infection was ultimately enhanced. Although PF4 was broadly active against a number of different retroviruses, the fact that it exhibited modest effects on VSV-G entry and no effect on H5N1 infection suggests that the chemokine functions in a relatively non-specific manner. Using an ELISA assay, we demonstrated that HIV-1 Env gp120 binds specifically and directly to immobilized PF4. However, exactly where PF4 binds on the viral envelope glycoprotein remains unclear—Auerbach et al. suggest that PF4 interacts with HIV-1 gp120 at a site proximal to (but not including) the CD4 binding site to inhibit infection (15). However, since not all

PF4-sensitive viruses require CD4 for entry, the precise site of PF4 binding on the viral envelope glycoprotein may have more generalizable characteristics, such as net charge or quaternary structure.

Auerbach et al. and Tsegaye et al. demonstrated potent antiviral activity of recombinant PF4 and activated platelet supernatant, respectively. Neither study reported the enhancing activity of PF4 we observed, though concentrations above 650nM were not tested. However, consistent with what we report here, previous research by Schwartzkopff et al. showed that high amounts of PF4 (4μM) potentiated HIV-1 infection in macrophage-colony stimulating factor (M-CSF)-derived macrophages (249). The observation that PF4, at higher concentrations, enhanced viral infection is not surprising, as the enhancing role of certain CC-chemokines has been well characterized (97, 135, 183, 274). Trkola et al. reported that RANTES oligomers increase viral infectivity *in vitro* (274). The similarities between how RANTES and PF4 enhance viral infection are striking in that high concentrations (presumably oligomers) of both chemokines interact with glycosaminoglycans (GAGs) to increase viral attachment to the cell surface and subsequent infection. As was reported with RANTES, we also show specific interaction between HIV-1 gp120 and PF4, but not with other cationic chemokines (stromal derived factor-1 (SDF-1) and IL-8). Additionally, the effects of RANTES are also relatively non-specific as infection of HIV-1 pseudovirus bearing the glycoprotein of MLV or VSV is also enhanced.

There are key differences, however, between the two chemokines that influences their role in modulating viral infection. First, RANTES and PF4 belong

to different subfamilies of chemokines; the former belongs to the CC-subfamily and the latter to the CXC-chemokine subfamily. Structurally, CC-chemokines are defined by the tandem arrangement of cysteine residues in the amino terminus, while CXC-subfamily cysteines are separated by a single amino acid. This subtle structural difference informs the quaternary structure of the chemokine and has implications not only for receptor recognition, but also oligomer formation (123, 237). It is well known that chemokines oligomerize in a variety of homo- and hetero-oligomeric forms at high concentrations both in solution and in physiological settings to carry out their effector functions (16, 184, 185, 284). However, studies have reported differences in the quaternary structures of CC- and CXC-chemokines and suggested that these differences may be important for how chemokine subfamilies are recognized by, and signal through, their cognate receptors (123, 195, 216, 220, 286). To illustrate this point, Trkola et al. report that one mechanism by which RANTES enhances infection is by signaling through its G protein-coupled receptor CCR5. In addition to RANTES, previous research has shown that the α-chemokine SDF-1 enhances R5-tropic, but not X4-tropic, HIV-1 and VSV-G infection (169). Specifically, SDF-1 was reported to enhance infection via signaling-mediated increase of Tat transactivation of the HIV-1 long terminal repeat. In our study, however, two lines of evidence suggest that signaling via the PF4 receptor CXCR3B plays no appreciable role in the chemokine's activity during infection; first, time-of-addition experiments showed that infection was inhibited or enhanced when PF4 was added either before or simultaneously with virus. It is reasonable to assume that if signaling was critical,

only with PF4 pretreatment of cells (presumably allowing sufficient binding and signaling events to occur) would PF4 possess anti- or proviral effects. Second, pretreating cells with an antibody to PF4's receptor CXCR3B inhibited agonist-induced phosphorylation of Akt and p44/42 (Erk1/2), but did not ablate the biphasic activity of PF4 during viral infection (data not shown). It is possible that longer (>24 hrs) PF4 pretreatment could result in signaling-mediated effects on viral infection as observed with RANTES and SDF-1.

Another significant difference between RANTES and PF4 that influences their role in modulating viral infection is the range of *in vivo* concentrations. The concentrations at which RANTES enhances infection is supraphysiological and unlikely to be found *in vivo* (274). However, PF4 concentrations that enhance viral infection (> 2μM) *in vitro* are likely to be present *in vivo*, where concentrations at local sites of platelet degranulation can be as high at 10μM (308). This suggests that, unlike RANTES, PF4 could modulate HIV-1 pathogenesis by enhancing HIV-1 infection *in vivo*. Therefore, while RANTES, SDF-1, and PF4 similarly inhibit and enhance viral infection in a relatively non-specific manner, understanding the differences in how these chemokines execute the same functions may elucidate a new paradigm for how we think about chemokines and their role during viral infection, and might reveal new virus-chemokine interactions that could influence virus infection *in vivo.*

It is well documented that chemokines require interaction with GAGs *in vivo* to carry out their primary effector function of directing the migration of leukocytes to sites of injury or during usual immune surveillance (125, 144, 213).

Chemokine binding to GAGs on endothelial cells and extracellular matrix serves as a gradient to direct cell migration (2, 41, 227). Without this anchoring interaction, the chemokine would freely disperse, effectively lowering the concentration needed to activate its cognate receptor (213). This is verified by the fact that chemokines that are engineered to lack the GAG-binding domain can still interact with their cognate receptors, but fail to induce cell migration *in vivo* (5, 206, 213). Thus, it was logical to assume that GAGs likely play a role in the activity of PF4 during viral infection. Like most chemokines, PF4 binds with high affinity to polyanionic GAGs such as heparin and heparan sulfate proteoglycans (HSP) (156, 257). Moreover, it is secreted from activated platelets in conjunction with chondroitin sulfate proteoglycans (20, 116, 155). We showed that a cell surface molecule was partially involved in the activity of PF4 as evidenced by the fact that washing cells thoroughly post PF4 pretreatment before infection still decreased viral entry. Furthermore, we found that the addition of soluble GAGs (heparan and chondroitin sulfate proteoglycans) significantly mitigated the enhancing, but not the inhibitory, effect of PF4 by decreasing viral attachment to cells. This is not entirely surprising, as PF4 tetramers have been shown to exhibit higher affinity for GAGs due to their favorable quaternary structure, which exposes a ring of basic amino acids (264, 305). Since we observed the greatest impact of soluble GAGs in the presence of high concentrations of PF4, one might predict that uncoupling GAG-binding from PF4 would attenuate or ablate the tetramer-related enhancement of infection.

PF4 exists in dynamic equilibrium in solution, where monomers aggregate into tetramers via dimer intermediates in a concentration dependent manner (20, 172, 179, 233). However, under physiological conditions PF4 exists predominantly as a tetramer complexed with chondroitin sulfate proteoglycans (20, 116, 172, 179). Noticing that the biphasic activity of PF4 correlated with its concentration, we postulated that oligomeric state might contribute to the anti- and proviral effects. To address this, we utilized PF4 mutants that differ in their ability to oligomerize, while still retaining functionality (217). At comparably high concentrations (4μM), wildtype PF4 enhanced infection by HIV-1 JRFL, the monomer-favoring $PF4^{K50E}$ decreased infection, and the complementary mutant $PF4^{E28R/K50E}$ restored the enhancing phenotype. From this, we concluded that the monomeric state of PF4 correlated with inhibition, while the presence of tetrameric PF4 (or absence of monomers) was associated with increasing viral infection. If monomers were fully responsible for the inhibitory effect we observed, we would predict that the monomer-favoring $PF4^{K50E}$ would be at least as potent as wildtype PF4 in inhibiting infection. However, we noted that the K50E mutation reduced the potency of PF4, while the complementary mutant $PF4^{E28R/K50E}$ partially restored the loss in potency. As mentioned above, $PF4^{K50E}$ and $PF4^{E28R/K50E}$ are both able to bind heparin comparable to wildtype PF4 (217), thus this decrease in potency is not due to a defect in chemokine function. We cannot, however, rule out the possibility that introducing a mutation(s) into PF4 may affect other properties/domains of the protein that are important for its anti- or proviral activity, while only subtly affecting anionic-binding functionality.

Increased platelet activation and heparin-induced thrombocytopenia and thrombosis (HITT) are complications associated with HIV-1 infection (66, 201, 251). PF4 is produced by megakaryocytes in the bone marrow and regulates hematopoetic stem cell cycle activity. The presence of PF4 in the bone marrow could enhance HIV-1 infection of stem cells and other progenitors, contributing to the well-documented hematopoietic abnormalities that are common in HIV-infected individuals. The heightened activation of platelets leads to enhanced release of platelet-derived chemokines such as RANTES (113) and PF4. Previous studies examining the role of PF4 in HIV-1 infection have suggested that the potent *in vitro* inhibitory effects of PF4 may be exploited *in vivo* to prevent transmission. Though an attractive option, it may not be feasible given the data we describe in project II. In this study, we demonstrate that the inhibitory capacity of PF4 was confined to a narrow concentration range characterized by the prevalence of PF4 monomers. As monomers aggregate to form tetramers at high concentrations, we found that the inhibitory capacity of PF4 waned and viral infection was ultimately enhanced. Collectively, these data suggest that *in vivo*, where PF4 exists primarily as a GAG-associated tetramer, the inhibitory effects of the chemokine are less likely to predominate. In fact, enhancement of viral infection is most likely the physiological impact of PF4 during viral infection. Whereas Auerbach et al. reported that three of 13 primary HIV-1 isolates were resistant to PF4 inhibition, none of the 23 primary viruses used in this study showed a resistance phenotype. This suggests that PF4 has perhaps not applied sufficient pressure *in vivo* to drive the development of widespread resistance.

In vivo studies on the role of chemokines in a disease setting are inherently difficult to perform and can only prove association, not causation. However, further studies could examine the *in vivo* dynamics of PF4 and HIV-1 pathogenesis. Specifically, measuring endogenous PF4 production levels (both locally and systemically) and/or genetic polymorphisms in PF4 and the impact on HIV-1 infection. Additionally, retrospective studies can be performed on HIV-1 patients with platelet dysfunction to assess whether PF4 (and other platelet chemokines such as RANTES) levels correlate with disease progression and pathology. These studies would be informative for whether endogenous PF4 levels (in addition to $CD4^+$ T cell count) could be used as a prognostic factor in HIV-1 disease.

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