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HIV Transmission, Entry, and Gene Therapy

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Abstract
For HIV to enter cells, the viral surface protein Envelope (Env) must sequentially bind the host protein CD4 and one of two coreceptors, either CCR5 or CXCR4. This triggers conformational changes in Env that result in fusion of the host and viral membranes. Our understanding of this process has led to the development of successful anti-viral drugs and provided insights into viral pathogenesis. One critical question is whether we can further exploit our knowledge of the HIV entry process to develop an effective therapeutic vaccine. To do this, we need to better understand HIV-1 transmission, which may reveal viral properties that could be targeted in future vaccine efforts. In chapter two, we conduct a phenotypic comparison of clade B transmitted/founder (T/F) and chronic HIV-1 Envs in an attempt to reveal viral properties associated with successful transmission. We demonstrate that T/F Envs are more sensitive to neutralization by CD4 binding site antibodies and that this correlates with antibody binding suggesting T/F Envs have subtle but potentially important structural differences that may have implications for HIV-1 transmission and vaccine design. A second critical question is how can we provide long-term viral control in the absence of anti-retroviral therapy. Recently, an HIV-1 infected individual was 'cured' after receiving a bone marrow transplant from an uninfected donor who had a naturally occurring mutation in CCR5. This suggests genetic disruption of the HIV coreceptors may provide clinical benefit. Previously *ccr5*-specific zinc finger nucleases (R5-ZFNs) were developed to disrupt *ccr5* and engineer HIV-resistant cells. ZFNs are DNA binding proteins that specifically bind and cleave a specific 24 base pair DNA target. After cleavage, error-prone host DNA repair pathways often introduce mutations resulting in a non-functional gene product. Since 50% of late-stage HIV-infected people harbor virus that can use CXCR4, we developed *cxcr4*-specific ZFNs (X4-ZFNs) that safely and efficiently disrupt *cxcr4* conferring resistance to X4 HIV both *in vitro* and in humanized mice *in vivo*. Genome editing with ZFNs results in HIV-resistant cells that can be re-infused into a patients own body and hopefully confer therapeutic benefit.

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ABSTRACT

HIV TRANSMISSION, ENTRY, AND GENE THERAPY

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For HIV to enter cells, the viral surface protein Envelope (Env) must sequentially bind the host protein CD4 and one of two coreceptors, either CCR5 or CXCR4. This triggers conformational changes in Env that result in fusion of the host and viral membranes. Our understanding of this process has led to the development of successful anti-viral drugs and provided insights into viral pathogenesis. One critical question is whether we can further exploit our knowledge of the HIV entry process to develop an effective therapeutic vaccine. To do this, we need to better understand HIV-1 transmission, which may reveal viral properties that could be targeted in future vaccine efforts. In chapter two, we conduct a phenotypic comparison of clade B transmitted/founder (T/F) and chronic HIV-1 Envs in an attempt to reveal viral properties associated with successful transmission. We demonstrate that T/F Envs are more sensitive to neutralization by CD4 binding site antibodies and that this correlates with antibody binding suggesting T/F Envs have subtle but potentially important structural differences that may have implications for HIV-1 transmission and vaccine design. A second critical question is how can we provide long-term viral control in the absence of anti-retroviral therapy. Recently, an HIV-1 infected individual was ‘cured’ after receiving a bone marrow transplant from an uninfected donor who had a naturally occurring

mutation in CCR5. This suggests genetic disruption of the HIV coreceptors may provide clinical benefit. Previously *ccr5*-specific zinc finger nucleases (R5-ZFNs) were developed to disrupt *ccr5* and engineer HIV-resistant cells. ZFNs are DNA binding proteins that specifically bind and cleave a specific 24 base pair DNA target. After cleavage, error-prone host DNA repair pathways often introduce mutations resulting in a non-functional gene product. Since 50% of late-stage HIV-infected people harbor virus that can use CXCR4, we developed *cxcr4*-specific ZFNs (X4-ZFNs) that safely and efficiently disrupt *cxcr4* conferring resistance to X4 HIV both *in vitro* and in humanized mice *in vivo*. Genome editing with ZFNs results in HIV-resistant cells that can be re-infused into a patients own body and hopefully confer therapeutic benefit.

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Chapter 1

Introduction to HIV entry

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Abstract

The first step of the HIV replication cycle - binding and entry into the host cell - plays a major role in determining viral tropism and the ability of HIV to degrade the human immune system. HIV utilizes a complex series of steps to deliver its genome into the host cell cytoplasm while simultaneously evading the host immune response. To infect cells, the HIV protein Envelope (Env) binds to the primary cellular receptor CD4 and then to a cellular coreceptor. This sequential binding triggers fusion of the viral and host cell membranes, initiating infection. Revealing the mechanism of HIV entry has profound implications for viral tropism, transmission, pathogenesis, and therapeutic intervention. Here, we provide an overview into the mechanism of HIV entry, provide historical context to key discoveries, discuss recent advances, and speculate upon future directions in the field.

HIV entry fundamentals

HIV entry, the first phase of the viral replication cycle, begins with adhesion of virus to the host cell and ends with the fusion of the cell and viral membranes with subsequent delivery of the viral core into the cytoplasm. The intricate series of protein-protein interactions that ultimately results in virus infection can be divided into several phases, some of which are essential and others that may serve to modulate the efficiency of the process. First, virions must bind to the target cell, with this being mediated either by the viral envelope (Env) protein or host cell membrane proteins incorporated into the virion with any one of a number of various cell attachment factors. Attachment can be relatively non-specific, with Env interacting with negatively charged cell surface heparan sulfate proteoglycans [1], or can result from more specific interactions between Env and α4β7 integrin [2,3] or pattern recognition receptors such as DC-SIGN [4](reviewed in [5]. HIV attachment to the host cell via any of these factors likely brings Env into close proximity with the viral receptor CD4 and coreceptor, increasing the efficiency of infection [6] (Figure 1-1). However, attachment factors differ from receptors in that they are not essential, and while they augment infection *in vitro* their physiologic role *in vivo* remains unclear.

The second step of virus entry and the first absolutely required for infection entails binding of Env to its primary receptor, the host protein CD4 [7,8]. Env is a heavily glycosylated trimer of gp120 and gp41 heterodimers. The gp120 subunit is responsible for receptor binding. The gp120 contains five relatively conserved domains (C1-5) and five variable loops (V1-V5), named for their relative genetic heterogeneity. Each of the variable regions is comprised of a loop structure formed by a disulfide bond at its base,

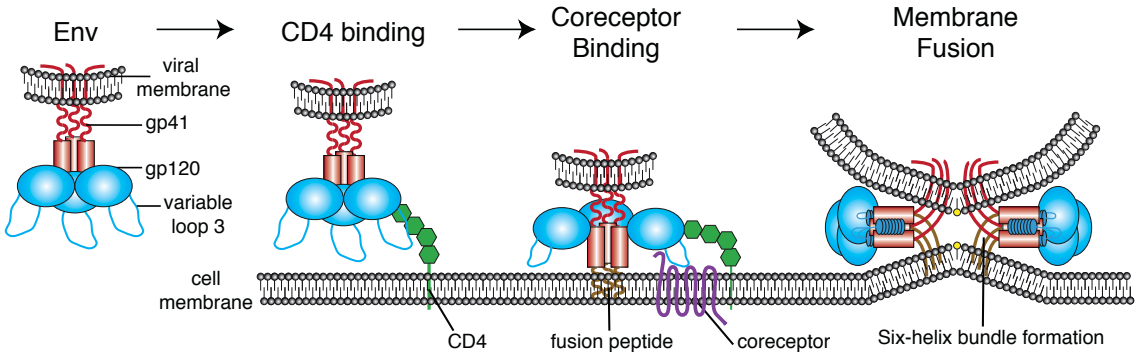

Figure 1-1. Overview of HIV entry. To deliver the viral payload into cells, HIV Env, comprised of gp120 and gp41 subunits (1) first attaches to the host cell, binding CD4 (2). This causes conformational changes in Env allowing coreceptor binding, which is mediated in part by the V3 loop of Env (3). This initiates the membrane fusion process as the fusion peptide of gp41 inserts into the target membrane, followed by six-helix bundle formation and complete membrane fusion (4).

with the exception of V5. The variable loops lie predominantly at the surface of gp120 and play critical roles in immune evasion and coreceptor binding, particularly the V3 loop (reviewed in [9]. CD4 is a member of the immunoglobulin superfamily that normally functions to enhance T cell receptor mediated signaling. Env interacts with the CD4 binding site (CD4bs) in gp120 [10]. Env binding to CD4 causes rearrangements of V1/V2 and subsequently V3. In addition, CD4 binding leads to formation of the bridging sheet, a four-stranded beta-sheet comprised of two double-stranded beta sheets that are spatially separated in the unliganded state [10,11]. The bridging sheet and repositioned V3 loop play critical roles in the next step of virus entry, coreceptor engagement.

The third step of virus entry, coreceptor binding, is widely thought to be the trigger that activates the membrane fusion potential of Env. HIV strains can be broadly classified based on their coreceptor usage. Viruses that use the chemokine receptor CCR5 are termed R5 HIV, those that use CXCR4 are termed X4 HIV, and viruses that can utilize both coreceptors are called R5X4 HIV [12]. There is no compelling evidence that coreceptors other than CCR5 and CXCR4 play important roles in supporting infection of HIV-1 *in vivo*. With rare exception, only R5 and R5X4 viruses are transmitted between individuals [13], likely due to multiple imperfect but overlapping host restrictions on X4 HIV transmission (reviewed in [14]). Interestingly, despite identification at earlier time points and despite high levels of CXCR4 expression on circulating HIV target cells, X4 or even R5X4 HIV rarely predominate until late in infection [15,16,17]. In addition, X4 viruses are less common in clade C HIV and SIV infection [18,19,20,21]. Several non-mutually exclusive models may explain this. First, clade B Envs may be different in their ability to adapt to CXCR4-tropism. Second, there may be differences in clade B host

biology. For instance, clade B hosts may have mitigated neutralizing antibody or cytotoxic T lymphocyte responses against X4 HIV compared to R5 HIV. Finally, clade B hosts most often live in developed countries and may face different environmental stresses including fewer or different chronic co-infections, which may increase target cell CCR5 expression. Elucidating the mechanism of coreceptor switch is a critical next step as it has implications for disease progression and therapy with HIV entry inhibitors.

A fourth step of virus entry is movement of the virus particle to the site where productive membrane fusion occurs. A series of recent studies have shown that a number of viruses usurp cellular transport pathways to reach specific destinations that are either needed for infection or that make entry more efficient, and that HIV might likewise utilize the host cell machinery to reach sites where membrane fusion can occur [22,23,24]. Some viruses have been shown to 'surf' along the cell surface, moving from distal sites of attachment to more proximal regions of the cell body where virus entry occurs. Retroviruses, including HIV, have been shown to utilize this process on some cell lines [24]. In addition, HIV may need to be internalized by the host cell's endocytic machinery in order for productive membrane fusion to occur, as will be discussed in a later section [25].

The fifth and final step of virus entry is membrane fusion mediated by Env. Coreceptor binding induces exposure of the hydrophobic gp41 fusion peptide, which inserts into the host cell membrane. This tethers the viral and host membranes, allowing the fusion peptide of each gp41 in the trimer to fold at a hinge region, bringing an N-terminal helical region (HR-N) and a C-terminal helical region (HR-C) from each gp41 subunit together to form a six-helix bundle (6HB) [26,27]. As the HR-N domain is in

close proximity to the host cell membrane due to the fusion peptide and the HR-C domain is in close proximity to the viral membrane due to the gp41 transmembrane domain, formation of the 6HB is the driving force that brings the opposing membranes into close apposition, resulting in the formation of a fusion pore (reviewed in [28]. Whether one or multiple HIV Env trimers are needed for complete membrane fusion is not yet clear. In summary, coreceptor binding unlocks the potential energy of the gp41 fusion complex resulting in 6HB formation, opening and stabilization of the membrane fusion pore, and subsequent delivery of the viral contents into the host cell cytoplasm.

Discovery of the HIV receptors

In 1981, several years before the discovery of HIV, Gottlieb and colleagues reported CD4+ T cell decline in four men who presented with Pneumocystis pneumonia and mucosal candidiasis, among other opportunistic infections [29]. Three years later it was demonstrated that HIV preferentially infects CD4+ T cells [30], and that infection is potently inhibited by CD4-specific antibodies (reviewed in [31]. CD4 was then shown to coimmunprecipitate with Env [7] and CD4 expression could rescue infection in some non-permissive cells [8]. However, CD4 transfection into mouse cells rescued binding of virus to the cell surface but not membrane fusion and virus infection, suggesting there were other required cofactors [8].

While the discovery of CD4 as the primary HIV receptor occurred shortly after the onset of the epidemic, it took more than a decade to discover the first coreceptor. In 1993, CD26 was reported as the elusive HIV coreceptor [32]; however, this was later disproved by several groups [33,34]. In 1995, Feng and colleagues conclusively

identified CXCR4 as a major HIV coreceptor by the use of an expression cloning strategy. A critical finding of this study was that CXCR4, then termed fusin, functioned as a coreceptor for what had been termed T cell line tropic strains of HIV but not for virus strains that could infect human macrophages but that failed to enter T cell lines [35,36]. The seminal discovery of CXCR4 as a G- protein coupled receptor in combination with the identification of the inhibitory effect of the β-chemokines CCL3 (MIP-1α), CCL4 (MIP1β), and CCL5 (RANTES) [37] on some virus isolates led to the simultaneous and rapid discovery of CCR5 as the coreceptor for macrophage tropic virus strains by five different groups [38,39,40,41,42].

The importance of the viral coreceptors for HIV infection *in vivo* was demonstrated by the discovery of a 32 base-pair deletion in *ccr5*, termed *ccr5Δ32*, that has an allelic frequency of approximately 10% in Caucasians [43,44,45]. The Δ32 mutation results in a premature stop codon in the second extracellular loop of CCR5 and subsequent retention of the mutant protein in the endoplasmic reticulum. Homozygosity for this polymorphism results in profound resistance to HIV infection, though several Δ32 homozygotes have been infected with X4 viruses [46,47,48]. In addition, heterozygosity confers partial protection to infection [43,45] and disease progression [45,49].

Elucidating the mechanism of HIV entry has directly translated into therapeutic benefit. Currently, there are two FDA-approved entry inhibitors, enfuvirtide and maraviroc, while others are in various stages of development. In 2003 enfuvirtide became the first licensed entry inhibitor; it is a 36 residue long peptide whose sequence is based on that of the HR-C in gp41. As a result, enfuvirtide behaves much like HR-C in that it binds to the HR-N prehairpin intermediate and inhibits 6HB formation and subsequent membrane fusion

[50,51]. While enfuvirtide is a highly specific and effective membrane fusion inhibitor [52,53], its use has been limited since it must be injected due to its lack of oral bioavailability. Recently, protease-resistant D-peptide fusion inhibitors have been developed that also prevent 6HB formation, which may overcome this limitation [54,55,56]. In addition to enfuvirtide, the CCR5 inhibitor maraviroc has been approved for clinical use. Maraviroc is a small molecule allosteric inhibitor that binds within the CCR5 transmembrane cavity resulting in conformational changes in the extracellular loop domains of the chemokine receptor that interact with Env [57]. Similar CCR5 small molecule inhibitors are in various stages of testing (reviewed in [58].

Key recent advances

Our understanding of the HIV entry process is derived largely from structural and *in vitro* studies. As the field has evolved, there is now increased emphasis on placing the now rather well understood membrane fusion reaction in a cellular context, asking where and when virus entry takes place as well as on how virus particles are transferred between cells. Increased structural detail continues to provide insight into the entry process and suggests targets for small molecule inhibitors and neutralizing antibodies. Finally, attempts to recapitulate the *ccr5Δ32* phenotype have been developed with some being brought forward to early stage clinical development [59].

New structural information. A full understanding of the HIV entry process requires detailed structural information. The structure of CD4 alone and in complex with a gp120 core-fragment has been solved for HIV [10,60] and SIV [11]. The structure of the post-fusion 6HB in gp41 has also been determined [26,27]. What has been lacking is

a structure of the native Env trimer and the HIV coreceptors. However, Wu et al recently described five independent structures of CXCR4 bound to two different small molecule antagonists which have given insight into both the tertiary and quaternary structure of the native protein [61] (Figure 1-2). First, both chemokines and Env have have been reported to engage CCR5 and CXCR4 in a two-site model with the chemokine receptor N-terminus as site one and the extracellular loops (ECLs), particularly ECL2 as site two. While the orientation of the CXCR4 N-terminal domain could not be solved due to structural flexibility, the crystal structure provides high-resolution insight into the ECL2 binding site. Second, all five structures portray CXCR4 as a homodimer, which is consistent with biochemical studies that have suggested CXCR4 exists as an oligomer in the host cell membrane [62]. While the implications of CXCR4 dimerization remain unclear for HIV infection, it may explain the dominant phenotype of a C-terminal CXCR4 human mutation that results in WHIM syndrome that is characterized by warts, hypogammaglobulinemia, infections, and myelokathexis (retention of neutrophils in the bone marrow) [63]. Finally, the identified homodimer interface may represent a novel CXCR4 or potentially CCR5 drug target, as CCR5 and CXCR4 have been reported to heterodimerize *in vivo* [64,65]. Further structural studies are needed to better define the precise interactions of Env and coreceptor and to assess the mechanisms of signaling and heterodimerization with other chemokine receptors.

Where does virus entry occur? The entry of viruses into cells is controlled in both time and space, with these parameters being regulated by host cell factors that serve

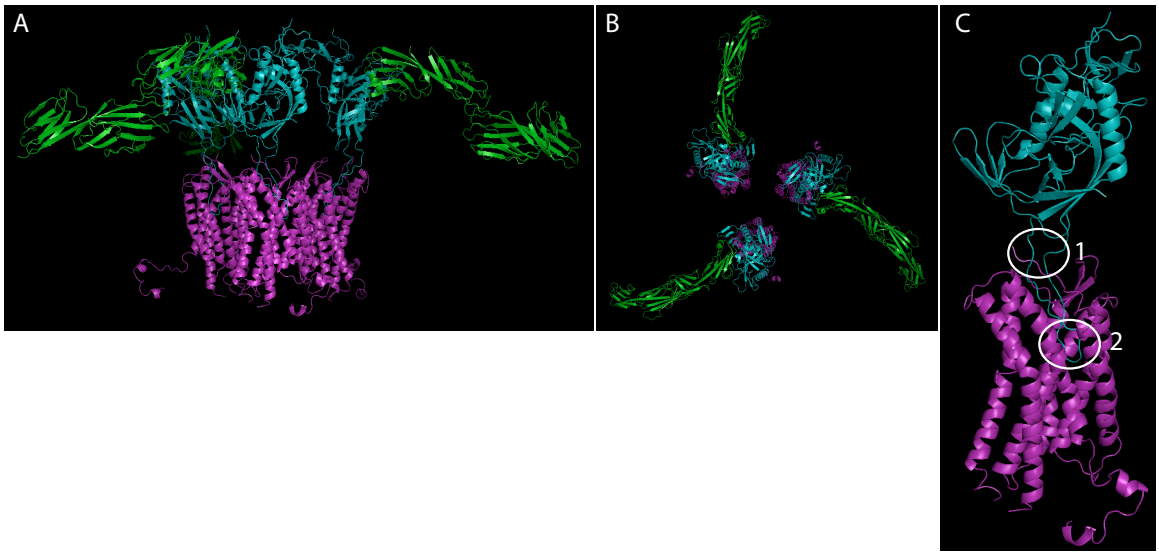

Figure 1-2. Model of gp120 engagement of CD4 and CXCR4. Recent structural studies have enhanced our understanding of the molecular interactions between gp120 (cyan) and its receptors. Here CD4 (green) and CXCR4 (purple), shown as monomers for clarity, are shown simultaneously binding to gp120. (A) Lateral view (B) Top view. However, the number of CD4 and coreceptor molecules required to interact with Env to mediate productive fusion remains unknown. (C) Gp120 has two key interactions with coreceptor. (1) The base of the V3 loop binds to the N-terminal domain of the coreceptor while the tip of the V3 loop binds to the second extracellular loop (ECL2). While both interactions are important, viral strains differ on their dependency of each interaction. Structural model generated by [61].

to unlock the membrane fusion potential of viral membrane proteins. Many viruses require delivery by the host cell into an acidic, intracellular compartment where low pH triggers membrane fusion-inducing conformational changes (reviewed in [66]. HIV entry does not require low pH, instead being triggered by receptor engagement [67]. The fact that HIV does not require low pH for cellular entry does not imply that fusion occurs at the cell surface. In fact, no spatial information is provided by the triggering mechanism. Despite this, it was often assumed that HIV fuses at the cell surface due to several observations (reviewed in [68]}. First, Env expression on the cell surface can mediate cell-to-cell fusion, indicating not only that Env is the only viral membrane protein needed to elicit fusion but that low pH is clearly not required. Second, very early studies on HIV entry showed that lysomotropic agents, which increase endosomal pH, do not inhibit HIV infection [69]. Third, inhibiting endocytosis of CD4 in cell lines by mutating its cytoplasmic domain does not affect HIV infection [70]. Together, these studies show that HIV entry is not pH dependent, but provide no definitive information as to whether fusion occurs at the cell surface or from within endocytic vesicles, albeit in a pH-independent fashion.

The question of where HIV-membrane fusion occurs has recently been re-examined [25]. By combining lipid and content mixing assays with single virion fluorescent imaging, Miyauchi et al tracked the location of virus membrane fusion in HeLa cells over-expressing CD4, CCR5, and CXCR4. They found that while lipid mixing can occur at the cell surface, content mixing only occurred in intracellular perinuclear compartments, and thus concluded that complete fusion requires endocytosis. Whether

this is always the case remains to be determined as the genetic variability of HIV and the diverse cell types it can infect make generalization difficult.

An interesting question is whether the site of entry matters. With regards to the use of entry inhibitors, probably not: both coreceptor antagonists and fusion inhibitors block virus infection *in vitro* and *in vivo*, and neutralizing antibodies clearly function as well. However, the site of entry is more likely to have an impact on the likelihood of a productive infection actually occurring. For instance, after cellular attachment, HIV can actively 'surf' along the cellular membrane from filopodia or microvilli to the cell body. This actin-dependent process requires receptor engagement and serves to enhance infection efficiency. Surfing towards the cell body may have several favorable consequences for the virus. First, it may facilitate endocytic HIV uptake. Second, it may bring the virus to a membrane region that has higher levels of coreceptor or important downstream signaling molecules [71]. Third, it may allow the fusion event to occur closer to the nucleus, which is the ultimate target of HIV. Thus, the site of initial HIV attachment is likely random; however, HIV hijacks the cellular machinery to traverse the cell membrane to a more favorable site of entry, be it at the plasma membrane or endosome, which ultimately serves to augment infection efficiency [24].

Cell-cell transfer and the virological synapse. *In vitro*, the rate-limiting step of virus infection is attachment to the host cell. *In vivo*, newly produced virions may well encounter an immediately adjoining, uninfected cell. In some cases, transfer of virus from one cell to another is a specialized process, as in the case of dendritic cells (DCs), which are professional antigen presenting cells that scavenge the periphery, sampling antigen. They are commonly found in the mucosa, and thus may be encountered by HIV

during transmission. Upon antigen binding, DCs migrate to the lymph nodes, process, and present the antigen to T cells to trigger an adaptive immune response. DCs are relatively resistant to productive HIV infection due to a combination of low CD4 and coreceptor expression, host restriction factors, post-integration HIV transcription blocks, and other unknown factors [72]. However, they express a diverse range of attachment factors that facilitate the internalization and processing of pathogens prior to antigen presentation. HIV, along with other viruses [73,74], can take advantage of this pathway to augment infection efficiency and dissemination (reviewed in [75]; Figure 1-3). Cameron et al. first demonstrated that DCs could catalyze HIV infection of co-cultured CD4+ T cells without themselves getting productively infected [76]. Each DC can bind up to several hundred virions [71] most likely via a C-type lectin such as DC-SIGN [4,77]. After binding, the virions are endocytosed into a trypsin-resistant compartment [4], and then upon DC binding to a T cell, internalized virus migrates to the DC:T cell interface [71] where it encounters the T cell membrane forming the infectious synapse, analogous to the immunologic synapse that forms upon MHC-T cell receptor binding (reviewed in [78]). In addition to efficiently concentrating and presenting HIV at the site of T cell contact, the infectious synapse is characterized by recruitment of CD4, CCR5, and CXCR4.

Recent advances in electron microscopy have enabled 3D-structural studies of the infectious synapse that have shed light on this mechanism [79]. DCs produce membranous protrusions that engulf the surrounding extracellular environment, trapping virions in a surface-accessible but protected compartment. It remains unclear as to whether this occurs before or after virion binding and whether it is Env-induced. When

CD4+ T cells contact DCs, they extend filopodia, enriched for CD4 and coreceptor, into the invaginated DC compartments that contain bound virions (Figure 1-3). Together, the efficient binding of HIV, relocalization to the point of CD4+ T cell contact, and the recruitment of the requisite HIV entry receptors promote HIV infection at the infectious synapse [71,80].

A novel attachment factor: α4β7 integrin. While cell-to-cell transmission of HIV augments infection efficiency, the mechanism of virological synapse formation remains unclear. α4β7 integrin has been reported to bind gp120, induce activation of LFA-1 (αLβ2 integrin), which contributes to formation of the immunologic synapse (reviewed in [81]), and subsequently augment infection efficiency *in vitro* [2,3].

α4β7 is a heterodimeric protein comprised of an α4 and β7 subunit that when expressed on CD4+ T cells facilitates homing to the gut and other mucosal tissues. Its activation and expression are upregulated by retinoic acid *in vitro,* which may also be locally secreted by mucosal DCs *in vivo*. The discovery of α4β7 as an attachment factor is of particular interest since HIV disrupts the integrity of the mucosal barrier and preferentially depletes gut CD4+ T cells, which are more activated and express higher levels of CCR5 than peripheral CD4+ T cells. α4β7 is thought to bind an LDV tripeptide motif on the second variable loop (V2) of gp120, with this resulting in LFA-1 activation. In addition, α4β7 colocalizes with CD4 and CCR5 at the virological synapse, which may further enhance infection. Blockade of α4β7 with monoclonal antibodies or a peptide delays replication of HIV *in vitro* further supporting its role in HIV infection [3]. Future work is needed to assess whether there are protective effects of inhibiting HIV-α4β7 interactions *in vivo* and to validate this novel attachment factor as a therapeutic target.

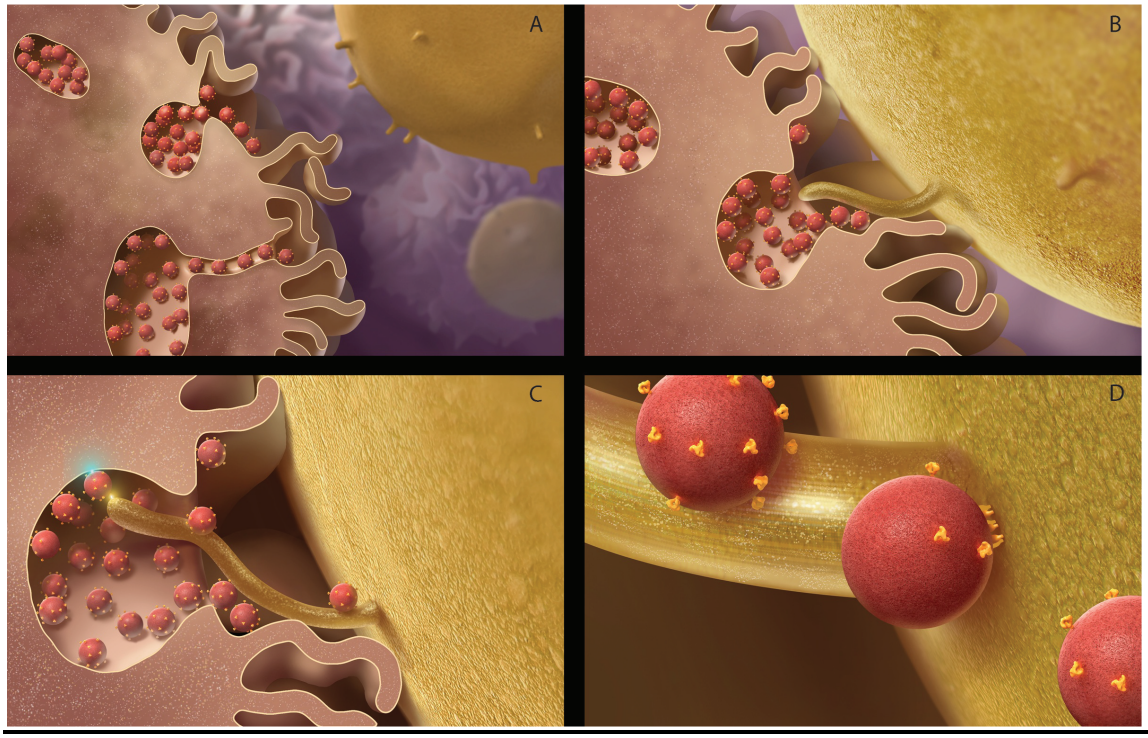

Figure 1-3. Model of DC-mediated *trans*-infection of CD4+ T cells. (A) DCs capture and concentrate virions in trypsin-resistant surface accessible compartments. (B) CD4+ T cells, containing membrane protrusions, bind DCs (C) The CD4+ T cell protrusions invade the virus-containing compartments and efficiently bind HIV. (D) Virus then migrates towards the cell body to initiate infection. Figure reproduced with permission [79].

Inhibitors of HIV entry

While studying HIV entry has informed us about basic principles of virology and cell biology, the overarching goal should be to develop novel and effective therapeutics to limit the morbidity and mortality of the HIV/AIDS pandemic. Currently, two entry inhibitors, the CCR5 inhibitor maraviroc and the fusion inhibitor enfuvirtide, are FDA-approved for the treatment of HIV infection. A number of other compounds that have targeted nearly every step of the entry pathway have also been tested in the clinic. For purposes of this discussion on therapeutics, HIV entry will be divided into three components: (1) attachment and CD4 binding, (2) coreceptor binding, and (3) membrane fusion.

Attachment and CD4 binding inhibitors. Attachment and CD4 binding inhibitors include relatively non-specific anionic polymers, CD4 binding site inhibitors, soluble CD4 mimetics, and CD4 down-modulators. Anionic polymers, which act by preventing the favorable electrostatic interactions between the positively charged Env and negatively charged cell surface, have been predominantly studied for use in vaginal microbicides. PRO2000, a naphthalene sulfonate polymer, inhibits soluble gp120 and CD4 binding *in vitro* [82]. However, in a phase III clinical microbicide trial, PRO2000 demonstrated no efficacy [83]. Other anionic polymers including cellulose sulfate and Carraguard, derived from seaweed, demonstrated no efficacy and cellulose sulfate may have actually increased the risk of HIV transmission [84,85,86].

Another approach involves targeting the CD4 binding site on gp120. Several small molecules that bind to gp120, such as BMS-378806 and BMS-488043, have anti-viral activity *in vitro* and prevent CD4-gp120 binding [87,88]. However, for at least BMS-

378806, HIV quickly adapts resistance limiting its therapeutic potential [88]. Furthermore, soluble CD4 (sCD4) demonstrated anti-HIV activity *in vitro,* but early stage clinical trials were unable to demonstrate antiviral activity, most likely due to insufficient circulating concentrations of sCD4 [89]. An additional class of compounds inhibits Env-CD4 interactions by down-regulating CD4. These drugs, derivatives of cyclotrizadisulfonamide (CADA) [90] reduce CD4 expression by an unknown mechanism. However, they do not alter CD4 mRNA levels suggesting they exert their function in a post-transcriptional manner [91].

Coreceptor binding inhibitors. Discovery of the ccr5Δ32 mutation demonstrated that CCR5 is not essential for normal human growth and development, suggesting that it could be safely targeted by small molecule inhibitors [43,44,45]. Several small molecule CCR5 antagonists have been developed and shown to have antiviral activity in vivo, including maraviroc, which received FDA approval in 2007 for use in treating HIV-infected individuals. Most small molecule CCR5 inhibitors, including maraviroc, function by binding to a hydrophobic pocket within the transmembrane domains of the protein - a region of the receptor not thought to directly interact with the viral Env protein [58,92,93]. As a result, CCR5 antagonists likely function by an allosteric mechanism, inducing conformational changes in the ECL domains of the receptor that subsequently prevent Env binding. Viral resistance to such compounds occurs by one of two pathways. In vivo, it appears that the most common resistance pathway is outgrowth of CXCR4-using viruses, even when present below the limit of detection in standard assays at the initiation of therapy. A second, less common pathway results from mutations in Env that enable it to utilize the drug-bound conformation of the receptor [94]. In at least

some cases, it appears that enhanced utilization of the CCR5 N-terminal domain is associated with this phenotype. In addition to traditional small molecule inhibitors, CCR5 blocking antibodies are being explored for therapeutic purposes. One such antibody, PRO140, blocks HIV utilization of CCR5 while preserving CCR5 ligand function. PRO140 is currently in phase II clinical trials [58,95].

Unlike for CCR5, inhibiting CXCR4 has been met with limited success primarily due to the concerns of systemic toxicity. Several CXCR4 inhibitors advanced to early stage clinical trials but none are currently ongoing for the treatment of HIV. One CXCR4 inhibitor, plerixafor, was recently FDA approved to mobilize hematopoetic stem cells to the peripheral blood for harvesting prior to bone marrow transplantation [96].

Fusion inhibitors. Membrane fusion is the net result of Env-receptor interactions, and is the target of the first entry inhibitor ever approved, enfuvirtide. Enfuvirtide, previously known as T20, is a 36 amino acid mimetic of the HR-C domain. The peptide binds the central coiled coils comprised of three HR-N molecules and inhibits 6HB formation, thus preventing fusion [50,51]. Despite the in vivo efficacy of enfuvirtide, resistance mutations in a ten amino acid region of HR-N that prevents enfuvirtide binding have been well documented [97]. Importantly though, resistance to enfuvirtide does not confer cross-resistance to other classes of entry inhibitors [98]. Novel fusion peptide-based inhibitors have been designed to combat enfuvirtide resistant viruses and some of these molecules have synergistic effects with enfuvirtide [99,100,101].

While these peptide-based fusion inhibitors exhibit efficacy, they are limited by the fact that are not orally bioavailable and therefore must be injected, a significant hindrance in maintaining patient adherence. One potential solution is the development of orally

bioavailable small molecules that recapitulate enfuvirtide's mechanism of action by blocking the hydrophobic "knob-into-hole" interactions. The knobs are hydrophobic HR-C residues, specifically tryptophans and isoleucines, that pack into large hydrophobic holes present in the HR-N central coil [26]. D-peptide inhibitors of gp120 represent one such exciting new class of compounds. They have potent in vitro activity and are not degraded by intestinal proteases and thus have the potential to be orally bioavailable [56].

Critical remaining questions

Work on the mechanisms of HIV entry has led to the discovery of human mutations affecting HIV susceptibility and disease progression as well as the development of new antiviral agents, such as enfuvirtide and maraviroc. In addition to the continued development of entry inhibitors, a critical future challenge is translating our molecular understanding of HIV entry into therapeutically useful information. Two critically important goals are the development of a preventative HIV-1 vaccine and a treatment that can functionally "cure" HIV-1 infected individuals. In this thesis, we attempt to make progress towards both of these goals.

HIV transmission is a relatively inefficient process characterized by infection in roughly 1 per 1,000 coital acts [102,103]. In approximately 80% of cases, transmission is mediated by a single virus, called the transmitted/founder (T/F) virus, despite significant viral diversity in the donor inoculum [13]. This results in a profound genetic bottleneck [13,104,105,106,107,108,109,110]. Questions of critical importance are what causes this bottleneck, what viral properties facilitate transmission, and can this knowledge be exploited for therapeutic gain. In chapter two, we assess the phenotypic properties of

transmitted/founder Env glycoproteins in an attempt to to better understand HIV transmission and pathogenesis and find HIV-1's Achilles' heal to aid in vaccine design.

In chapter three, we discuss a novel therapeutic strategy to engineer HIV-resistant CD4+ T cells with a long-term goal of achieving a functional "cure," that is, virologic control in the absence of long-term anti-retroviral therapy (ART). Despite the success of ART, there are significant toxicities associated with its chronic use, and thus improved treatment is needed. One such approach involves genetically disrupting the HIV coreeceptors, *ccr5* and *cxcr4,* with gene-specific zinc-finger nucleases (ZFNs). An individual's own cells can be removed, genetically modified *ex vivo*, and then reinfused creating a population of cells resistant to HIV infection. These cells should have a survival advantage *in vivo* and may serve to maintain a functional immune system and prevent progression to AIDS.

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Chapter 2

Phenotypic and Immunologic Comparison of Clade B Transmitted/Founder and Chronic HIV-1 Envelope Glycoproteins

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Abstract

Sexual transmission of human immunodeficiency virus type 1 (HIV-1) across mucosal barriers is responsible for the vast majority of new infections. This relatively inefficient process results in the transmission of a single transmitted/founder (T/F) virus -- from a diverse viral swarm in the donor -- in approximately 80% of cases. Here we compared the biological activity of 24 clade B T/F envelopes (Envs) with that of 17 chronic controls to determine whether the genetic bottleneck that occurs during transmission is linked to a particular Env phenotype. To maximize the likelihood of an intact mucosal barrier in the recipients and to enhance the sensitivity of detecting phenotypic differences, only T/F Envs from individuals infected with a single T/F variant were selected. Using pseudotyping to assess Env function in single round infectivity assays, we compared coreceptor tropism, CCR5 utilization efficiency, primary CD4+ T cell subset tropism, dendritic cell *trans*-infection, fusion kinetics, and neutralization sensitivity. T/F and chronic Envs were phenotypically equivalent in most assays; however, T/F Envs were modestly more sensitive to CD4 binding site antibodies b12 and VRC01, as well as pooled human HIV Ig. This finding was independently validated with a panel of 14 additional chronic HIV-1 Envs controls. Moreover, the enhanced neutralization sensitivity was associated with more efficient binding of b12 and VRC01 to T/F Env trimers. These data suggest that there are subtle but significant structural differences between T/F and chronic clade B Envs that may have implications for HIV-1 transmission and the design of effective vaccines.

Introduction

Sexual transmission of HIV-1 across mucosal barriers is a relatively inefficient process and is most often due to the transmission of a single transmitted/founder (T/F) virus from the swarm of viral variants present in the donor, resulting in a profound genetic bottleneck [1,2,3,4,5,6,7,8]. As discussed in chapter one, a question of central importance is whether T/F viruses have particular phenotypic properties, which favor their transmission. If so, viruses with these properties should logically be targets of vaccination and microbicide efforts. The viral envelope (Env) protein is a likely candidate for transmission-related signatures. For example, viruses expressing Envs that utilize the CCR5 coreceptor (R5-tropic) are transmitted far more frequently than those expressing Envs that utilize CXCR4 (X4-tropic) [1,6,9,10]. Variations in Env have also been linked to differences in the utilization of CD4 and coreceptor, the rate and efficiency of membrane fusion, as well as binding to C-type lectins such as DC-SIGN that are expressed on dendritic cells (DCs) and can function as virus attachment factors [11,12,13,14].

Studies to characterize the properties of transmitted HIV-1 strains face several challenges. First, it is difficult to identify individuals during the acute phase of HIV-1 infection, particularly before the onset of immune responses (that is, at early Fiebig stages [15]), thus limiting sample sizes. Second, individual viruses cloned from the peripheral blood or plasma of acutely infected individuals within weeks of transmission may have already evolved away from the actual T/F virus and may thus have acquired phenotypic changes [16]. Third, in the absence of extensive sampling of the early viral quasispecies by single genome amplification (SGA), it is impossible to know if one or

more virus strains established the clinical infection, making it difficult to assess the integrity of the mucosal barrier [1]. Infection with multiple T/F viruses may reflect a different mechanism of transmission, with these T/F Envs likely facing different or reduced transmission selection pressure [17,18]. Nonetheless, small numbers of Envs cloned from acutely infected individuals have been obtained and compared to Envs cloned from corresponding donors or from other chronically infected individuals. Derdeyn et al. examined clade C Envs from eight heterosexual transmission pairs and concluded that transmitted Envs have fewer putative N-linked glycosylation sites (PNGs), more compact variable loops, and enhanced neutralization sensitivity to donor plasma [2], although subsequent phenotypic studies of a subset of viruses bearing these Envs did not reveal functional differences [19,20]. Analysis of clade A and D transmission pairs also identified shorter recipient Envs with a lower V3 charge, although no differences in the number of PNGs were noted [21]. For clade B Envs, initial studies suggested that transmission was independent of variable loop length and PNGs [22,23,24]; however, more recent comparisons of thousands of clade B T/F and chronic *env* sequences confirmed significantly fewer total PNGs and a trend towards fewer in the V1/V2 loops of transmitted Envs (Gnanakaran et al, submitted). Finally, several studies have investigated neutralization sensitivities of acute or T/F Envs compared to chronic control Envs, but reported conflicting results [1,23,25,26]. These discrepancies may have resulted from differences in sample size, demographics of acutely infected individuals and chronic controls, cloning strategy, and whether the Envs under investigation represented true T/F viruses.

The use of SGA of plasma viral RNA during the earliest stages of infection has allowed the inference of the nucleotide sequence of T/F viruses from an increasingly large number of individuals [1,27,28,29]. Recent analyses of a large number of clade B T/F Env sequences led to the identification of transmission signatures in the CCR5 binding site, certain PNGs, and sites in the signal peptide and gp41 cytoplasmic domain that could affect Env processing and localization (Gnanakaran et al, submitted). These results suggested that T/F Envs might differ in some phenotypic properties from chronic Envs. To examine this, we conducted a comprehensive phenotypic analysis of T/F and chronic clade B HIV-1 Envs in the context of viral pseudotypes. Specifically, we assessed coreceptor tropism, CCR5 utilization efficiency, CD4+ T cell subset tropism, DC-mediated *trans*-infection efficiency of T cells, and membrane fusion kinetics. In addition, we examined the sensitivity T/F and chronic Envs to neutralization by purified immunoglobulin from infected patients (HIV Ig) and a panel of broadly neutralizing monoclonal antibodies (MAbs) and assessed the binding efficiencies of these MAbs to trimeric Env on the cell surface. Our results failed to identify a major transmission phenotype, but uncovered subtle functional differences between T/F and chronic Envs that may be of biological significance.

Materials and Methods

Pseudovirus production. Pseudotyped virus was produced by calcium phosphate co-transfection of 6 μg of pcDNA3.1+ containing *env* with 10 μg of HIV-1 core (pNL43-ΔEnv-vpr+-luc+ or pNL43- ΔEnv -vpr+-eGFP) into 293T17 cells. Virus was harvested 72 hours post-transfection, filtered through a 0.45 μm filter, aliquoted, and stored at -80°C. For the primary CD4+ T cell infection, pseudovius was concentrated by ultracentrifugation through a 20% sucrose cushion. Pelleted pseudovirus was then resuspended in PBS. All luciferase-encoding pseudoviral stocks were serial diluted and used to infect NP2 cells to define the linear range of the assay. A viral dilution was chosen in the middle of the five-fold linear range of the assay to maximize sensitivity.

***Env* cloning and sequence analysis.** The derivation of most T/F Env clones used in this study has been described [1]. THRO.F4.2026, SUMAd5.B2.1713, 9010-09.A1.4924, and PRB959-02.A7.4345 were cloned from SGA amplicons known to contain the nucleotide sequence of the corresponding T/F *env* sequence into pcDNA3.1 according to manufacturer's instructions (Invitrogen). The AD17.1 *env* gene was subcloned from a full-length infectious molecular T/F clone described elsewhere [17]. Chronic Envs HEMA.A4.2125 and HEMA.A23.2143 were also cloned in pcDNA3.1; briefly, viral RNA was extracted from plasma of chronically infected patients and amplified using SGA methods. Individual *env* genes were then either cloned at random, or selected, to maximize within-patient *env* sequence diversity. Env clones were sequenced to confirm that they did not contain *Taq* polymerase errors, but represented *env* genes of viruses circulating in the patient. The nucleotide sequences of all T/F and chronic Envs have previously been reported (Gnanakaran et al, submitted). PNGs were

determined with N-glycosite (hiv.lanl.org) [30]. To assess lengths of the V1/2, V3, V4, V5, and V1-4 regions, sequences were aligned to HXB2, then boundaries were identified for each region and non-gap residues were counted.

Coreceptor tropism testing and cell line infections. NP2 cells stably expressing CD4 and either CCR5 (NP2/CD4/CCR5) or CXCR4 (NP2/CD4/CXCR4) were infected with HIV-1 pseudoviruses expressing luciferase by spinoculating in 96-well plates at 450 g for 90 minutes at 25°C. Cells were lysed with Brite-Glo (Promega) 72 hours post-infection and analyzed on a Luminoskan Ascent luminometer. Coreceptor tropism was arbitrarily defined by mean relative light units (RLUs) greater than 1 (approximately 100-fold over background). To assess sensitivity to coreceptor inhibitors, NP2/CD4/CCR5 or NP2/CD4/CXCR4 cells were pre-incubated for 30 minutes with saturating concentrations of the CCR5 inhibitor maraviroc (1 μM), the CXCR4 inhibitor AMD3100 (2 μM), or the fusion inhibitor enfuvirtide (10 μ/ml) prior to infection. To assess sensitivity to broadly neutralizing MAbs, viral pseudotypes were pre-incubated with 10 μg/ml of antibody for 30 minutes at 37°C. Virus and antibody mixes were then used to infect NP2/CD4/CCR5 or NP2/CD4/CXCR4 cells. All NP2 cell line infections were done in at least triplicate in at least three independent experiments using R5-tropic JRFL as a positive control and Env-deficient pseudotypes as a negative control.

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: pNL4-3-deltaE-eGFP (Cat# 11100) from Drs. Haili Zhang, Yan Zhou, and Robert Siliciano [31], bicyclam JM-2987 (hydrobromide salt of AMD-3100; Cat# 8128) [32,33,34], maraviroc (Cat #11580)

[35,36,37], and HIV-1 gp120 MAb IgG1 b12 (Cat# 2640) from Dr. Dennis Burton and Carlos Barbas [38,39,40,41].

Primary human CD4+ T cell tropism assay. Primary human CD4+ T cells, purified by negative selection, were obtained from the University of Pennsylvania's Human Immunology Core. $2x10^6$ cells per virus were stimulated with plate-bound anti-CD3 (clone OKT3) (eBiosciences) and anti-CD28 (clone 28.2, BD biosciences) and 20 units (U)/ml recombinant IL-2 in RPMI containing 10% FBS. Three days post-stimulation cells were transferred to 96-well V-bottom plates prior to infection. Five µl/well of concentrated HIV-GFP was used to infect cells in triplicate. Plates were then spinoculated at 1200 g for 2 hrs. Cells were then transferred to new 24-well plates and new media containing 20 U/ml IL-2 was added. Three days post-infection, cells were stained for flow cytometry.

Determination of alternative coreceptor use. Primary human CD4+ T cells from two different *ccr5Δ32* homozygous donors were obtained and purified as previously described. Prior to infection, cells were pre-incubated with 50 µM AMD3100 for 30 min. Cells were infected as previously described. Two hours after spinfection, enfuvirtide (1 µg/ml final concentration) was added to all samples to prevent additional fusion prior to transferring cells to a 24-well plate for further incubation. Samples were stained and analyzed as previously described.

Flow cytometry. 1-$2x10^6$ cells were stained per tube for flow cytometry. All incubations were done at RT and in Facs Wash Buffer (PBS, 2.5% FBS, 2mM EDTA), and all antibodies were from BD Biosciences, unless otherwise noted. To stain 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 in Facs Wash Buffer was added and incubated for 30 min. Cells were then washed in Facs Wash Buffer before staining with anti-CD3 Qdot 655 (Invitrogen), anti-CD4 Alexa Fluor 700, anti-CD45RO PE-Texas Red (Beckman Coulter), and anti-IgM PE (Invitrogen) for 30 min. Cells were then washed in Facs Wash Buffer and resuspended in 1% PFA. Samples were run on a LSRII (BD) and analyzed with FlowJo 8.8.6 (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 CD3+ cells (SSC-A by CD3), then by memory markers (CCR7 by CD45RO).

DC *trans*-infection assay. To differentiate DCs, freshly isolated monocytes from the University of Pennsylvania's Human Immunology core were treated with 50 ng/ml GM-CSF (R&D systems) and 100 ng/ml IL-4 (R&D systems) in AIM V serum free media (Invitrogen). New media containing GM-CSF and IL-4 was added on day 3. Six days post-stimulation DCs were washed and plated at $3x10^4$ cells per well in a V-bottom 96 well plate. $3x10^4$ CD4+ T cells alone, three days post-stimulation with plate-bound anti-CD3/anti-CD28 were used as a negative control. Viral stocks were first titered by RLUs on NP2/CD4/CCR5 or CXCR4 cells. Virus sufficient to generate 80 RLUs was added to DCs or a CD4+ T cell control and allowed to bind for two hours at 37°C. Cells were washed three times with fresh media to remove cell-free virus. Then, $3x10^5$ stimulated heterologous CD4+ T cells were added to each well containing $3x10^4$ HIV-bound DCs or CD4+ T cells. As an additional control, and equal amount of virus was added to $3x10^5$ stimulated CD4+ T cells to ensure there was no differential infection of CD4+ T cells. For CD4+ T cell luciferase infection, cells were spinoculated at 450 g for 90 minutes and then incubated without washing off virus. Cells were then transferred to

a flat bottom 96-well plate for three days prior to take down with Brite Glo. Each condition was done in triplicate and each viral pseudotype was used in at least three independent experiments with cells from different healthy donors.

Enfuvirtide time-of-addition assay. To assess entry kinetics of T/F and chronic Envs, NL43vpr+luc+ pseudotypes were chilled to 4°C and added to NP2/CD4/CCR5 (or NP2/CD4/CXCR4 for the one X4-tropic Env) cells on metal blocks embedded in ice covered by a moist towel. Cells were then spinoculated at 1300 rpm for 90 minutes at 4°C to enhance viral binding. Immediately post-spinoculation, cold supernatant was aspirated off and all wells were flooded with 270 μl of pre-warmed 37°C media and transferred to a 37°C incubator. 30 μl of 10 μg/ml enfuvirtide (final concentration of 1μg/ml) was then added at 0, 5, 10, 20, 40, 80, or 160 minutes post-warming. A no drug control was also included to normalize percent infection. Cells were then incubated for 72 hours and assessed for RLUs. At least three wells per virus per time point were included in each experiment, and all Envs were examined in at least three independent experiments. Data was analyzed with Prism 4.0 (GraphPad Software, Inc.) by fitting a best-fit sigmoidal line to each independent experiment prior to averaging the Hill slopes and time to half-max fusion.

Neutralization sensitivity. Neutralization assays were performed using both NP2 and TZMbl cells in two independent laboratories. To assess sensitivity to MAbs b12, VRC01, PG9, and PG16, viral pseudotypes were pre-incubated with 10 μg/ml of antibody for one hour prior to infection of NP2 cells. To assess sensitivity to HIV Ig, pseudotypes were pre-incubated with two-fold serial dilutions of clade B HIV Ig from 1500-23 μg/ml. This mix was then added to NP2 cells and spinoculated as described

previously. For MAbs, neutralization was assessed by determining the maximum percent inhibition (MPI) compared to a no antibody control. Clade B HIV Ig (lot 12 100158) was obtained from the AIDS Repository.

Neutralization sensitivity was assessed on TZMbl cells as previously described [42,43]. Briefly, 8 x 10^3 TZMbl cells were plated overnight. Five-fold dilutions of MAbs (b12, VRC01, PG9, PG16, and clade B HIV Ig) were incubated in the presence of 40 μg/ml DEAE-Dextran and 2000 infectious units (as measured on TZMbl cells) of pseudovirius for one hour at 37°C. After media was removed from TZMbl cells, the virus/MAb dilutions were added to the cells and incubated for 48 hours before being analyzed for luciferase expression (Promega). The highest concentration tested for b12, VRC01, PG19 and P16 was 10 μg/ml. The highest concentraion of clade B HIV Ig was 1500 μg/ml. Samples were tested in duplicate with all experiments repeated at least two times. IC_{50} values were calculated as described previously [43].

Cell-Based Enzyme-Linked Immunosorbent Assay (CELISA). The binding of MAbs to HIV-1 Env trimers expressed on cells was measured using a cell-based ELISA system, as previously described [44]. Briefly, COS-1 cells were seeded in 96-well plates (1.8×10^4 cells/well) and transfected the next day with 0.1 μg of a plasmid expressing Env and 0.02 μg of a Rev-expressing plasmid per well using Effectene transfection reagent. Three days later, cells were incubated with the indicated MAb suspended in blocking buffer (35 mg/ml BSA, 10 mg/ml non-fat dry milk, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Tris, pH 7.5 and 140 mM NaCl) for one hour at room temperature. Cells were then washed four times with blocking buffer and four times with washing buffer (140 mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM Tris, pH 7.5). A horseradish

peroxidase-conjugated antibody specific for the Fc region of human IgG was then incubated with the samples for 45 minutes at room temperature. Cells were washed five times with blocking buffer and five times with washing buffer. HRP enzyme activity was determined after the addition of 33 μl per well of a 1:1 mix of Western Lightning oxidizing and luminol reagents (Perkin Elmer Life Sciences) supplemented with 150 mM NaCl. Light emission was measured with a Mithras LB 940 luminometer (Berthold Technologies). To correct for the level of cell surface expression of each envelope glycoprotein, binding of the antibodies is expressed as percent binding of the CD4-Ig probe at saturating concentrations (5 μg/ml). We decided a priori to exclude all envelope glycoproteins that bound CD4-Ig at less than 20% of the binding measured for the SC05.8H2.3243 control isolate. Five of the 57 Envs were thus not analyzed, including three T/F and two chronic Envs. Measurements of antibody binding and neutralization were performed under code to prevent potential bias.

Statistical analyses. T/F and chronic Envs were compared with Mann-Whitney tests and correlations were assessed by Spearman tests. P-values less than 0.05 were considered significant. Data was analyzed with Prism 4.0 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

Panels of T/F and chronic Envs. To determine if there are functional differences between T/F Envs and those that predominate during chronic infection, we assembled a panel of 24 clade B T/F Envs previously inferred and cloned from plasma viral RNA of 24 individuals with acute HIV-1 infection as defined by the Fiebig staging system, in which patients are classified from Stage I (viral RNA positive, antibody and antigen negative) to Stage VI (ELISA and western blot positive with multiple bands) [1,17,45] (Table 2-1). Twelve individuals were sampled during Fiebig stage II, five during Fiebig stage III, two during in Fiebig stage IV, and five during Fiebig stage V. Acutely infected individuals were predominantly males (22 of 24) from the southeastern United States (18 of 24) with a variety of sexual risk factors, all denying intravenous drug use (IDU). All T/F Envs were inferred from SGA-derived sequences, which are devoid of PCR-induced errors and cloning bias [1]. Env clones identical to this inferred T/F sequence were then chosen for phenotypic analysis. Importantly, all T/F Envs were selected from subjects with single variant transmissions. This was done to increase the likelihood that the viruses encoding these Envs were transmitted across an intact mucosal barrier, thereby maximizing our chances of observing properties required for this process [17,18].

To generate chronic clade B control Envs, we used SGA to amplify *env* genes from plasma viral RNA of two groups of anti-retroviral therapy naïve individuals. The first group consisted of 11 individuals sampled 14-83 months post-infection (mean 42 months). A test set of 17 Env clones was derived from this group consisting predominantly of males (8 of 11) from the southeastern United States (10 of 11) all

Table 2-1. Description of T/F and chronic Envs

Env type	Subject	*env* Clone name	Fiebig Stage, or minimal time since infection	Viral load (copies/ml)	Gender	Risk behavior[c]	Geographic location	Sampling date	Coreceptor tropism[d]
T/F	REJO4541	REJO.D12.1972	V	722,349	M	Heterosexual	Alabama	9/28/01	R5
	RHPA4259	RHPA.A19.2000	V	1,458,354	F	Heterosexual	Alabama	12/5/00	R5
	SUMA0874	SUMAd5.82.1713	II	939,260	M	MSM	Alabama	5/13/91	R5
	THRO4156	THRO.F4.2026	V	5,413,140	M	MSM	Alabama	8/1/00	R5
	WEAU0575	WEAUd15.410.5017	II	216,415	M	MSM	Alabama	5/30/90	R5X4
	WITO4160	WITO.B10.2062	II	325,064	M	Heterosexual	Alabama	8/4/00	R5
	700010040	CH40.C9.4520	V	298,026	M	MSM	North Carolina	7/27/06	R5
	700010058	CH58.A4.4375	III	394,649	M	Unknown	North Carolina	9/8/06	R5
	700010077	CH77.SA2.6559	V	144,145	M	Unknown	North Carolina	9/31/06	R5X4
	1006-11	1006-11.C3.1601	III	1,600,000	M	SPD	North Carolina	6/5/97	R5
	1018-10	1018-10.A5.1732	III	270,000	M	SPD	South Carolina	6/20/97	R5
	1053-07	1053-07.B15.1648	III	1,400,000	M	SPD	South Carolina	12/3/97	R5
	1056-10	1056-10.TA11.1826	II	140,000	M	SPD	South Carolina	1/14/98	R5
	1058-11	1058-11.B11.1550	IV	550,000	M	SPD	South Carolina	3/18/98	R5X4
	9010-09	9010-09.A1.4924	II	146,954	F	SPD	South Carolina	11/25/97	R5
	9015-07	9015-07.A1.4729	II	500,000	M	SPD	South Carolina	12/27/97	R5
	9021-14	9021-14.B2.4571	II	143,379	M	SPD	California	6/10/98	R5
	9032-08	9032-08.A1.4685	III	40,815	M	SPD	Alabama	7/30/98	R5
	PRB956-04	PRB956-04.B22.4267	II	600,000		SPD	Virginia	8/19/97	R5
	AD17	AD17.1	II	47,600,000	M	MSM	New York	6/14/99	R5
	PRB959-02	PRB959-02.A7.4345	II	>2,000,000		SPD	South Carolina	11/17/99	R5
	TT35P	TT35P.11H8.2874	II	1,849,301	M	Heterosexual	Trinidad	1/26/99	R5
	SC20	SC20.8A8.2437	IV	2,789,313	M	Heterosexual	Trinidad	2/18/98	R5
Chronic 1[a]	SC05	SC05.8C11.2344	II	9,980,952	M	Heterosexual	Trinidad	6/28/93	R5
		SC05.A10.2362	5 y 5 m	19,514				12/9/98	R5
		SC05.8H2.3243							R5
		SC05.8A11.2363							R5
	SHKE4761	SHKE.A26.4112	1 y 2 m	544,000	M	MSM	Alabama	8/2/06	R5
		SHKE.A7.2118							R5
		SHKE.A4.2116							R5
	HEMA4284	HEMA.A4.2125	1 y 10 m	49,755	M	MSM	Alabama	10/2/02	R5
		HEMA.A23.2143							R5
	WICU4248	WICU.B4.2973	5 y 11 m	8,424	M	MSM	Alabama	10/6/05	R5
		WICU.C1.2992							R5
	CRPE4571	CRPE.B28.4072	2 y	21,917	F	Heterosexual	Alabama	11/30/01	R5X4
	JOTO5278	JOTO.TA1.2247	1 y 6 m	404,180	M	Heterosexual	Alabama	2/13/04	X4
	OLLA4645	OLLA.A14.1923	2 y 1 m	382,000	F	Heterosexual	Alabama	2/22/02	R5
	SAMI4303	SAMI.A8.1863	3 y 11 m	116,000	M	MSM	Alabama	8/5/04	R5
	SMRE4166	SMRE.A13.4127	1 y 4 m	135,858	F	Heterosexual	Alabama	11/16/01	R5
	TALA4022	TALA.A2.1780	6 y 11 m	228,200	M	MSM	Alabama	12/9/03	R5
	YOMI4024	YOMI.F2.4137	6 y 1 m	14,178	M	MSM	Alabama	2/15/06	R5
Chronic 2[b]	1632	1632.TA9	2 y 5 m	97,800	M	MSM	Washington	9/14/05	R5
		1632.A17							R5
		1632.A6							R5
		1632.TA1							R5
		1632.A7							R5
		1632.A23							R5
	1451	1451.D17	20 y 3 m	532,000	M	MSM	Washington	3/1/05	R5
		1451.C16							R5
		1451.D1							R5
		1451.C8							R5
	1588	1588.TA7	7 y 2 m	99,600	M	MSM/IDU	Washington	7/6/05	R5
	1470	1470.D27	4 y 3 m	492,200	M	MSM/IDU	Washington	3/15/05	R5
	1599	1599.B11	6 y 7 m	112,000	M-F	IDU	Washington	7/26/05	R5
	1444	1444.A21	7 y	86,300	M	MSM	Washington	2/22/05	R5

[a] Original panel of chronic Envs

[b] Chronic Envs from Washington state assessed only in neutralization assays.

[c] Men who have sex with men (MSM); Serial plasma donor (SPD); Intravenous drug user (IDU)

[d] Coreceptor tropism was assessed on NP2 cells for T/F and Chronic 1 Envs. Tropism for chronic 2 Envs was previously assessed on TZMbl cells (Gnanakaran, et al submitted).

denying IDU (Table 2-1). An additional 14 clade B control Envs were SGA amplified and cloned from six chronically infected individuals residing in the northwestern United States. This second group of chronic Envs served as a validation set to confirm differences in neutralization sensitivity observed with the first test set. A phylogenetic tree of the 31 chronic Envs is depicted in Figure 2-1 along with the 24 T/F Envs. None of the Envs were from epidemiologically linked infections.

Previous studies noticed fewer PNGs in the gp120 region of T/F compared to chronic Envs [46] (Gnanakaran et al, submitted). To determine whether our selected subset of T/F and chronic Envs differed from this much larger group, we compared variable loop length as well as the number and distribution of putative PNGs. There were no differences in V1/2, V3, V4, V1-4 lengths between T/F and chronic clade B Envs. Further, the median gp120 PNGs in T/F Envs was 26.0 compared to 27.0 for the chronic controls ($p=0.16$) and 26.0 for clade B Envs in general [31]. Thus, the panel of T/F Envs selected for our functional analyses exhibited no statistically significant differences in patient demographics, virus phylogeny, variable loop length, or PNGs relative to the panel of chronic Envs we assembled or to clade B Envs in general.

Determination of coreceptor tropism. R5-tropic viruses represent the vast majority of transmitted viruses, with dual (R5X4)-tropic viruses being transmitted less frequently [1,6,9,10]. On rare occasions, X4-tropic viruses can be transmitted [47,48,49]. To determine the coreceptor usage in our panel, we characterized the CCR5 and CXCR4 utilization of the 24 T/F and 17 chronic Envs by producing viral pseudotypes and using these to infect NP2 cell lines expressing CD4 and either CCR5 (NP2/CD4/CCR5) or

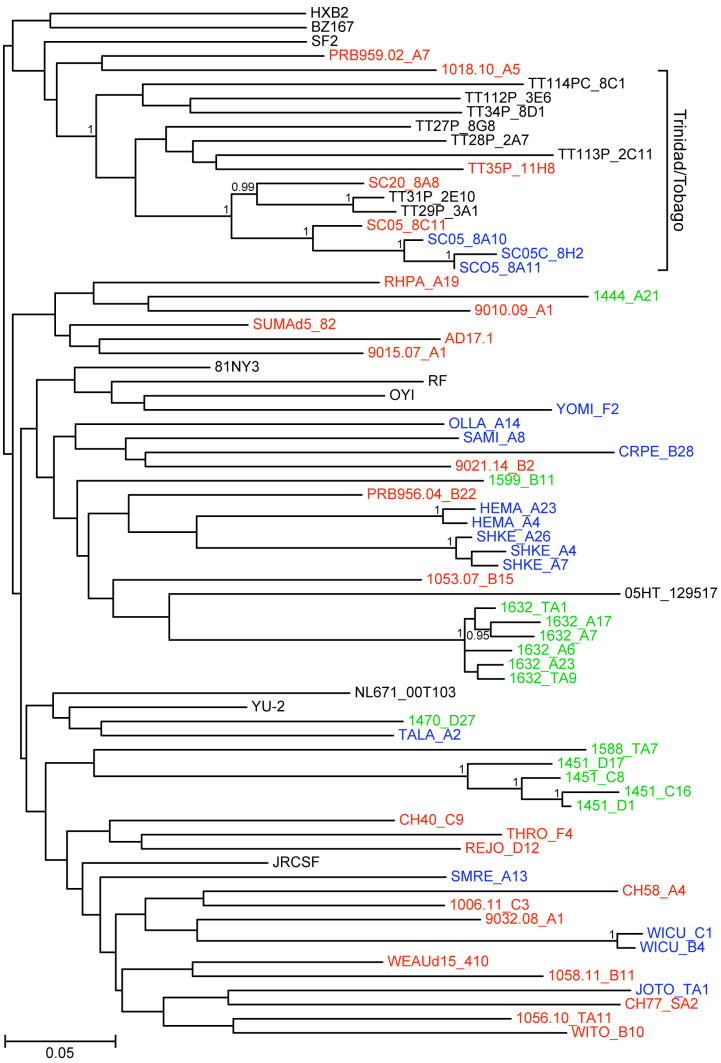

Figure 2-1. Phylogenetic relationships of T/F and chronic Envs selected for phenotypic analyses. The tree was constructed from Env amino acid sequences of T/F (red), original chronic (blue), and Washington state chronic (green) control viruses (subtype B reference sequences from the database are shown in black). All sequences were derived by SGA methods; Env sequences from the same individuals form discrete subclusters. A bracket indicates epidemiologically linked infections from Trinidad and Tobago [50]. The tree was inferred using maximum likelihood methods [51]; Numbers on nodes indicate posterior probabilities (only values above 0.95 are shown). The scale bar represents 0.05 amino acid substitutions per site.

CXCR4 (NP2/CD4/CXCR4), as well as primary human CD4+ T cells. NP2 cells were selected because they provide a 5-6 log linear range of infection, approximately 2-3 logs greater than that of the TZMbl assay. We found that of the 24 T/F Envs, 21 were R5-tropic and three were R5X4-tropic, while of the 17 chronic Envs, 15 were R5-tropic, one was R5X4-tropic, and one was X4-tropic (Table 1). This is consistent with previous results with the exception of T/F Envs 1058-11.B11.1550 and CH77.SA2.6559, which were R5X4-tropic on NP2 cells and R5-tropic on TZMbl cells [1] (Gnanakaran et al, submitted). This discrepancy is likely due to differences in CXCR4 expression, as the NP2 cells used stably express high levels of CXCR4 compared to the HeLa-derived TZMbl cells, which express lower endogenous CXCR4 levels. All four R5X4-tropic Envs utilized CCR5 and CXCR4 with approximately equivalent efficiency as assessed by a less than two-fold difference in RLUs between the NP2/CD4/CCR5 and NP2/CD4/CXCR4 cells. To assess coreceptor use on human CD4+ T cells, we infected *ccr5Δ32* or *ccr5wt* CD4+ T cells in the presence or absence of saturating concentrations of the CXCR4 inhibitor AMD3100. The results paralleled those obtained with the NP2 cell lines. R5-tropic Envs mediated infection of *ccr5wt* but not *ccr5*Δ*32* CD4+ T cells, while R5X4 Envs mediated infection of both cell types. Infection of *ccr5*Δ32 CD4+ T cells by three of the R5X4 Envs was completely inhibited by AMD3100, while Env CRPE.B28.4072 could infect *ccr5Δ32* cells in the presence of AMD3100, though with reduced efficiency (data not shown). However, we found that AMD3100 inhibited infection of NP2/CD4/CXCR4 cells by viruses bearing the CRPE.B28.4072 Env by only 50%. In addition, this Env was unable to infect NP2 cells expressing CD4 alone or in combination with any of 17 different putative alternative coreceptors, indicating that this

Env can utilize the drug-bound conformation of CXCR4 (data not shown). Several other HIV-1 Env proteins have been shown to exhibit this property [52]. In su mmary, all T/F Envs utilized CCR5, while three were R5X4-tropic. Thus, there were no differences in coreceptor tropism between the T/F and chronic Envs with the exception of the one X4-tropic chronic Env, and there was no evidence for utilization of coreceptors other than CCR5 or CXCR4 to infect human CD4+ T cells.

Sensitivity to coreceptor antagonists and CCR5 utilization efficiency.

Mucosal transmission of HIV-1 is dependent upon CCR5. Hypothesizing that the ability to use low levels of CCR5 may confer selective advantage to viruses at the moment of transmission, we determined the sensitivity of each Env to the CCR5 inhibitor maraviroc (MVC) as a surrogate for CCR5 utilization efficiency. High MVC IC_{50} values indicate that an Env can mediate infection at low levels of CCR5, while low IC_{50} values suggest an Env requires high CCR5 expression for viral entry. We found no significant difference in median MVC IC_{50} values between the T/F (2.4 nM) and chronic Envs (2.3 nM) ($p=0.79$; Mann-Whitney) (Figure 2-2A). In addition, we determined the maximal percent inhibition (MPI) of infection by MVC. While uncommon, several *in vivo* derived MVC-resistant R5-tropic viruses have been identified that can utilize the drug-bound form of CCR5 [53,54]. Such viruses engage the coreceptor differently, relying predominantly upon the N-terminus for entry whereas most viruses require the N-terminus as well as the extracellular loops of CCR5. Furthermore, determining the MVC sensitivity of T/F viruses has implications for microbicides and pre-exposure prophylaxis. All 41 Envs examined had MPIs greater than 85%, with the vast majority greater than 95%. There were no significant differences ($p=0.17$ Mann-Whitney) between the T/F

(median= 99.1%) and chronic Envs (median=98.3; Figure 2-2B). Together, these data indicate that the HIV-1 transmission bottleneck does not impose a selection pressure for viruses capable of using low concentrations of CCR5.

Primary CD4+ T cell tropism. CD4+ T cells, the major target and source of HIV-1 *in vivo* [55,56], can be broadly divided into four subsets: naïve (CCR7+CD45RO-), central memory (T_{CM}) (CCR7+CD45RO+), effector memory (T_{EM}) (CCR7-CD45RO+), and CD45RA+ in part to variation in coreceptor expression [57], cellular activation [58], and tissue localization [59]. T_{EM} and T_{EMRA} cells are found predominantly in effector sites including the rectal and cervicovaginal mucosa, while naïve and T_{CM} cells are most abundant in the lymph nodes. T_{EM} cells, the most abundant subset in mucosal effector sites, are preferentially infected and massively depleted during acute infection (reviewed in [60]). Since potential target cells in the mucosa may be limiting during transmission, we hypothesized that T/F Envs may infect T_{EM} and T_{EMRA} cells preferentially relative to the matched chronic controls.

Peripheral blood CD4+ T cells from three normal uninfected donors were purified by negative selection and stimulated with anti-CD3/anti-CD28 and IL-2 for three days prior to infection with HIV-1 pseudotypes expressing a GFP reporter. Three days post-infection, viability and expression of CD3, CD4, CCR7, CD45RO, and GFP was assessed by FACS analysis. Productively infected cells were defined as CD3+ GFP+ since CD4 was down-regulated in the majority of infected cells [61]. The gating strategy is shown in Figure 2-3A. In all three donors, infected cells were predominantly T_{EM} (~65%),

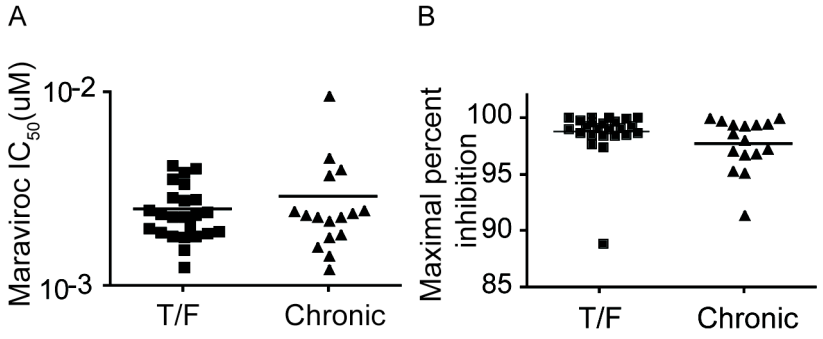

Figure 2-2. CCR5 utilization efficiency. (A) Viral pseudotypes were used to infect NP2/CD4/CCR5 cells in the presence of serial dilutions of the CCR5 antagonist maraviroc (MVC). Higher IC_{50} values correspond to Envs that can utilize CCR5 more efficiently, and vice versa. T/F and chronic clade B Envs have similar MVC IC_{50} values ($p=0.79$) suggesting they engage CCR5 comparably. (B) Since some Envs can utilize the MVC-bound conformation of CCR5 and since MVC is a candidate microbicide, we assessed the maximal percent inhibition (MPI) of MVC for each Env. All Envs were sensitive to MVC and there was no difference in MPI between the T/F and chronic Envs ($p=0.17$). All infections were done in at least triplicate in each of at least three independent experiments. Data was analyzed by a Mann-Whitney test.

tropic viruses have been previously reported to readily infect naïve CD4+ T cells compared to R5-tropic viruses [62,63,64], this assay contains an important internal validation: the five viruses that could utilize CXCR4 for entry (one X4-tropic Env shown in cyan; four R5X4-tropic Envs shown in red) preferentially infected naïve cells. With the exception of these five Envs, no other pseudotypes were reproducibly outliers in their ability to mediate entry into any of the subsets, and there were no statistically significant differences or trends between the T/F and chronic Envs for any of the four cell subsets in any of the three donors examined (Figure 2-3B). In addition, there was no statistically significant difference in overall infectivity between the T/F and chronic Env pseudotypes in any of the three donors examined suggesting comparable Env fitness in peripheral CD4+ T cells (Figure 2-3C). Together, this suggests that transmission and early expansion is not due to differential infection of CD4+T cells or their subsets between T/F and chronic Envs.

DC-mediated *trans*-infection. DCs can enhance HIV-1 infection in *trans* by efficiently capturing virus particles and presenting them to CD4+ T cells. *In vitro*, co-culture of monocyte-derived DCs with CD4+ T cells results in enhanced virus infection, particularly at low virus inocula (reviewed in [65]). To assess whether DCs preferentially bind and transfer T/F compared to chronic Env pseudoviruses, we performed DC:CD4+ T cell co-culture experiments. Viral pseudotype stocks were normalized for infectivity on NP2 cells to control for differences in viral titer. A relatively limiting amount of virus (80 RLUs on NP2 cells) was bound to DCs, which were then washed to remove cell-free virus and co-cultured with CD4+ T cells. All Envs were assessed in at least three

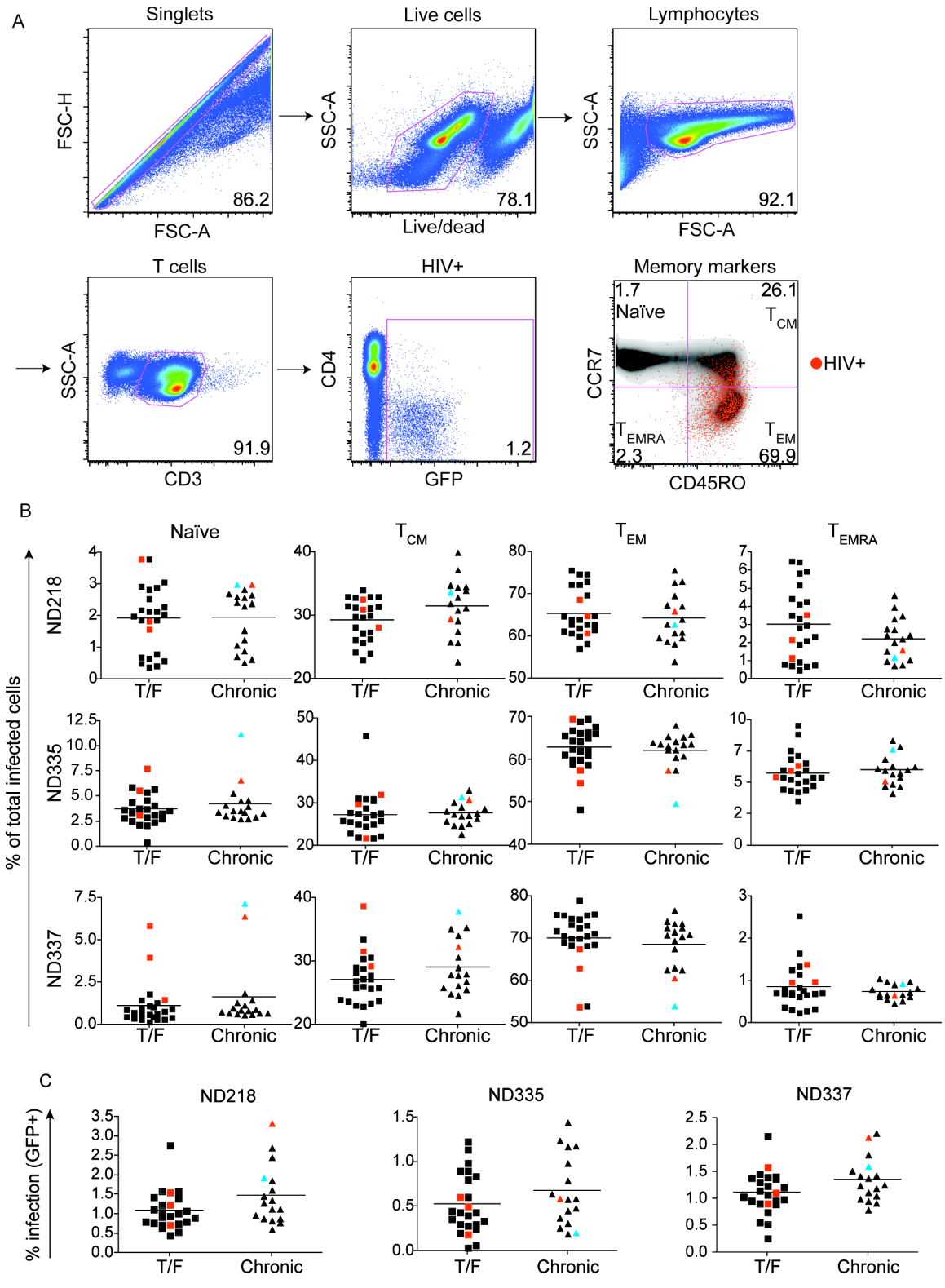
A
Singlets
FSC-H
FSC-A
86.2
Live cells
SSC-A
Live/dead
78.1
Lymphocytes
SSC-A
FSC-A
92.1
T cells
SSC-A
CD3
91.9
HIV+
CD4
GFP
1.2
Memory markers
1.7
Naïve
26.1
T CM
T EMRA
2.3
T EM
69.9
CCR7
CD45RO
HIV+
B
Naïve
T CM
T EM
T EMRA
ND218
ND335
ND337
% of total infected cells
T/F
Chronic
C
ND218
ND335
ND337
% infection (GFP+)
T/F
Chronic

Figure 2-3. CD4+ T cell subset tropism. To assess human CD4+ T subset tropism of the T/F and chornic Envs, cells were infected with Env pseudotypes expressing GFP and then stained and analyzed by flow cytometry. (A) Cells were gated as shown. Infected cells (GFP+) were then back-gated on the memory markers CCR7 and CD45RO to evaluate differential subset infection. Naïve (CCR7+CD45RO-); central memory (T_{CM}) (CCR7+CD45RO+); effector memory (T_{EM}) (CCR7-CD45RO+), effector memory RA (T_{EMRA}) (CCR7-CD45RO-). (B) T/F and chronic Envs infected all four CD4+ T cell subsets comparably. T_{EM} and T_{CM} cells were infected most readily followed by naïve and T_{EMRA} cells. As expected, Envs that could utilize CXCR4 preferentially infected naïve cells compared to Envs that used exclusively CCR5. (C) T/F and chronic Env pseudotypes have comparable overall CD4+ T cell infection frequency in each of the three donors examined. R5X4-tropic Envs are shown in red and the one X4-tropic Env is shown in cyan. R5X4-tropic Envs are shown in red and the one X4-tropic Env is shown in cyan. Tropism was assessed in cells obtained from three different, uninfected normal donors as indicated (ND218, ND335, and ND337). The horizontal lines indicate the mean value for each group of Envs.

independent experiments, each time using DCs and CD4+ T cells from different normal donors. Adding this amount of virus to $3x10^4$ CD4+ T cells, then washing as with the DCs, resulted in infection at background levels. However, adding virus associated with DCs markedly increased infection. Nonetheless, the magnitude of DC:CD4+ T cell *trans*-infection was not different between T/F and chronic Envs (Figure 2-4; p=0.44 Mann-Whitney). In addition, there was no difference in CD4+ T cell infectivity in the absence of DCs and there was no detectable infection of DC control cultures in the absence of CD4+ T cells (data not shown). The absence of any difference between T/F and chronic pseudoviruses in this *trans*-infection assay suggests that, at least when presented with an equal amount of infectious pseudovirus, DCs bind and transfer T/F and chronic Env pseudotypes similarly.

Entry kinetics and enfuvirtide sensitivity. Productive entry of HIV-1 into cells may occur following internalization and delivery to endosomes, albeit in a pH-independent manner [66]. If so, then the rate at which a virus is internalized, fuses, and enters cells could impact viral tropism. In addition, the rate at which a virus fuses is a measure of how well it productively engages CD4 and coreceptor. Hypothesizing that faster-fusing viruses may preferentially overcome mucosal barriers to transmission, we indirectly assessed the entry kinetics of the T/F and chronic pseudoviruses using a time-of-addition experiment with the fusion inhibitor enfuvirtide. As enfuvirtide is not membrane permeable, time to enfuvirtide escape may reflect the rate of viral endocytosis, fusion, or some combination thereof. HIV-1 pseudotypes were added to NP2 cells on ice.

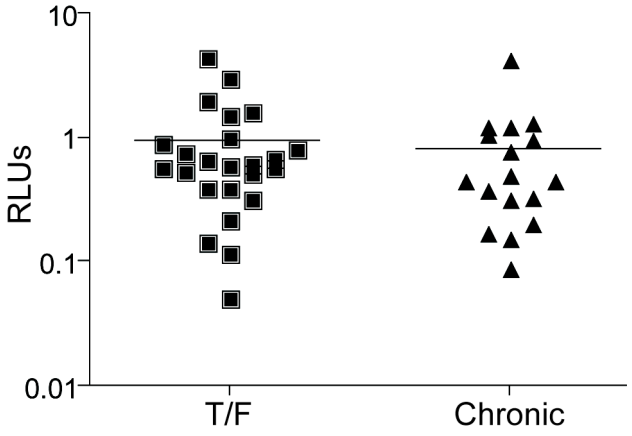

Figure 2-4. Dendritic cell (DC) *trans*-infection. To assess differential DC-binding and CD4+ T cell *trans*-infection of T/F and chronic Envs, we pulsed DCs with luciferase expressing Env pseudotypes and then washed off unbound virus and added CD4+ T cells. Relative light units (RLUs) were then measured as a surrogate for infection. DC *trans*-infection efficiency was comparable between the T/F and chronic Envs (p=0.44). Viral input was normalized based upon infectivity on NP2 cell lines. Data shown is from one of at least three independent experiments with cells from different donors, each done in at least triplicate. Data was analyzed by a Mann-Whitney test.

Cells were spinoculated at 4°C to facilitate HIV-1 binding and then cold media was removed and replaced immediately with pre-warmed media. Saturating concentrations of enfuvirtide were then added at 0, 5, 10, 20, 40, 80, and 160 minutes post-warming, and then infectivity was normalized to a no-drug control. To control for experimental variation, the prototypic R5-tropic virus JRFL was included in all experiments. There was no significant difference or trend in the rate at which T/F and chronic Env pseudotypes productively entered NP2 cells, thus becoming resistant to enfuvirtide addition. The median time to half maximal resistance (t ½ max) post-warming was 32.5 minutes for the T/F and 31.4 minutes for the chronic Envs (p=0.55 Mann-Whitney). Interestingly, JRFL became resistant to enfuvirtide significantly faster (t ½ max= 15.9 minutes) than all 41 T/F and chronic Envs (Figure 2-5A). In addition, we assessed enfuvirtide potency, a measure of pre-hairpin bundle exposure that also reflects kinetics of CD4/coreceptor engagement and endocytosis [67]. There was no difference between T/F and chronic Env sensitivity to enfuvirtide (mean IC_{50} 0.10 vs 0.13 μg/ml; p=0.53; Figure 2-5B). Together this suggests that the kinetics of viral entry/endocytosis are comparable between T/F and chronic Envs.

Sensitivity to broadly neutralizing antibodies and HIV Ig. It has previously been reported that Envs derived from acutely infected individuals may exhibit enhanced sensitivity to antibody-mediated neutralization because of changes in glycosylation and/or variable loop length [2]. This finding raised the possibility that such Envs might be able to bind to CD4 and coreceptor more efficiently. To examine this, we measured the sensitivity of the T/F and chronic Envs to four broadly neutralizing MAbs. MAbs b12 [41] and VRC01 [68] neutralize Env by engaging the CD4 binding site (CD4bs), while

the epitopes for PG9 and PG16 [69], distinct germ-line variants from the same individual, are glycosylation-dependent and include parts of the V1/2 and V3 loops [70]. To assess neutralization sensitivity, pseudoviruses were pre-incubated with a single concentration (10 μg/ml) of each MAb for 60 minutes prior to infection of NP2 cells. Maximal percent inhibition was then determined by normalizing to a control without antibody.

Interestingly, T/F Envs were more sensitive than chronic Envs to both b12 (mean MPI 66% vs. 17%; p=0.0003; Figure 2-6A) and VRC01 (mean MPI 89% vs. 50%; p=0.0077; Figure 2-6B compare T/F to Chronic 1). There was also a trend towards enhanced sensitivity to neutralization by PG9 (Figure 2-6C) and PG16 (Figure 2-6D). To confirm these differences, the neutralization sensitivity of T/F Envs was independently examined using a different backbone (SG3) and HIV-1 reporter cell line (TZMbl), with both MPI and IC_{50} values being determined. The results confirmed the NP2 cell data in that the T/F Envs were more sensitive to neutralization by b12 (Figure 2-6E) and VRC01 (Figure 2-6F). In addition, the T/F Envs exhibited a trend towards increased neutralization sensitivity to both PG9 (Figure 2-6G) and PG16 (Figure 2-6H). While this did not reach statistical significance, it is consistent with a more neutralization sensitive phenotype of T/F compared to chronic Envs.

To assess whether the neutralization sensitive phenotype of our T/F Envs depended on the particular panel of chronic Envs used, we examined the neutralization sensitivity of 14 clade B control Envs derived from six additional chronically infected individuals (Chronic 2 in Figure 2-6A-D). Similar to the initial test set of chronic Envs

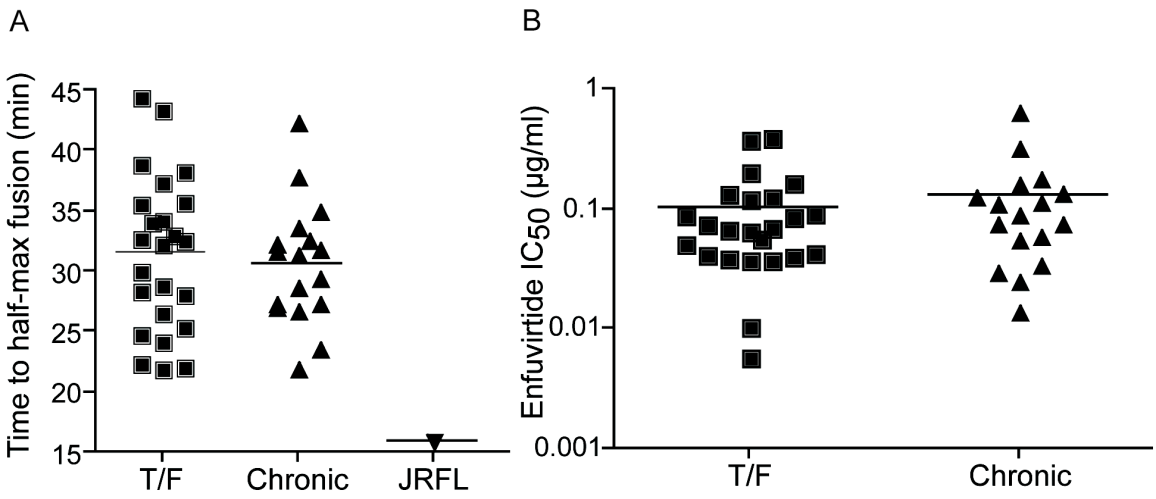

Figure 2-5. Entry kinetics and enfuvirtide sensitivity. (A) To examine differences in T/F and chronic Env endocytosis/fusion kinetics, we employed an indirect assay in which viral pseudotypes were bound to NP2 cells in the cold prior to the addition of pre-warmed media. A saturating concentration of enfuvirtide was added at various times post-warming. The time to half-maximal resistance to enfuvirtide (t ½ max) was then calculated. The T/F and chronic Envs became resistant to enfuvirtide at equal rates ($p=0.55$), with all of the Envs acquiring resistance to enfuvirtide more slowly than a prototypic R5-tropic HIV-1 control, JRFL. (B) Enfuvirtide potency, a compound measure of fusion kinetics and affinity, was assessed for all T/F and chronic Envs. There was no difference in enfuvirtide IC50 between the T/F and chronic Envs ($p=0.53$) further suggesting there is no difference in endocytosis/fusion rates between T/F and chronic Envs. Each infection condition was done in triplicate (A) or duplicate (B) for each Env in each of at least three independent experiments. Data was analyzed by a Mann-Whitney test.

(Chonic 1 in Figure 2-6A-D), this validation set exhibited increased resistance to b12 compared to T/F Envs (p=0.0001 Mann-Whitney). However, unlike the initial chronic Env panel, the validation Envs were similar to the T/F Envs in their sensitivity to VRC01 (p=0.14 Mann-Whitney). Finally, there were no differences in PG9 and PG16 sensitivity between the T/F and the validation Envs (Figure 2-6C, D).

While broadly neutralizing MAbs are useful tools in examining neutralization sensitivity, they are rare in HIV-1-infected individuals and thus may give a biased view of HIV-1 neutralization. Thus, we examined neutralization sensitivity of the T/F and chronic Envs to pooled sera from patients infected with clade B HIV-1 strains (clade B HIV Ig). The T/F Envs (median IC_{50} 741 μg/ml) were approximately two-fold more neutralization sensitive than the chronic test panel (median IC_{50}= 1179 μg/ml p=0.062), the chronic validation panel (median IC_{50} =1500 μg/ml p=0.0095), and the combined clade B chronic panel (median IC_{50} 1324 μg/ml p=0.0078; Figure 2-6I).

To examine the basis for the enhanced b12 and VRC01 neutralization sensitivity of T/F Envs, we measured the binding of the two MAbs to both T/F and chronic Envs. Binding to the trimeric form of the Env expressed on the surface of cells was measured using a cell-based ELISA system [44]. To obtain an accurate measure of antibody binding affinity, we corrected binding measurements for the level of cell surface expression of the different Envs. For this purpose, the binding efficiency of b12 and VRC01 was expressed as a fraction of the binding of a CD4-Ig probe added at saturating concentrations. CD4-Ig is a fusion protein that consists of two copies of the two N-terminal domains of CD4 that are linked to the Fc region of human IgG1.

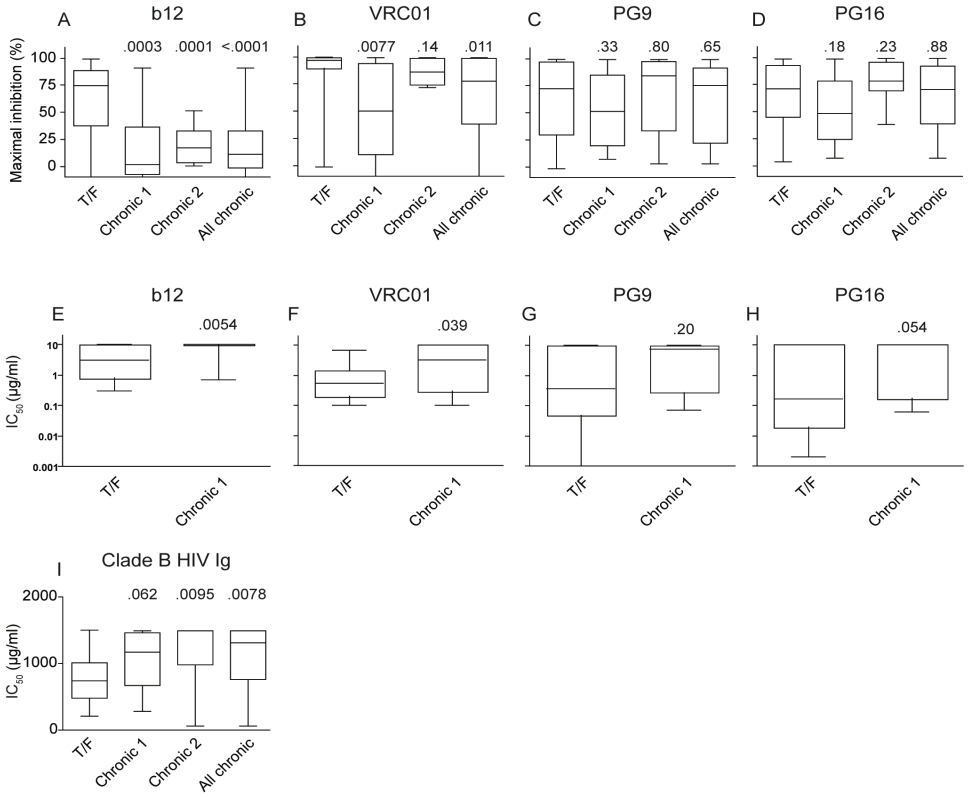

Figure 2-**6. Neutralization sensitivity.** The sensitivity to monoclonal antibodies b12, VRC01, PG9, and PG16 was assessed on both NP2 cells (A-D) and TZMbls (E-H). Neutralization sensitivity on NP2 cells was assessed by determining the maximal percent inhibition (MPI) to 10µg/ml of the indicated MAb. IC_{50} values were determined in the TZMbl assay. Clade B T/F Envs were more sensitive to b12 and VRC01 compared to the geographically-matched panel of chronic Envs (Chronic 1). To confirm this finding, we assessed an independent panel of clade B chronic Envs from Washington state (Chronic 2). "All chronic" includes clade B chronic panels 1 and 2. (I) Clade B T/F Envs are also more sensitive to clade B HIV Ig on NP2 cells as measured by IC_{50}. P-values shown are from Mann-Whitney tests with the corresponding T/F Envs. NP2 and TZMbl experiments were performed in at least three and two independent experiments, respectively.

For the entire group of Envs (i.e., T/F and both chronic Envs groups combined), a very strong correlation was observed between the binding of the MAbs to the trimeric Envs and their sensitivity to inhibition. Spearman rank-order correlation coefficients of 0.62 ($p<0.0001$) and 0.77 ($p<0.0001$) were obtained for b12 and VRC01, respectively (Figure 2-7A and B). Comparison of MAb binding to the T/F and chronic Envs showed clear differences between the two groups for both b12 and VRC01. Binding of b12 to the T/F Envs was significantly increased relative to both groups of chronic Envs (Figure 2-7C). Binding of VRC01 to the T/F Envs was increased relative to the original group of chronic Envs ($p=0.004$; Figure 2-7D). The differential formation/exposure of these epitopes suggests the existence of at least modest structural differences within or near the CD4-binding site of T/F and chronic Envs. No significant differences were observed between VRC01 binding to the T/F and the Washington Envs ($p= 0.21$).

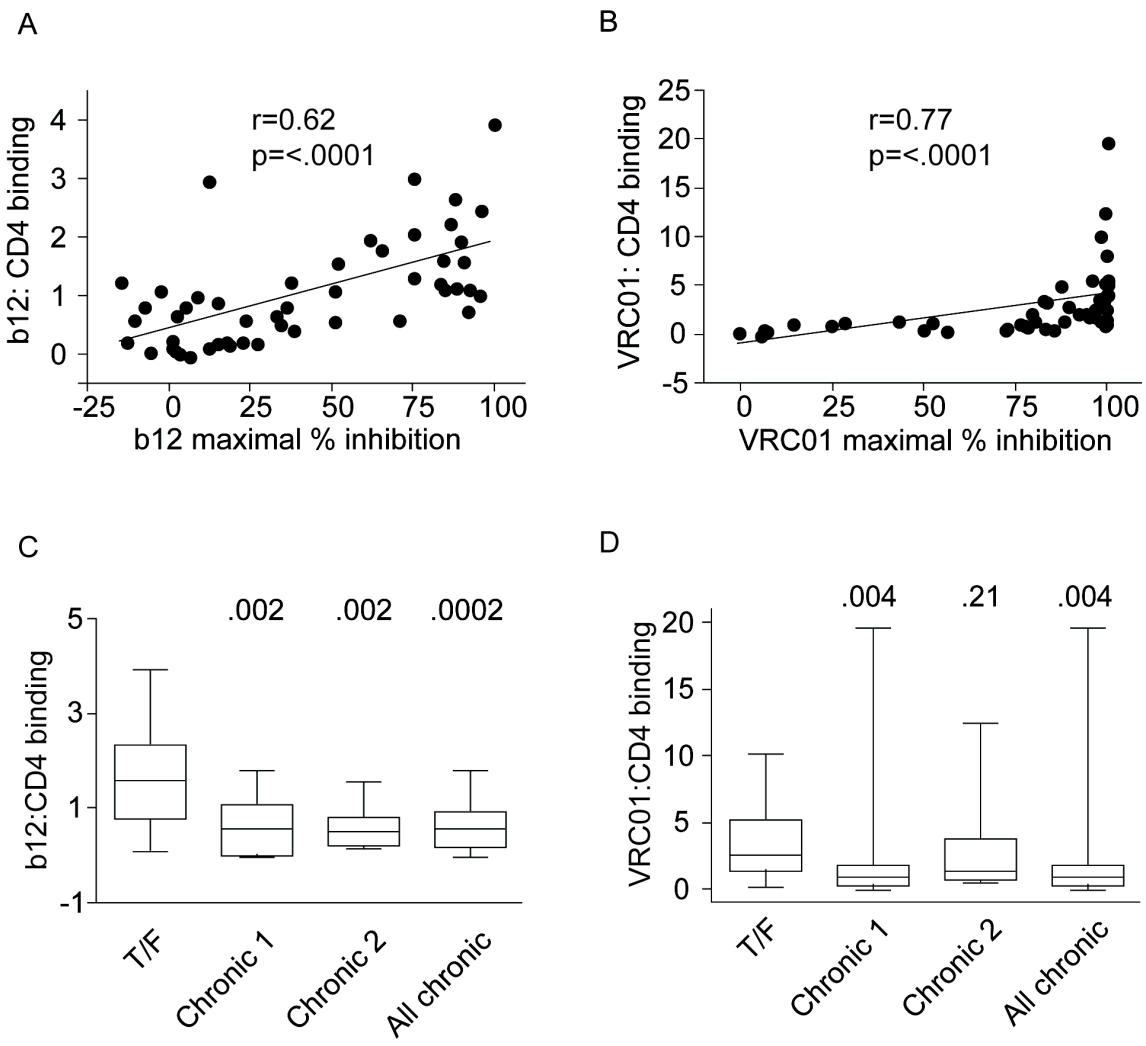

Figure 2-**7. Correlation between MAb binding and neutralization.** Env was expressed on the surface of cells and then binding to b12 (A) and VRC01 (B) was assessed relative to a CD4 control by ELISA. There is a strong positive correlation between binding and Env pseudotype neutralization sensitivity for both b12 and VRC01 for the T/F and both panels of chronic Envs serving to validate the assay. To assess the mechanism of enhanced neutralization sensitivity, we compared b12 (C) and VRC01 (D) binding between T/F and chronic Envs. This suggests that differences in MAb binding explain neutralization differences between T/F and chronic Envs. Data shown is the mean of two independent experiments.

Discussion

The genetic bottleneck that occurs during mucosal transmission of HIV-1 results from the fact that most often only a single founder virus is successfully transmitted from amongst a diverse swarm of viruses present in the donor [1]. It is evident that a significant degree of selection is manifest at this step since transmission of R5-tropic virus strains is far more efficient than that of X4-tropic and even R5X4-tropic viruses [6,71]. Whether there is selection for additional viral phenotypes beyond coreceptor use or whether viral transmission is essentially a stochastic process, in which any reasonably fit R5-tropic HIV-1 strain can be transmitted, has not yet been determined. Addressing this question is of practical importance since properties associated with preferential viral transmission could potentially be exploited by vaccine or other antiviral approaches.

Genetic, immunologic, and phenotypic signatures associated with transmitted HIV-1 Envs have been sought in a number of previous studies, most entailing Envs obtained from early infections (acute Envs) [2,3,6,19,20,24,26] as opposed to true T/F Envs obtained by SGA analyses [1,17,28]. Several studies concluded that T/F and acute Envs have on average shorter variable loops and fewer PNGs than Envs derived from chronically infected individuals [2,3]. While such differences have been noted for Envs from multiple HIV-1 clades, they are relatively subtle, variable in location and far from predictive, with some being evident only when larger numbers of sequences are compared. The 24 T/F Envs examined here, for example, exhibited no consistent genetic differences from the chronic controls. Nonetheless, a much larger sequence comparison that included all but one of the *envs* examined here identified a small number of sequence signatures associated with transmission, including specific sites in the signal sequence

and gp41 cytoplasmic domain that could affect Env processing, localization, and incorporation into virus particles as well as changes in the receptor binding regions in gp120 and in N-linked glycosylation sites (Gnanakaran, et al, submitted). Thus, existing evidence points to an array of genetic features that may be associated with enhanced HIV-1 transmission across mucosal surfaces by unknown mechanisms.

The identification of genetic motifs in *env* that are enriched in T/F viruses is consistent with the possibility that specific phenotypic properties can be identified that might provide a selective advantage to transmitted viruses. This is clearly the case at a global level, in that T/F Envs are almost invariably R5-tropic and replicate well in CD4+ T cells but poorly in monocyte-derived macrophages (with the exception of clade D viruses, G.M. Shaw and J. Baalwa, unpublished data) [28]. More detailed phenotypic studies of recently transmitted viruses are generally lacking, although donor and recipient Envs from eight transmission pairs exhibited no differences in CD4 or CCR5 utilization, while a second study using some of these same Envs did not find consistent differences in primary cell infection or use of receptors other than CCR5 and CXCR4 [19,20]. As genetic signatures associated with transmission can be both variable and subtle, we employed a more detailed series of functional assays to seek differences between viral pseudotypes bearing the T/F Envs and those expressing Envs from chronic controls. We found no phenotypic differences between the T/F and chronic Envs examined here in assays designed to probe the efficiency and rate of membrane fusion, the efficiency of coreceptor use, the ability to infect primary CD4+ T cell subsets from different donors, and the ability of virus to be captured by DCs and transferred to adjoining CD4+ T cells. One could ask whether the assays we employed are sufficiently sensitive to detect

functional differences between viruses bearing different Env glycoproteins. We feel that they are, as we and others have used these and similar assays to identify significant functional differences between Envs at the level of primary CD4+ T cell tropism, membrane fusion kinetics, the efficiency of CD4 and co-receptor utilization, and attachment to C-type lectins such as DC-SIGN [11,12,13,14]. Even single amino changes in Env can impact these properties to extents that can be easily detected. The CD4+ T cell subset tropism assay that we have developed, which can determine the efficiency with which a given virus infects T_{CM}, T_{EM}, T_{EMRA} and naïve T cells, is a particularly sensitive measure of CD4 and coreceptor use, as these receptors are expressed differently on various CD4+ T cell subsets [57,72,73,74,75]. The fact that that 24 T/F Envs here were functionally equivalent to the chronic Env controls in all of the assays employed argues that any phenotypic differences between these and chronic Env controls are apt to be slight in magnitude.

A second consideration regarding the presence or absence of phenotypic traits associated with enhanced virus transmission is whether the assays we employed effectively recapitulate the key events during the earliest stages of HIV-1 transmission (reviewed in [76]). Following mucosal transmission of HIV-1, virus is not detected in the circulation for about 10 days, a period termed the eclipse phase (reviewed in [77]). Detailed studies in the macaque model show that after vaginal exposure small clusters of infected cells are found in the endocervical region, which is lined by a single layer of epithelial cells [56]. The recruitment of plasmacytoid DCs, T cells, and macrophages over several days transforms the initial focus of infection into a CD4+ T cell-rich environment. Similar studies have not yet been conducted assessing penile or rectal

transmission in the rhesus model, the likely mode of transmission in the predominantly male cohort assessed in this study. Conceivably, Env properties that promote entry into resting and activated CD4+ T cells in the submucosa as well as transmission between cells could increase the possibility that an initial focus of infection will successfully propagate and eventually lead to dissemination to regional lymph nodes and a systemic infection. The CD4+ T cell subset tropism assay we employed, while more detailed and sensitive than bulk CD4+ T cell infection assays, may not produce CD4+ T cells with properties identical to those found in the rectal or cervicovaginal mucosa. In addition, the DC:CD4+ T cell transmission assay we used is but a surrogate for the likely more complex cell-cell interactions found in the initial foci of infection. It is important to keep in mind that since virus appears to replicate locally for a period of at least a few days to a week, even a relatively subtle change in an Env property that might enhance infection could result in a significant selective advantage over the course of multiple rounds of infection. The single-cycle assays we employed, while sensitive and well-validated, cannot capture the impact of more subtle differences in Env fitness over time. Future studies employing T/F infectious molecular clones in both primary cell and tissue explant cultures might be better suited for the identification of early fitness differences associated with T/F viruses.

In addition to genetic signatures, differences at the level of sensitivity to antibody-mediated neutralization have been found in some studies of recently transmitted viruses [1,2]. We found that the panel of clade B T/F Envs was more sensitive to the CD4 binding site MAbs b12 and VRC01 as well as clade B HIV Ig, but not to the broadly neutralizing antibodies PG9 and PG16. These differences were approximately two-fold in

magnitude and partially dependent upon the control group employed. Specifically, when a second panel of chronic Envs was used as a control, enhanced sensitivity to VRC01 was not observed, though MAb b12 and clade B HIV Ig continued to neutralize the T/F Envs more efficiently. The relatively modest differences that were observed, along with the fact that enhanced neutralization was not seen between all study groups raises several important questions: do T/F Envs exhibit features that generally enhance their sensitivity to certain types of neutralizing antibodies, and if so, what is the basis for these differences and what are the implications for virus transmission?

One limitation of this study is the selection of chronic control Envs. Ideally, chronic control Envs would be selected from longitudinal samples or confirmed transmission pairs; however, such samples are difficult to find in sufficient numbers, especially since the great majority of acute clade B infections are treated with anti-retroviral therapy. It would also be preferable to obtain chronic Envs from semen or genital secretions, the likely source of the viral inoculum, but again such samples are exceedingly scarce. In addition, the majority of Envs used in this study were from males who likely acquired HIV by penile or rectal transmission. Thus, further work is needed to characterize the transmission bottleneck that occurs during vaginal transmission. Our results emphasize the importance of selecting appropriate matched chronic controls since the chronic test and validation sets differed in their neutralization profiles to VRC01 (though not to MAb b12 and clade B HIV Ig) despite no obvious differences in length of infection, transmission risk factor, patient demographics, or phylogenetic relationships to the T/F Envs. Of course, since we are unable to reliably predict neutralization sensitivity from sequence information alone, a control group could by chance differ

immunologically from the T/F Envs despite being otherwise well-matched. To mitigate this, selecting chronic Env controls from geographically-matched individuals may be important. For example, we previously reported that clade B T/F Envs are more resistant than chronic Envs to b12 and the membrane proximal external region (MPER) antibodies 2F5 and 4E10 [1], seemingly in contradiction with our current findings. However, re-examination of the data in Keele et al. showed that this was due to the predominance of neutralization-sensitive Envs derived from chronically-infected individuals in Trinidad. These Trinidad Envs form a subcluster within the other clade B Envs used in this study (Figure 2-1), have a Thr deletion in the V3 loop compared to the clade B consensus, were over-represented in the chronic controls and were more sensitive to neutralization by MAbs b12, 2F5 and 4E10 [50]. Thus, the previous 2F5 and 4E10 neutralization difference between T/F and chronic Envs was due to bias resulting from disproportionate representation of Envs from Trinidad in the chronic controls.

Several other studies that have assessed neutralization sensitivity of clade B Envs did not use geographically-matched chronic controls, raising the possibility that the results from theses studies could be complicated by genotypic differences linked to geographic location [25,69,78]. In addition to the location, it may also be important to match the time of sample collection when developing well-matched chronic control groups. For example, Bunnik et al. reported that HIV-1 has become more neutralization resistant over the course of the epidemic and thus patient sampling times may bias comparisons between T/F and chronic Envs [79]. Here, the chronic Envs were sampled four calendar years before the T/F Envs on average. However, this difference is significantly shorter than the 14-21 year time-span between contemporary and historic

Envs assessed in Bunnik et al. In addition, we detected no correlation between sampling time and neutralization sensitivity, and thus this cannot account for the neutralization differences between the T/F Envs and the chronic controls. It is also of note that multiple chronic Envs from the same individual were treated as independent events in this study. Reanalyzing the data to include only one chronic Env value (mean of the multiple Envs) per individual did not change the magnitude of the neutralization difference, though it did decrease the p-values above the level of significance for VRC01 and HIV Ig, but not b12, likely due to decreased sample size. In summary, more detailed studies involving larger numbers of T/F Envs with appropriately matched control Envs, including Envs derived from the same individuals over time, and a greater number of broadly neutralizing MAbs and human sera, will be needed to draw definitive conclusions about the neutralization sensitivity of transmitted virus strains.

When our data are considered along with other published studies on T/F and acute Envs, several conclusions can be drawn. First, we believe that HIV-1 transmission is in part stochastic, with any reasonably fit R5-tropic virus being capable of initiating an infection [1,6,9,10]. With a now relatively large number of T/F and acute Envs having been examined, it is evident that no single major genetic, phenotypic or immunologic signature is required for transmission beyond the use of CCR5. Second, an array of genetic traits including but not limited to shorter variable loops and reduced numbers of N-linked glycosylation sites are associated with enhanced virus transmission. The structural implications of these signatures are not well understood, and it is not yet clear if these or as yet unidentified other genetic traits are responsible for the modestly enhanced sensitivity to antibody-mediated neutralization that is characteristic of some

T/F and acute Envs. Third, the presence of genetic signatures linked to transmission implies some impact on function that enhances transmission. If so, then the functional impact is apt to be modest given the variable nature of the genetic signatures and the fact that neither we, nor others, have observed clear differences between T/F and acute Envs with chronic controls. However, the possibility exists that relatively subtle alterations of Env function, perhaps in the context of full-length T/F viral genomes, could provide a sufficiently robust selective advantage during the eclipse phase of HIV-1 transmission to result in preferential transmission of viruses with specific properties. The growing application of SGA technology coupled with increasingly sophisticated cell-to-cell and *ex vivo* tissue systems will make it possible to more rigorously identify immunologic and phenotypic traits associated with HIV-1 transmission.

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Chapter 3

Engineering HIV-Resistant Human CD4+ T Cells with CXCR4-Specific Zinc-Finger Nucleases

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Abstract

HIV-1 entry requires the cell surface expression of CD4 and either the CCR5 or CXCR4 coreceptors on host cells. Individuals homozygous for the *ccr5*Δ*32* polymorphism do not express CCR5 and are protected from infection by CCR5-tropic (R5) virus strains. As an approach to inactivating CCR5, we introduced CCR5-specific zinc-finger nucleases into human CD4+ T cells prior to adoptive transfer, but the need to protect cells from virus strains that use CXCR4 (X4) in place of or in addition to CCR5 (R5X4) remains. Here we describe engineering a pair of zinc finger nucleases that, when introduced into human T cells, efficiently disrupt *cxcr4* by cleavage and error-prone non-homologous DNA end-joining. The resulting cells proliferated normally and were resistant to infection by X4-tropic HIV-1 strains. CXCR4 could also be inactivated in *ccr5*Δ*32* CD4+ T cells, and we show that such cells were resistant to all strains of HIV-1 tested. Loss of CXCR4 also provided protection from X4 HIV-1 in a humanized mouse model, though this protection was lost over time due to the emergence of R5-tropic viral mutants. These data suggest that CXCR4-specific ZFNs may prove useful in establishing resistance to CXCR4-tropic HIV for autologous transplant in HIV-infected individuals.

Introduction

For HIV to infect cells, the viral envelope (Env) protein must bind to the host protein CD4 and then to a coreceptor, most commonly CCR5 (R5 HIV) (reviewed in [1]). The importance of CCR5 for HIV-1 pathogenesis is shown by the fact that individuals who are homozygous for an inactivating 32 base pair deletion in *ccr5* (*ccr5*Δ*32*) are highly resistant to HIV infection [2, 3], while heterozygotes typically live longer after HIV infection due to reduced CCR5 expression levels [4, 5]. Recently, an HIV infected patient with acute myelogenous leukemia received a bone marrow transplant from a *ccr5*Δ*32* homozygous donor [6]. This patient's viral load remains undetectable even in the absence of anti-retroviral therapy more than three years post-transplant, suggesting that this individual's HIV infection has been eradicated. In theory, the success of this approach could be recapitulated by inhibiting CCR5 with an orally bioavailable small molecule such as maraviroc, which binds to CCR5 and prevents its use by most R5 HIV-1 strains. However, virus strains that can utilize CXCR4 either in place of (X4 HIV) or in addition to CCR5 (R5X4 HIV) are found at significant levels in roughly 50% of late-stage infected individuals [7, 8], supporting the need for therapies targeted to CXCR4 [9]. Ideally, an approach to target CXCR4 would complement CCR5-specific therapy, but the broad expression pattern of CXCR4 has made systemic inhibition of this coreceptor by small molecules problematic [10, 11]. In addition, resistance to CCR5 and CXCR4 antagonists can arise in patients by mutations in the viral envelope protein (Env) that enable it to utilize the drug-bound forms of these coreceptors [12-16]. The ability of HIV-1 to adapt to new selective pressures and the plasticity with which Env interacts with its coreceptors argues for approaches that reduce or eliminate coreceptor expression rather

than simply altering coreceptor conformation. If approaches could be developed that specifically target expression of both CCR5 and CXCR4 on CD4+ T cells, virus entry should be inhibited more effectively.

Several genetic approaches have been taken to reduce or eliminate CCR5 expression in human cells, including the use of ribozymes [17, 18], single-chain intracellular antibodies [19], trans-dominant coreceptor mutants [20], and RNAi [21, 22]. However, these studies are limited by the requirement for stable expression of an exogenous gene. To circumvent this, a CCR5 specific zinc-finger nuclease pair (R5-ZFNs) has been developed [23]. Zinc finger proteins that recognize a specific 24bp DNA sequence are fused with a monomeric cleavage domain from *FokI* endonuclease that functions only as a dimer. For DNA cleavage to occur, two zinc finger proteins must bind, each to specific, adjoining sequences in the CCR5 gene, leading to *FokI* dimerization and subsequent DNA cleavage resulting in a double strand break [24-26]. The double strand break then can be repaired by error-prone non-homologous end joining (NHEJ) often introducing insertions and deletions leading to a non-functional gene product when this break is placed within the coding region of the targeted gene [27]. Following introduction into human CD4+ T cells [23] or hematopoietic stem cells [28] via an adenovirus vector or DNA nucleofection, respectively, the *ccr5* gene was efficiently and specifically disrupted. This confers protection *in vitro* and in humanized mice to infection by HIV-1 isolates that require CCR5 (but not CXCR4). Several early stage clinical trials using autologous infusions of ZFN-generated CCR5-modified CD4+ T cells are currently underway (clinicaltrials.gov identifiers NCT00842634, NCT01252641, NCT01044654).

In this study we describe the design and pre-clinical evaluation of a CXCR4-specific ZFN pair (X4-ZFNs) that specifically and efficiently disrupts *cxcr4*, rendering human CD4+ T cells permanently resistant to HIV-1 strains that require CXCR4 for infection. We also demonstrate that *cxcr4* can be safely and efficiently disrupted in CD4+ T cells obtained from *ccr5*Δ*32* homozygotes resulting in cells resistant to all strains of HIV-1 tested. This suggests that combined treatment of mature CD4+ T cells with X4-ZFNs and R5-ZFNs can provide permanent protection against HIV-1 infection.

Materials and Methods

Zinc-finger nuclease constructs. We designed ZFNs specific to the human and rhesus CXCR4 and CCR5 genes using a previously described approach [29]. One ZFN pair was used to target both the human and rhesus macaque CXCR4 genes since the 24 bp target sequences are identical. Zinc-finger proteins were optimized against the target gene sequence and assembled as described [30] from an archive of *in-vitro*-selected modules [31, 32]. The ZFP moieties (target gene; ZFP name; target sequence (5'→3'); recognition α-helices (finger number)) are as follows: CXCR4; X4-ZFN-L; GTAGAAGCGGTC, DRSALSR (1), RSDDLTR (2), QSGNLAR (3), QSGSLTR (4); CXCR4; X4-ZFN-R; GACTTGTGGGTG, RSDSLLR (1), RSDHLTT (2), RSDSLSA (3), DRSNLTR (4). Rhesus CCR5; rhR5-ZFN-L; GATGAGGACGAC, RSDNLAR (1), TSGNLTR (2), RSDNLAR (3), TSGNLTR (4); Rhesus CCR5; rhR5-ZFN-R; AAACTGCAAAAG; RSDNLSV (1), QKINLQV (2), RSDVLSE (3), QRNHRTT (4)., The human CCR5-specific ZFNs are described in Perez et al [23]. The Ad5/F35 adenoviral vectors were generated on an E1/E3 deleted backbone. The ZFNs targeting either the *cxcr4* or *ccr5* genes were linked via a 2A peptide sequence and cloned into the pAdEasy-1/F35 vector under control of the CMV TetO promoter, and the Ad5/F35 virus for each construct was generated using TREx 293T cells as described [33]. The Ad5/F35 vector encoding the X4-ZFNs is identical to that use by Nilsson, et al. [33] except for the ZFN inserts, promoter, polyA and linker sequences.

Cel1 (surveyor nuclease) assay. Genomic DNA was extracted with the MasterPure kit (Epicentre Biotechnologies) according to manufacturer's instructions. Frequency of gene modification by NHEJ was evaluated as described previously [23, 25,

28]. Briefly, the purified genomic DNA was used as a template to amplify a fragment of the *cxcr4* gene using the specific primers (human CXCR4: 5'-CAACCTCTACAGCAGTGTCCTCATC -3'and 5'-GGAGTGTGACAGCTTGGAGATG -3'; rhesus CXCR4: 5'-GGTGGTCTATGTTGGAGTCTGG -3'and 5'- GGAGTGTGACAGCTTGGAGATG -3') in the presence of a ^{32}P-dATP and dCTP. The PCR products were then heated, allowed to re-anneal followed by treatment with the mismatch-sensitive Surveyor nuclease as described in order to detect insertions and deletions caused by NHEJ. For humanized mice samples, whole genome amplification using the REPLI-g Mini Kit (Qiagen) was conducted prior to the surveyor nuclease assay due to limiting cell numbers.

Human CD4+ T cell stimulation and transduction. Fresh CD4+ T cells from normal human donors, purified by negative selection, were obtained from the Center for AIDS Research Human Immunology Core at the University of Pennsylvania. 2.5 million CD4+ T cells were seeded at a density of 0.8 x 10^6 cells/ml in RPMI containing 10% fetal calf serum, 1% penicillin/streptomycin, and 100U/ml interleukin-2 (IL-2). The cells were stimulated with anti-CD3/anti-CD28 coated magnetic beads at a 3:1 bead to cell ratio [34]. Approximately 18hrs post-stimulation, the cells were transduced with an Ad5/F35 vector encoding either the X4-ZFNs or R5-ZFNs at a multiplicity of infection (MOI) of 600. Beginning 72 hours post-stimulation, cells were counted every 48 hours using trypan blue dye exclusion on an automated hemocytometer (Countess, Invitrogen) and split to 0.8 x 10^6 with fresh media containing 100U/ml IL-2. Five days post-stimulation, the magnetic beads were removed and washed twice in fresh media. Cells

were counted and split until cell growth plateaued 10-14 days post stimulation. For longer experiments, cells were restimulated with beads and cultured for an additional 10-14 days.

***In vitro* HIV-1 challenge of CD4+ T cells treated with AdX4-ZFNs**. Five days post-stimulation the anti-CD3/anti-CD28 coated magnetic beads were removed from each of the three cultures (non-transduced (NTD), AdX4-ZFNs, and AdR5-ZFNs) and 2.5 million cells were seeded in each of four cultures that were subsequently infected with either Bk132 (primary X4 isolate), HxB2 (lab-adapted X4 isolate), R3A (R5X4 primary isolate), or media only (mock). 100ng p24 of HIV-1 was used per million cells.

Flow cytometry. All staining was done at room temperature in FACS Wash Buffer (1mM EDTA, 2.5% fetal calf serum in PBS) and all antibodies were from BD Biosciences unless otherwise noted. 0.5-1.0 $x10^6$ cells were washed in PBS and stained with Live/Dead Aqua (Invitrogen) for 10 min. Then, anti-CD4 PE Cy5.5 and anti-CXCR4 APC (clone 12G5) were added and cells were stained for 20-30 minutes. Cells were then washed and permeabilized per manufacturer's protocol using Cytofix/cytoperm (BD) and stained intracellularly for HIV gag with KC57-RD1 (Beckman Coulter). For compensation, ArC beads (Invitrogen) were used for live/dead, and CompBeads (BD) were used for all other fluorochromes. To detect wtCXCR4 and CXCR4Δ18 in 293T transient transfection experiments, anti-CXCR4 APC (clone 12G5) and anti-CXCR4 PE (clone 4G10) (Santa Cruz Biotechnologies) were used. All samples were run on an LSRII (BD) and analyzed using FlowJo 8.8.6 (Treestar Inc).

Events were gated as follows: singlets (FSC-A by FSC-H), live cells (SSC-A by Live/Dead), lymphocytes (FSC-A by SSC-A), CD3+CD4+ (CD3 by CD4), and then

events were divided into CXCR4+ and CXCR4- populations based upon a fluorescence minus one (FMO) control.

454 deep sequencing and *cxcr4* analysis. Genomic DNA was isolated from CD4+ T cells using the QIAamp DNA Micro Kit (Qiagen). For each condition, 200 ng genomic DNA was then PCR amplified using Platinum Taq High Fidelity (Invitrogen) using the following primers plus 454 adaptor sequences and 8 letter DNA barcodes: CAACCTCTACAGCAGTGTCCTCATC (forward) and GGAGTGTGACAGCTTGGAGATG (reverse). Cycle conditions were 95° for 5min, then 30 cycles of 95° for 30sec, 55° for 3 sec, 68° for 30 sec, followed by 68° for 2 min. Following PCR amplification the PCR product was analyzed on a 2% agarose gel and then extracted and gel purified using Wizard SV Gel and PCR Clean-Up System (Promega). Quant-iT dsDNA High-Sensitivity Assay Kit (Invitrogen) was then used to determine the concentration of each bar-coded amplicon. DNA samples were then pooled at an equimolar ratio and run on a Roche/454 GS FLX using standard chemistries at the University of Pennsylvania's DNA Sequencing Facility. Approximately 30,000-100,000 reads were obtained for each experiment. CXCR4 pyrosequencing data were assigned to samples by DNA barcode. Any reads containing ambiguous base calls or without a perfect match to barcode and primer were discarded. All remaining reads were aligned to the CXCR4 reference sequence using Mosaik (http://bioinformatics.bc.edu/marthlab/Mosaik). All deviations from the CXCR4 consensus sequence 40 base pairs up or downstream from the ZFN binding site were determined. Any reads that did not extend across this region or that failed to align were discarded. Reads containing only two or fewer substitutions were not classified as

mutations as these likely represent sequencing artifacts. Next, background pyrosequencing error, identified by an untransduced control sample, was subtracted from each group of reads. For frameshift analysis, the sequencing error was determined and subtracted for each individual insertion or deletion size.

To ensure sufficient sampling of diverse amplicons, at least 200ng gDNA was used for CXCR4 analysis and at least 400ng gDNA was used for off-target site amplification, representing the genomic DNA content of approximately 70,000 and 140,000 alleles, respectively. Determining genetic disruption frequency by both the Cel1 and 454 assays require the assumption that wild type and disrupted alleles are not differentially amplified.

Systemic evolution of ligands by exponential enrichment (SELEX) and determination of off-target sites. To empirically determine the DNA binding preference of the X4-ZFNs, we employed SELEX as previously described [23]. Briefly, each ZFP was HA-tagged and incubated with randomized DNA oligonucleotides and anti-HA Fab fragments. Any DNA bound to the ZFPs was then isolated and amplified. The newly amplified DNA was then used to repeat this process for a total of four rounds of enrichment. The DNA pool was then sequenced at approximately 50x coverage to generate a positional-weighted matrix. This matrix was then aligned to the human genome with the following criteria: putative off-target sites could have up to six mismatches compared to the SELEX consensus sequence, the ZFP pairs must be separated by either 5 or 6 bps, and both ZFP homo- and heterodimers were considered. Off-target sites were ranked and scored by multiplying the probability of each nucleotide at each of the 12 positions of the positional-weighted matrix. The highest scores were

then deemed most likely to be disrupted. 454 off-target site data was analyzed as discussed previously [23].

NSG mice. NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/Szj) mice, 8-9 weeks old at time of initial injection, were derived from breeders purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were maintained in a defined flora animal barrier facility at the University of Pennsylvania's Stem Cell and Xenograft Core.

Human CD4+ T cells were isolated and stimulated as previously described and then transduced with an Ad5/F35 vector expressing either the R5-ZFNs or the X4-ZFNs at an MOI of 600. Cells were maintained as previously described. Ten days post stimulation 10^7 modified cells resuspended in 100µL PBS were injected intravenously into the tail vein of each mouse. 23 animals received cells treated with X4-ZFNs and 22 mice received cells treated with R5-ZFNs. Animals were randomized by age, sex, and cage. Mice were maintained on the antibiotic Baytril (Bayer) for 24 hours post-injection.

To infect the mice with HIV-1, 10^5 autologous CD4+ T cells previously infected with X4 HIV-1 strain Bk132 were injected into the tail vein of each mouse. Autologous cells used to infect mice that were not transduced were obtained and stimulated simultaneously as the initially engrafted cells. Five days post-stimulation cells were infected with 100ng p24/million cells and then were cryopreserved four days post-infection. Cell engraftment was assessed 27 days post injection, and mice were infected with HIV-1 the following day.

To obtain whole blood, mice were anesthetized with isoflurane and a capillary tube was used to drain the retroorbital vein. Human CD4+ T cell counts were determined by staining 50µl of whole blood in Trucount tubes (BD) with anti-CD45 FITC

(Biolegend), anti-CD3 Qdot 655 (Invitrogen), anti-CD4 Alexa Fluor 700, anti-CD8 Pacific Blue (Biolegend), and anti-CXCR4 PE-Cy5. Human CD4+ T cells were defined as CD45+CD3+CD4+CD8-.

At the time of sacrifice, a cardiac puncture was performed to obtain maximal blood volume and then the spleen was harvested. Spleens were homogenized and erythrocytes were lysed with ACK lysis buffer (Invitrogen) before cell purification. Human CD4+ T cells were then isolated with the Human CD4 Positive Selection Kit using the Robosep robotic cell separator (Stem Cell Technologies).

Rhesus macaque CD4+ T cell modification. Whole blood from rhesus macaques (Macaca mulatta) housed at the Tulane National Primate Research Center was used for CD4+ T cell isolation and ZFN treatment. Peripheral blood mononuclear cells were isolated by centrifugation with 96% Ficoll (BD), followed by erythrocyte lysis with ACK lysis buffer. CD4+ T cells were then isolated by negative selection with a non-human primate CD4+ T cell selection kit (Miltenyi). Cells were then stimulated with 1:4 anti-CD3 (clone FN-18)/ anti-CD28 (clone L293) M-450 tosylactivated beads (Invitrogen) at a ratio of 1 bead per cell [35, 36].

Approximately 18 hours post-transduction, cells were transduced with an Ad5/F35 vector expressing either the X4-ZFNs or rhesus specific R5-ZFNs. Cells were maintained in culture as human CD4+ T cells. Surveyor nuclease assay was performed six-ten days post transduction to assess disruption efficiency.

Ethics statement. Human CD4+ T cells were obtained after written informed consent and approval by the University of Pennsylvania's institutional review board. All humanized mouse experiments were approved by the University of Pennsylvania's

Institutional Animal Care and Use Committee (Protocol 802436), and were carried out in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All rhesus macaque experiments were approved by the Tulane Institutional Animal Care and Use Committee approval (Protocol P0085; Project 3520) The Tulane National Primate Research Center (TNPRC) is an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility (AAALAC #000594). The NIH Office of Laboratory Animal Welfare assurance number for the TNPRC is A3071-01. All clinical procedures, including administration of anesthesia and analgesics, are carried out under the direction of a veterinarian. Blood was collected while the animals were anesthetized with Tiletamine-zolazepam with Burprenorphine given as an analgesic. All possible measures are taken to minimize discomfort of all the animals used in this study. The University of Pennsylvania and Tulane comply with NIH policy on animal welfare, the Animal Welfare Act, and all other applicable federal, state and local laws.

Results

Design and characterization of X4-ZFNs. To genetically disrupt the CXCR4 allele, we designed a pair of zinc-finger proteins (ZFPs) targeting the region of the *cxcr4* gene that encodes residues Asp 187 to Val 196 in the second extracellular loop (ECL2) of this seven-transmembrane domain receptor using methods previously described [29-32] (Figure 3-1). The ECL2 was chosen because this region is less well conserved amongst the CXC family of chemokine receptors, which should reduce the frequency with which other CXC receptors might be targeted, and because ECL2 is important in supporting interactions with the HIV-1 Env protein [37, 38]. Two ZFPs were designed to bind each of two 12bp targets separated by 6bp in this region of CXCR4. Each ZFP was then fused to a modified *FokI* cleavage domain, active preferentially as a dimer to reduce nonspecific DNA cleavage, resulting in zinc-finger nucleases (ZFNs) [25]. Upon binding of both X4-ZFNs, the *FokI* nuclease cleavage domains dimerize and then generate a double strand break that can subsequently be repaired by error-prone NHEJ resulting in mutations targeted to the cleavage site that can include missense mutations, deletions and insertions (Figure 3-1).

Efficiency of CXCR4 allele disruption in human CD4+ T cells. To determine the efficiency and specificity with which the *cxcr4* genes could be disrupted in human T cells, we produced a bicistronic Ad5/F35 vector to deliver the X4-ZFNs (AdX4-ZFNs). The Ad5/F35 vector is a serotype 5 virus with the fiber protein from a serotype 35 adenovirus that utilizes CD46 for entry as opposed to the coxsackie and adenovirus receptor (CAR), which is poorly expressed on human CD4+ T cells [39]. Primary human CD4+ T cells were stimulated with anti-CD3/anti-CD28 coated magnetic beads and

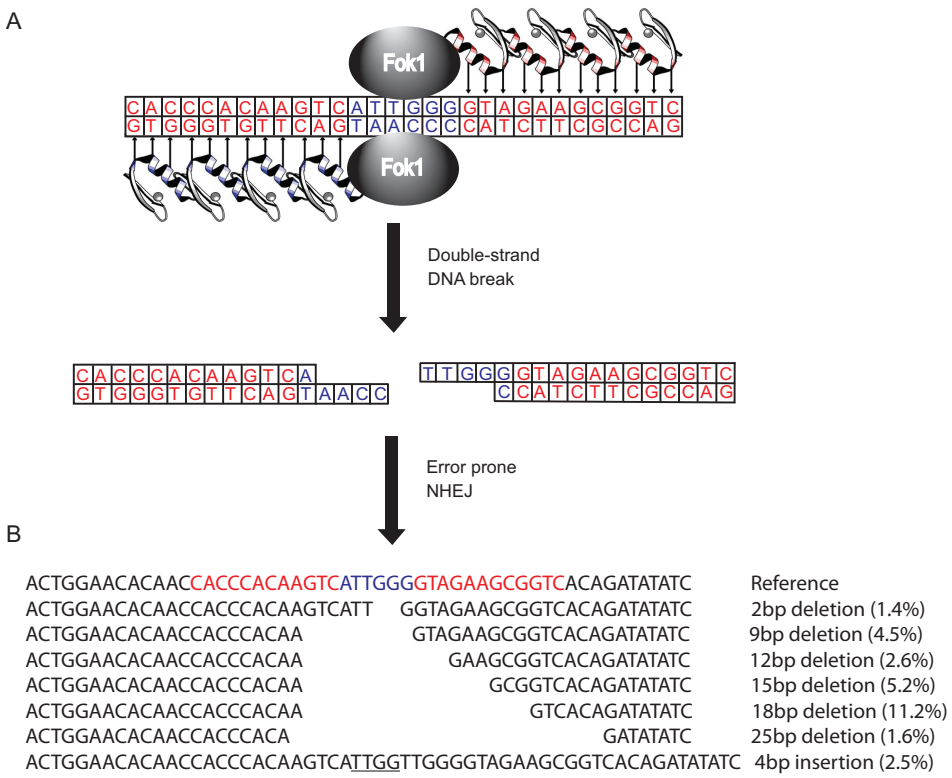


Figure 3-1. Zinc finger nucleases (ZFNs) bind, cleave, and disrupt *cxcr4.* (A) A CXCR4-specific ZFN pair was generated, comprised of two DNA-binding zinc finger proteins (ZFPs) each fused with a *FokI* endonuclease monomer. Each ZFP was designed to target 12 bp of *cxcr4* sequence (in red), separated by 6 bp (in blue), conferring 24 bp of total specificity. Upon binding of both ZFPs, the *FokI* domains can dimerize and cleave the double stranded DNA. The subsequent double strand break is then repaired by error prone non-homologous end-joining resulting in various targeted mutations and a non-functional protein product. (B) The most common mutations induced by the X4-ZFNs, as detected by 454 deep sequencing, are indicated with their frequencies among all ZFN-induced lesions. In-frame deletions were preferentially generated with the most common being an 18 bp deletion, referred to as CXCR4Δ18. Frequences were averaged across five independent experiments in the absence of HIV infection.

transduced 18 hours later with AdX4-ZFNs, AdR5-ZFNs which expresses previously described CCR5-specific ZFNs [23], or an Ad5/F35 vector that expresses green fluorescent protein (AdGFP). To identify optimal disruption conditions, multiplicities of infection ranging from 100 to 1000 were employed. Cell growth was monitored every 48 hours post-stimulation for approximately two weeks, and the efficiency of CXCR4 disruption was assessed at day five post-transduction by both the Surveyor nuclease assay and by deep-sequencing of the CXCR4 target site. As shown in Figure 3-2A, the Ad5/F35 vectors had a slight dose-dependent impact on cell growth at higher multiplicities of infection that was similar with the AdX4-ZFNs and AdGFP vectors.

Cxcr4 allelic disruption efficiencies as determined by either deep sequencing or the Surveyor nuclease assay were comparable, and were approximately 10% at an MOI of 100, 20% at an MOI of 300, 34% at an MOI of 600, and 38% at an MOI of 1000 (Figure 3-2B). For subsequent experiments we used an MOI of 600 as this provided near-maximal disruption efficiency with limited impact on cell growth. Notably, this is also the MOI being used in an adoptive therapy phase I clinical trial with R5-ZFNs. Importantly, the level of *cxcr4* disruption in cells from multiple donors was stable over nearly four weeks in culture (Table 3-1), indicating that CXCR4-disrupted cells continued to grow normally. Cell proliferation remained dependent on stimulation, and transformation has not been observed after treatment with ZFNs (data not shown).

Mutations introduced by cleavage with X4-ZFNs. Deep sequencing of the ZFNs target site 10 days after transduction made it possible to assess the mutations introduced by NHEJ reactions following cleavage with X4-ZFNs. Of the nearly 50,000 modified *cxcr4* alleles analyzed across five independent experiments, 81.1% (range 75.3-

81.7%) contained pure deletions from 1-64 bp in size with the most common deletions being 2, 9, 12, 15, 18, and 25 bp, while 13.5% (range 12.8-16.9%) of *cxcr4* alleles contained pure insertions ranging from 1 to 69 bp with more than 90% being 7 bp or less (Figure 3-1B). The remaining 5.3% (range 4.3-7.4%) of disruption events contained multiple insertions and deletions that may be due to more extensive DNA end-processing or multiple cycles of ZFN-mediated cleavage and subsequent NHEJ. Surprisingly, frameshift mutations occurred at a ratio of 0.90 in-frame per out-of- frame mutation as opposed to the expected frequency of 0.50 (1 in-frame per 2 out-of-frame mutations; Table 3-1). This unexpected bias likely resulted from microhomology-mediated joining that produced in-frame deletions. To our knowledge, preferential in-frame repair has not been reported or seen with other ZFNs [23, 40, 41].

To further characterize the consequences of disruption mediated by X4-ZFNs, we analyzed an unusually common lesion, an in-frame 18 bp deletion (CXCR4Δ18) that results in the deletion of DNA encoding amino acids R188 to D193 (Figure 3-1B). This deletion comprised 11.2% (range 9.8 and 11.9%) of all *cxcr4* disruptions across five independent experiments with cells from five different donors. The resulting CXCR4Δ18 protein, containing a six-residue deletion in ECL2, could potentially be expressed at the cell surface and support HIV infection. To examine this, we transiently expressed CXCR4Δ18 or wt CXCR4 as a control in 293T cells, which have low endogenous CXCR4 expression. CXCR4 cell surface and intracellular expression was detected by flow cytometry after co-staining with the N-terminal specific CXCR4 antibody 4G10 and the extracellular loop (ECL) specific antibody 12G5 whose epitope includes the CXCR4Δ18 deleted residues [42]. As expected, CXCR4 could be detected on the surface

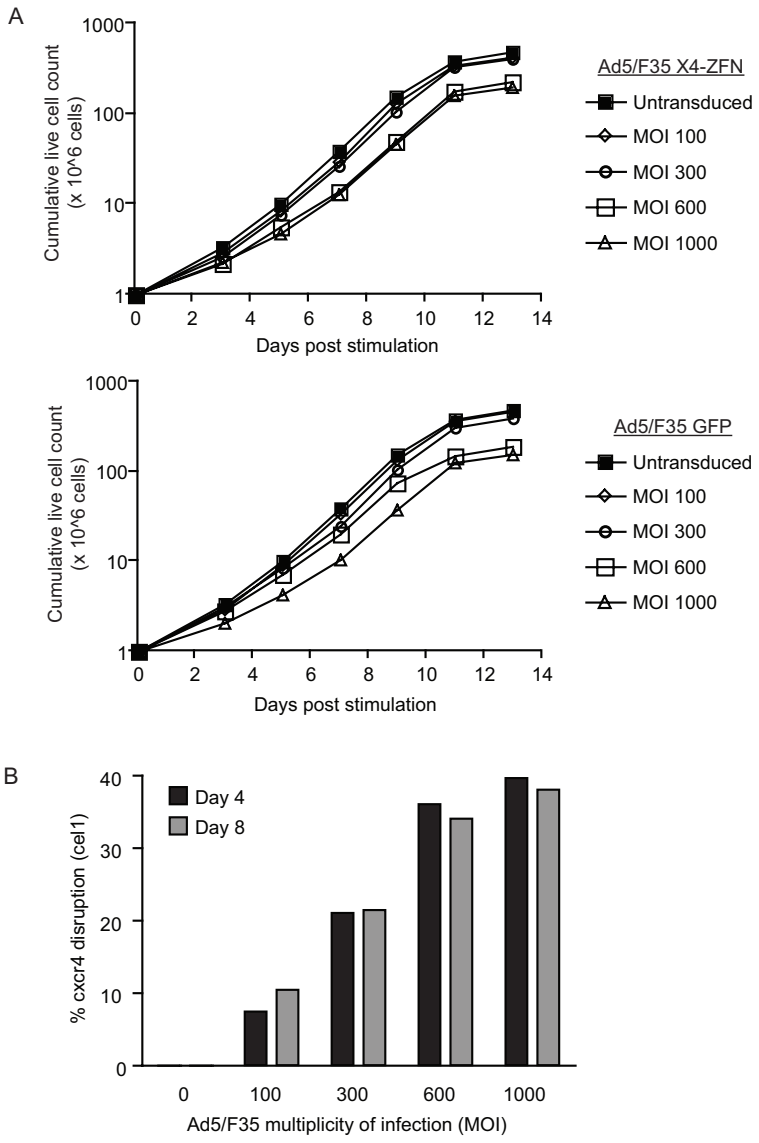


Figure 3-2. X4-ZFNs mediated disruption of *cxcr4* in primary human CD4+ T cells. (A) Primary human CD4+ T cells were stimulated and transduced with an Ad5/F35 vector expressing either the X4-ZFNs (top) or GFP (bottom) at MOIs from 100-1000. Total live cells were counted at different times after stimulation, and compared to an untransduced control. Data is from one of two independent experiments. (B) *Cxcr4* disruption was determined four and eight days post treatment with the X4-ZFNs by the surveyor nuclease assay (cel1).

Table 3-1. Deep sequencing results of *cxcr4* disruptions.

										In-frame to out-of-frame ratio			Most common disruptions induced by X4-ZFNs3						
CCR5 genotype	Donors	# Reads	Day[1]	Ad5/F35 X4-ZFN MOI	HIV	%NHEJ	%Insertions	%Deletions	%Multiple[2]	Total	Deletions	Insertions	CXCR4Δ25	CXCR4Δ18	CXCR4Δ15	CXCR4Δ12	CXCR4Δ9	CXCR4Δ2	CXCR4+4
wt/wt	ND306	4940	5	300	Mock	10.8	1.6	8.4	0.7	0.93	1.29	0.09	1.8%	9.5%	3.8%	3.0%	5.2%	0.3%	1.9%
		4431	5	600	Mock	23.6	3.8	18.3	1.3	0.89	1.25	0.05	1.2%	12.0%	5.2%	1.0%	5.2%	1.8%	1.8%
		4772	5	1000	Mock	28.3	4.8	21.6	1.9	1.00	1.44	0.11	1.9%	9.1%	5.7%	3.0%	5.0%	2.0%	3.0%
wt/wt	ND335	4801	13	600	Mock	24.3	4.1	18.3	1.8	0.88	1.27	0.08	1.3%	11.9%	5.8%	3.2%	5.8%	1.4%	3.5%
		5080	13	600	Bk132	42.1	6.9	32.7	2.5	0.92	1.31	0.08	1.6%	13.3%	5.1%	3.2%	6.2%	1.2%	3.8%
		5108	13	600	HxB2	29.0	4.2	22.5	2.3	0.87	1.14	0.12	1.5%	13.6%	5.2%	2.6%	3.8%	1.1%	2.8%
		4902	13	600	R3A (1)	38.2	5.3	30.1	2.8	0.94	1.27	0.07	1.8%	13.6%	5.7%	2.9%	4.5%	0.9%	3.0%
		4546	13	600	R3A (2)	40.9	5.2	33.0	2.6	1.07	1.42	0.09	1.5%	14.1%	7.1%	3.1%	7.4%	1.5%	2.8%
wt/wt	ND221	3261	11	600	Mock	19.6	2.8	15.6	1.0	1.08	1.42	0.20	1.5%	11.9%	4.5%	3.3%	5.9%	1.1%	1.8%
		3979	18	600	Mock	19.0	2.8	15.4	0.8	0.98	1.39	0.11	0.3%	12.8%	6.4%	2.7%	4.8%	1.0%	3.0%
		3503	24	600	Mock	17.9	2.5	14.3	0.9	1.09	1.53	0.16	0.4%	15.0%	4.5%	3.2%	5.1%	1.5%	2.3%
		3291	11	600	Bk132	18.1	2.5	14.8	0.8	0.92	1.23	0.08	1.4%	13.9%	5.2%	3.5%	5.8%	1.2%	3.5%
		2832	18	600	Bk132	70.2	9.6	57.3	3.3	1.05	1.44	0.07	1.8%	14.9%	6.6%	3.4%	7.1%	1.0%	3.4%
		2475	24	600	Bk132	83.3	12.1	67.5	3.6	1.16	1.62	0.11	1.3%	15.6%	6.9%	4.0%	7.4%	1.9%	3.1%
		2997	11	600	HxB2	20.2	3.7	15.6	0.9	0.94	1.46	0.07	1.2%	12.0%	4.5%	3.5%	6.8%	1.2%	3.6%
		2839	18	600	HxB2	48.9	6.9	39.4	2.7	1.04	1.45	0.07	0.8%	13.7%	5.8%	3.9%	6.9%	1.0%	2.0%
		3070	24	600	HxB2	61.9	7.7	51.1	3.1	1.12	1.51	0.09	1.5%	14.2%	6.7%	4.3%	6.5%	1.3%	2.0%
		3640	11	600	R3A	21.5	2.6	17.9	1.0	1.06	1.44	0.09	0.3%	13.0%	5.9%	2.8%	6.2%	0.9%	2.5%
		2784	18	600	R3A	61.2	7.7	50.0	3.5	1.18	1.62	0.10	1.1%	15.6%	6.7%	4.2%	6.0%	0.6%	3.1%
		2793	24	600	R3A	75.4	9.5	62.4	3.5	1.21	1.63	0.11	1.2%	16.8%	5.7%	4.0%	7.0%	0.9%	2.0%
wt/wt	ND336	734	5	600	Mock	38.1	5.6	30.6	1.9	0.84	1.09	0.13	4.2%	10.2%	4.5%	1.6%	2.9%	1.0%	2.6%
		1924	16	600	Mock	28.8	3.7	23.2	1.8	0.74	0.94	0.09	3.4%	8.4%	7.2%	1.9%	1.9%	1.1%	2.8%
		1827	26	600	Mock	29.3	3.7	23.8	1.8	0.71	0.95	0.00	2.7%	10.4%	5.1%	2.6%	3.1%	1.4%	3.1%
		1636	16	600	Bk132	58.6	7.2	48.2	3.2	0.68	0.83	0.09	2.6%	9.0%	3.8%	2.2%	3.5%	1.6%	2.9%
		1587	26	600	Bk132	87.3	8.6	74.9	3.7	0.74	0.88	0.08	3.3%	8.6%	5.3%	3.3%	3.3%	1.5%	0.9%
		704	16	600	HxB2	40.4	5.8	32.2	2.3	0.81	1.02	0.19	1.9%	12.7%	4.7%	1.3%	3.0%	1.2%	2.8%
		1294	26	600	HxB2	91.3	10.5	77.2	3.5	0.85	1.05	0.06	2.1%	10.8%	5.7%	1.9%	2.6%	1.3%	2.4%
		922	16	600	R3A	44.4	3.4	40.0	1.0	0.72	0.79	0.16	3.0%	12.3%	5.5%	2.0%	2.9%	0.9%	2.5%
		1887	26	600	R3A	88.2	7.6	75.7	4.8	0.80	0.97	0.05	2.9%	12.6%	5.6%	3.7%	2.5%	1.5%	2.5%
		2666	16	600	Tybe	36.8	4.5	30.3	2.0	0.75	0.93	0.10	2.1%	12.2%	6.3%	2.1%	2.7%	2.1%	2.9%
		2929	26	600	Tybe	92.2	7.1	80.3	4.7	0.87	1.02	0.05	2.4%	13.2%	7.5%	2.7%	2.3%	1.2%	2.8%
Δ32/Δ32	ND235	2720	5	600	Mock	32.9	5.9	25.3	1.7	0.77	1.18	0.05	1.6%	10.0%	4.1%	2.7%	4.6%	1.7%	1.2%
		2583	13	600	Mock	31.8	5.1	24.9	1.9	0.68	0.98	0.10	1.0%	11.3%	4.1%	2.9%	3.2%	1.5%	2.7%
		2341	26	600	Mock	32.7	4.7	26.7	1.4	0.78	1.08	0.04	1.2%	11.8%	4.3%	2.8%	3.8%	1.9%	2.2%
		2035	13	600	Bk132	42.3	7.7	33.0	1.6	0.69	1.00	0.06	1.0%	12.1%	4.9%	2.8%	3.2%	0.6%	2.6%
		2105	26	600	Bk132	89.2	13.4	72.4	3.4	0.86	1.19	0.08	1.7%	14.2%	7.0%	2.9%	4.4%	1.1%	2.6%
		2398	13	600	HxB2	31.7	4.1	26.0	1.7	0.84	1.10	0.14	1.4%	12.5%	5.6%	2.3%	4.2%	0.7%	2.4%
		4416	26	600	HxB2	83.1	10.6	68.3	4.1	0.89	0.83	-0.04	1.9%	13.0%	4.8%	2.6%	4.4%	1.3%	2.1%
		2165	13	600	R3A	58.0	8.9	46.0	3.0	0.81	1.16	0.06	1.8%	11.8%	5.2%	3.0%	4.8%	1.5%	2.5%
		1686	26	600	R3A	89.7	13.2	72.6	3.7	0.83	1.16	0.07	2.1%	15.1%	5.0%	3.2%	4.4%	1.5%	3.5%

[1]Days refers to days post stimulation. Cells were transduced with the X4-ZFNson day 1 and HIV infected on day 5.

[2]Multiple refers to sequences with multiple insertions and deletions.

[3]Refers to disruptions shown in Figure 1B.

of control cells by both the N-terminal and ECL antibodies. However, CXCR4Δ18 was not detected at the cell surface, though it was detected intracellularly by the N-terminal antibody (Figure 3-3). In addition, cells expressing CXCR4Δ18 along with CD4 did not support HIV-1 infection. These findings indicate that CXCR4Δ18, the most common in-frame deletion resulting from the X4-ZFNs, does not readily traffic to the cell surface and does not function as an HIV-1 coreceptor.

Specificity of cleavage by X4-ZFNs. Potential off-target genome modification comprises the predominant safety concern with ZFNs. Although ultra-deep full genome sequencing could best identify off-target effects, it is impractical and cost-prohibitive with current technology. Instead, we took a more targeted approach that used an experimentally derived binding site for each X4-ZFP to guide the identification of potential off-target cleavage sites. We conducted *in vitro* selection, or SELEX (**s**ystemic **e**volution of **l**igands by **ex**ponential enrichment) to determine the actual binding site preference of each X4-ZFP (Figure 3-4) [43, 44]. A positional-weighted matrix was then generated of the 12bp binding site and 1bp flanking region for each ZFP. A BLAST search against the human genome was then used to determine the top 15 off-target binding sites by allowing up to six mismatches per ZFP binding site, a 5 or 6 bp gap between ZFPs, and formation of hetero or homodimers (Table 3-2) [23]. To assess low frequency disruption events, we conducted 454 deep sequencing on all 15 sites in both control CD4+ T cells and those treated with X4-ZFNs, yielding approximately 7,500-26,000 reads per site in the ZFN-treated samples (Table 3-2). In a sample with 26.9% of CXCR4 alleles disrupted, NHEJ events were detected at a frequency of 2.3% (170/7531 reads) in an extragenic region on chromosome 12 and 0.8% (84/10531) in 20,312 reads

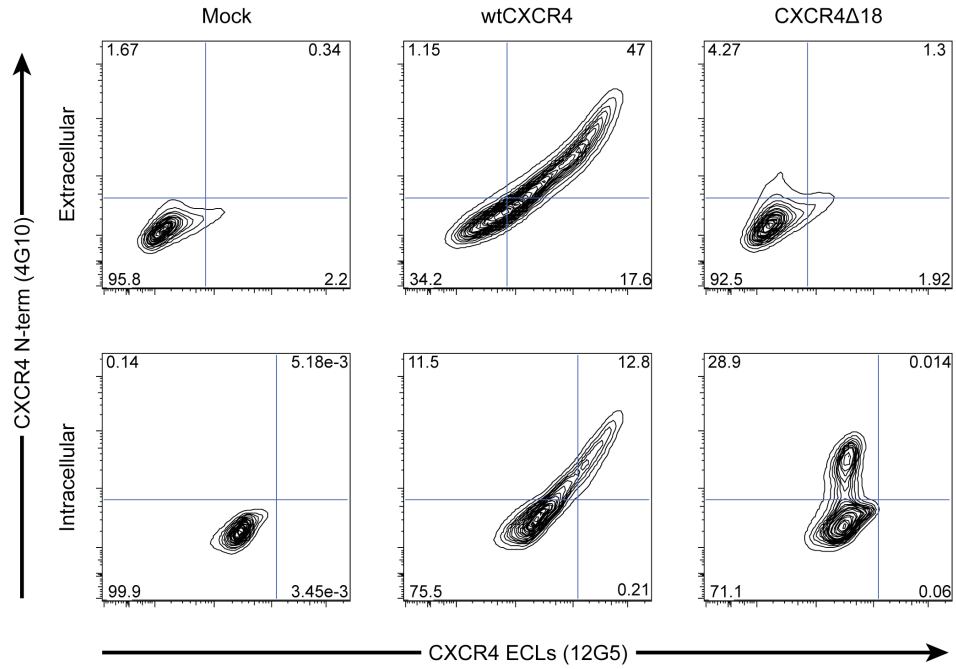


Figure 3-3. X4-ZFNs preferentially generate in-frame deletions resulting in the absence of CXCR4 cell surface expression. The most common lesion induced by the X4-ZFNs was an 18bp deletion, *cxcr4Δ18*, that results in deletion of the amino acid sequence RFYPND from the second extracellular loop of CXCR4 (see Figure 3-1B). To determine if CXCR4Δ18 was expressed on the cell surface, a mock, wild type *cxcr4*, or *cxcr4Δ18* plasmid was transiently transfected into 293T cells that have low endogenous CXCR4 expression. Cells were then analyzed by flow cytometry after being stained simultaneously with anti-CXCR4 clone 4G10, which recognizes the N-terminus, and clone 12G5 whose epitope includes the second extracellular loop that is disrupted by the X4-ZFNs. WtCXCR4 was detected equally by both antibodies on the cell surface (middle panel, top row) and intracellularly (middle panel, lower row). However, CXCR4Δ18 was not detected by the N-terminal antibody on the cell surface (right panel, top row), but was detected when cells were permeabilized (right panel, bottom row) suggesting the 18bp deletion prevents its expression on the cell surface.

found in DEC1 (a putative tumor suppressor [46]) and the single mutation out of 21,139 reads found in an extragenic region of chromosome 11 could be due to PCR and sequencing errors or to very low levels (< 0.02%) of ZFN-mediated cleavage events. Overall, the X4-ZFNs are highly specific for *cxcr4* with low frequency disruption clearly seen at 2 of 15 putative off-target sites with the highest homology to the intended target.

X4-ZFNs confer *in vitro* protection to human CD4+ T cells from HIV challenge. Disruption of both *cxcr4* alleles should render human CD4+ T cells resistant to X4- and perhaps some R5X4- viruses as well, while cells harboring a single disrupted allele might express lower levels of CXCR4 and so be more resistant to virus entry. To determine whether ZFN-mediated disruption of *cxcr4* indeed protects CD4+ T cells from an *in vitro* HIV challenge, human CD4+ T cells from three different *ccr5* wild type donors were stimulated and transduced with AdX4-ZFNs or an AdR5-ZFNs control. Four days post-transduction, the cells were infected with three diverse HIV-1 strains: BK132 (primary X4 HIV), HxB2 (lab-adapted X4 HIV), or R3A (primary R5X4 HIV). Approximately two weeks post-transduction the cells were restimulated with anti-CD3/anti-CD28 beads, and cultures were maintained for an additional two weeks.

In the absence of HIV infection, there was no detectable growth difference between the X4-ZFNs treated, R5-ZFNs treated, and non-transduced controls over the course of the experiment. However, upon infection with the X4- or R5X4- HIV-1 strains, X4-ZFNs treated cells maintained exponential growth compared to profound cell death seen in the R5-ZFNs and untransduced controls. Despite the ability of R3A to utilize both CCR5 and CXCR4 to infect cell lines, in human CD4+ T cells stimulated with anti-CD3/anti-CD28 coated magnetic beads, CCR5 is downregulated causing transient

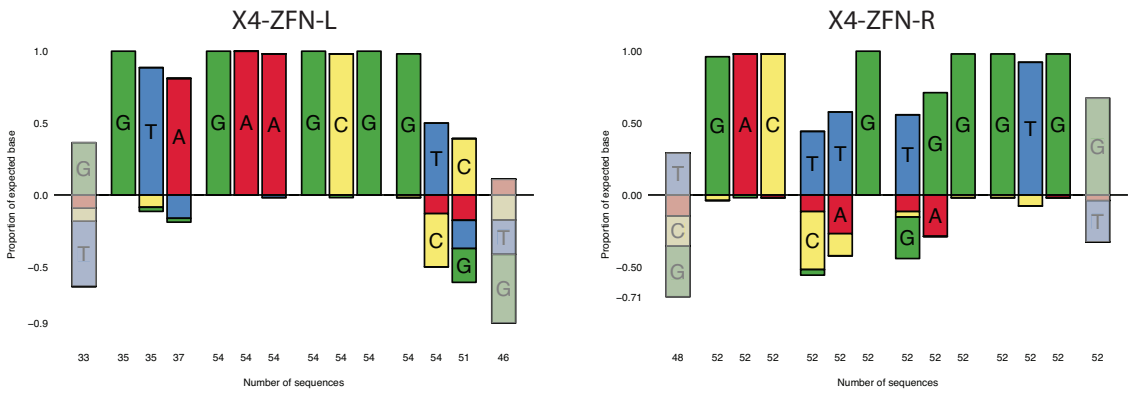


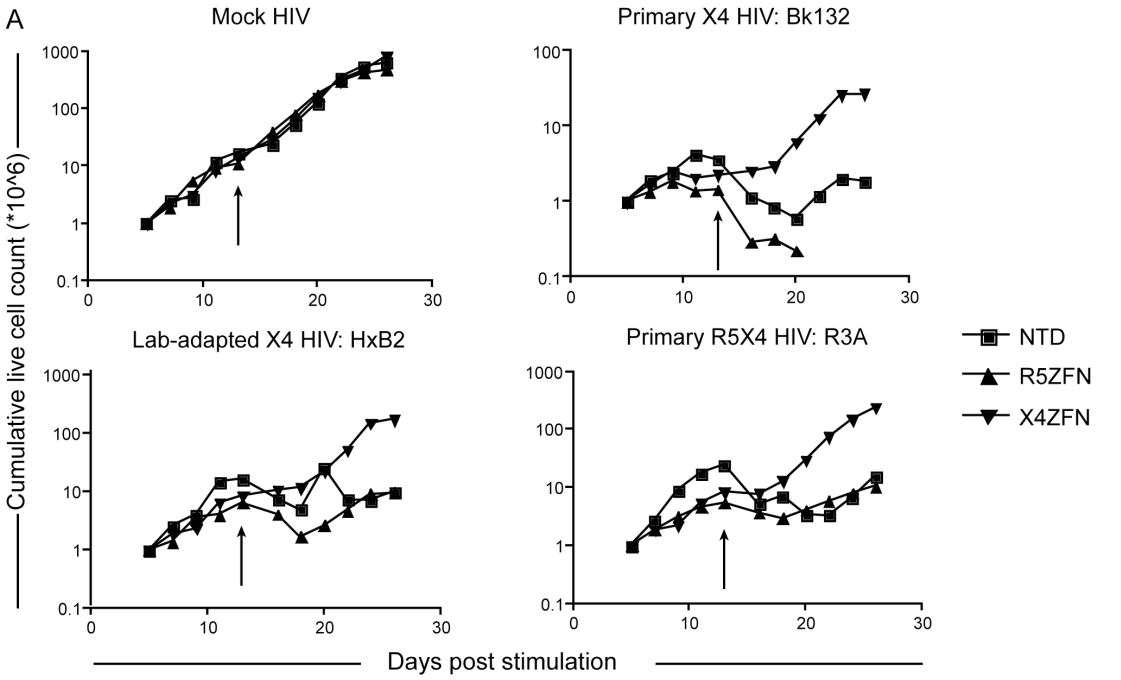
Figure 3-4. Determination of putative off-target sites. The DNA binding preference of the X4-ZFP left and X4-ZFP right was determined empirically by systemic evolution of ligands by exponential enrichment (SELEX). Briefly, a random pool of oligonucleotides was mixed with each ZFP. Unbound oligos were washed and bound oligos were amplified. After four rounds of selection, the enriched oligo pool was sequenced, and a position weighted matrix was generated for the 12 bp target site and one flanking residue per side (faded). Nucleotides corresponding to the wild type *cxcr4* sequence are shown above the horizontal line.

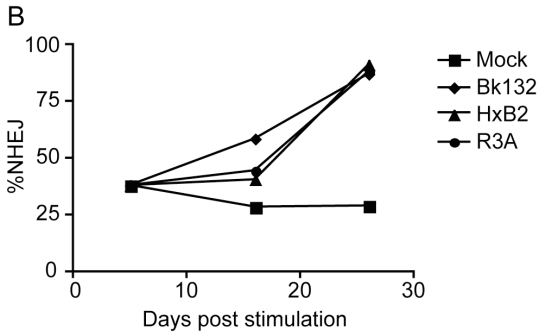
Table 3-2. Analysis of X4-ZFNs specificity

								X4-ZFN treated			Untransduced control		
Rank	**Chr**	**Location**	**Site**	**Mismatch**	**Arrangement[1]**	**Homo(+)/Hetero(-)dimer**	**Gene**	**# reads**	**NHEJ events**	**%NHEJ**	**# reads**	**NHEJ events**	**%NHEJ**
0	chr2	136872909	CCACCCACAAGTCATTGGGGTAGAAGCGGTCA	0	X4-ZFN-R_N6_X4-ZFN-L	-	CXCR4	27485	7404	26.94	23430	0	0
1	chr6	25025407	CCACCCACAGGTCCTGGTGtACaTGTGGGTGG	2	X4-ZFN-R_N6_X4-ZFN-R	+		13346	0	0	9605	0	0
2	chr14	69258063	CCACCtCCtAGTCAATAGCGgaTTGGGGGTGG	4	X4-ZFN-R_N6_X4-ZFN-R	+	ZFP36L1	20874	0	0	15219	1	0.007
3	chr5	98363253	ACtCCaCTTCTACCTCTCTGTAGAAGCaGTGG	3	X4-ZFN-L_N6_X4-ZFN-L	+		14811	0	0	13290	0	0
4	chr19	8595061	CCACCCACtAGTgCCTCACcTtGAAGCGGCGG	4	X4-ZFN-R_N6_X4-ZFN-L	-	MYO1F	20356	0	0	23645	0	0
5	chr20	49900265	CCACCttCAAGTCCTCCAGtCaaGGaGGTGG	6	X4-ZFN-R_N5_X4-ZFN-R	+		19266	0	0	294	0	0
6	chr18	48000536	GAGCCtCTcCTACAAAATGACCaGTGGGTGG	3	X4-ZFN-L_N5_X4-ZFN-R	-		11140	0	0	13193	0	0
7	chr9	118148132	CCACCCtCAGGcCAGAGCGGgCCTGaaGGTGG	5	X4-ZFN-R_N6_X4-ZFN-R	+	DEC1	20312	4	0.02	22408	0	0
8	chr4	166114452	CatCCCCCAAGTCAACTGAGACaTGTGGGTGG	3	X4-ZFN-R_N6_X4-ZFN-R	+		19520	0	0	7429	0	0
9	chr3	129336995	TCACCaACAAGTCCTGCGGcCTTGGGGGTGG	2	X4-ZFN-R_N5_X4-ZFN-R	+		10754	0	0	9157	0	0
10	chr7	5334982	CCACCCCCtGGTCAGCACccCaTGTGGGTGT	4	X4-ZFN-R_N5_X4-ZFN-R	+	SLC29A4	21471	1	0.005	35104	1	0.003
11	chr1	237162073	CCACCtCCtGGTCCTGCAGtcCCaGTGGGTGG	5	X4-ZFN-R_N6_X4-ZFN-R	+		25621	1	0.004	26879	2	0.007
12	chr11	47621508	GGGCCcCTTCTgCAGATGGACCaGGaGGTGA	4	X4-ZFN-L_N5_X4-ZFN-R	-		21139	1	0.005	22902	0	0
13	chr15	100765052	CCACCCACAGGTtTTAAGGGTAGAAGCaGCAA	2	X4-ZFN-R_N6_X4-ZFN-L	-	ADAMTS17	10531	84	0.8	7682	2	0.03
14	chr5	150448685	CCACCCACtAGcaGGCAAGcACTTGTGGGTGG	4	X4-ZFN-R_N6_X4-ZFN-R	+	TNIP1	24996	0	0	32148	1	0.003
15	chr12	30679386	CCACCtACttGTCTTGTGTGTtGAAGCaGaCA	6	X4-ZFN-R_N6_X4-ZFN-L	-		7531	170	2.26	13573	0	0

[1]N5 and N6 refer to the number of nucleotides between each putative ZFN binding site.

resistance to R5 HIV [47]. Thus, R5X4 HIV strains are likely to function predominantly as X4 HIV strains under these conditions [47]. The growth advantage conferred by treatment with X4-ZFNs in the presence of HIV was magnified upon restimulation. (Figure 3-5A). This likely resulted from increased cell activation, which increases the ability of HIV to infect and replicate in CXCR4 positive cells.To determine whether the growth advantage conferred by X4-ZFNs treatment in the presence of X4- and R5X4-HIV resulted from a survival advantage of CXCR4 disrupted cells, we performed flow cytometry at various time points post infection as well as deep sequencing of the X4-ZFNs target site on HIV-infected and uninfected cultures. In the absence of HIV infection, the *cxcr4* disruption frequency remained stable over time in four independent experiments testing four different *ccr5* wild type donors as measured by deep sequencing. A representative experiment is shown in Figure 3-5B and CXCR4 disruption data from all experiments is shown in Tables 3-1 and 3-3. While CXCR4 gene disruption remained stable over time at approximately 30%, CXCR4 gene disruption in HIV-infected cultures increased to 87%, 91%, and 88% in the presence of BK132, HxB2, and R3A respectively after 21 days of infection. FACS analysis showed that at day 19 post-HIV challenge, the frequency of CXCR4 negative cells amongst all live mock HIV-infected CD4+ lymphocytes was 13.0% in untransduced cells, 14.1% in cells transduced with R5-ZFNs, and 35.0% in cells transduced with X4-ZFNs compared to greater than 98%, 97%, and 99% of Bk132, HxB2, and R3A infected cultures transduced with the X4-ZFNs, (Figure 3-5C). We also found that after 19 days post-HIV infection, reduced but significant cell growth was detectable in several of the HIV-infected control cultures,


A
Mock HIV
Primary X4 HIV: Bk132
Lab-adapted X4 HIV: HxB2
Primary R5X4 HIV: R3A
Cumulative live cell count (*10^6)
Days post stimulation
NTD
R5ZFN
X4ZFN


B
%NHEJ
Days post stimulation
Mock
Bk132
HxB2
R3A

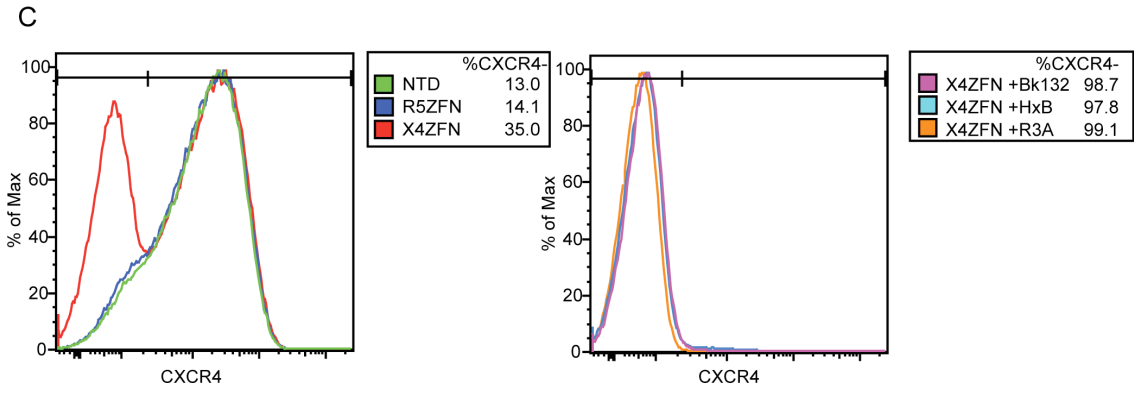

C
% of Max
CXCR4
%CXCR4-
NTD 13.0
R5ZFN 14.1
X4ZFN 35.0
X4ZFN +Bk132 98.7
X4ZFN +HxB 97.8
X4ZFN +R3A 99.1

Figure 3-5. Treatment of human CD4+ T cells with X4-ZFNs confers protection to HIV-1 challenge *in vitro.* (A) Human CD4+ T cells were treated with the X4-ZFNs or R5-ZFNs expressed by Ad5/F35 vectors or were non-transduced (NTD). Four days later cells were infected with a primary X4 HIV-1 (Bk132), lab-adapted X4 HIV-1 (HxB2), primary R5X4 HIV-1 (R3A) or mock infected. The number of viable cells were measured at various times after stimulation. Cells were re-stimulated on day 13 (arrows). (B) The proportion of disrupted *cxcr4* alleles was determined at the indicated times post-stimulation by 454 deep sequencing. The frequency of *cxcr4* disruption was relatively constant in the mock-treated cells, but increased dramatically in the presence of HIV-1. (C) FACS analysis using a CXCR4-specific monoclonal antibody was performed at 19 days post infection (24 days post-stimulation). Mock HIV-infected cultures are shown on the left and HIV infected cultures on the right. Data shown is one of three independent experiments.

Table 3-3. Surveyor nuclease data after treatment with X4-ZFNs and challenge by HIV.

CCR5 genotype	Donor	Day	MOI	HIV	%NHEJ
wt/wt	ND304	29	600	mock	36.3
		29	600	BK132 (100ng)[1]	81.9
		29	600	Bk132 (10ng)[1]	82.4
		29	600	Bk132 (1ng)[1]	78.7
Δ32/Δ32	ND235	5	600	Mock	34.4
		15	600	Mock	35.0
		18	600	Mock	30.1
		28	600	Mock	37.2
		15	600	Bk132	55.2
		18	600	Bk132	73.0
		28	600	Bk132	77.2
		15	600	HxB2	32.5
		18	600	HxB2	60.7
		28	600	HxB2	80.2
		15	600	R3A	54.8
		18	600	R3A	64.7
		28	600	R3A	81.5

[1]The number refers to the amount of HIV p24 used to infect per million cells. All experiments used 100ng unless specified.

untransduced and treated with R5-ZFNs. However, greater than 95% of these cells, compared to approximately 10% of cells treated with X4-ZFNs, were CD3+CD4- suggesting that the surviving cell population was protected from HIV infection by down-regulating CD4 (Figure 3-6). Thus, CXCR4 disruption had no impact on cell viability, but conferred a significant survival advantage in the presence of HIV strains that can use CXCR4 to infect cells. Furthermore, in control cultures that were untransduced or treated with R5-ZFNs, viral titers exponentially increased until extensive cell death began approximately 8-10 days post infection. In contrast, in cultures treated with X4-ZFNs viral titers steadily decreased after peak viremia while cell growth remained exponential suggesting there was not significant viral production (data not shown).

***Ccr5*Δ*32* CD4+ T cells treated with X4-ZFNs are resistant to R5 and X4 HIV.** Given the ongoing adoptive therapy trial of CD4+ T cells treated with R5-ZFNs and the anti-viral success of the recent *ccr5*Δ*32* bone marrow transplant in an HIV-infected, we sought to determine if *cxcr4* could be genetically disrupted simultaneously with *ccr5*. Human CD4+ T cells from a *ccr5*Δ*32* homozygote were transduced with AdX4-ZFNs or AdR5-ZFNs and subsequently infected with HIV-1 strains Bk132, HxB2, and R3A as described above. Representative data from one of two independent experiments conducted in cells from the same donor is shown in figure 3-7 and data from both experiments is shown in Tables 3-1 and 3-3. As seen in *ccr5* wild type CD4+ T cells, exponential cell growth was preserved in cultures treated with X4-ZFNs compared to control cultures that were untransduced or treated with R5-ZFNs (Figure 3-7A). In addition, disruption frequency in cultures treated with X4-ZFNs as determined by deep

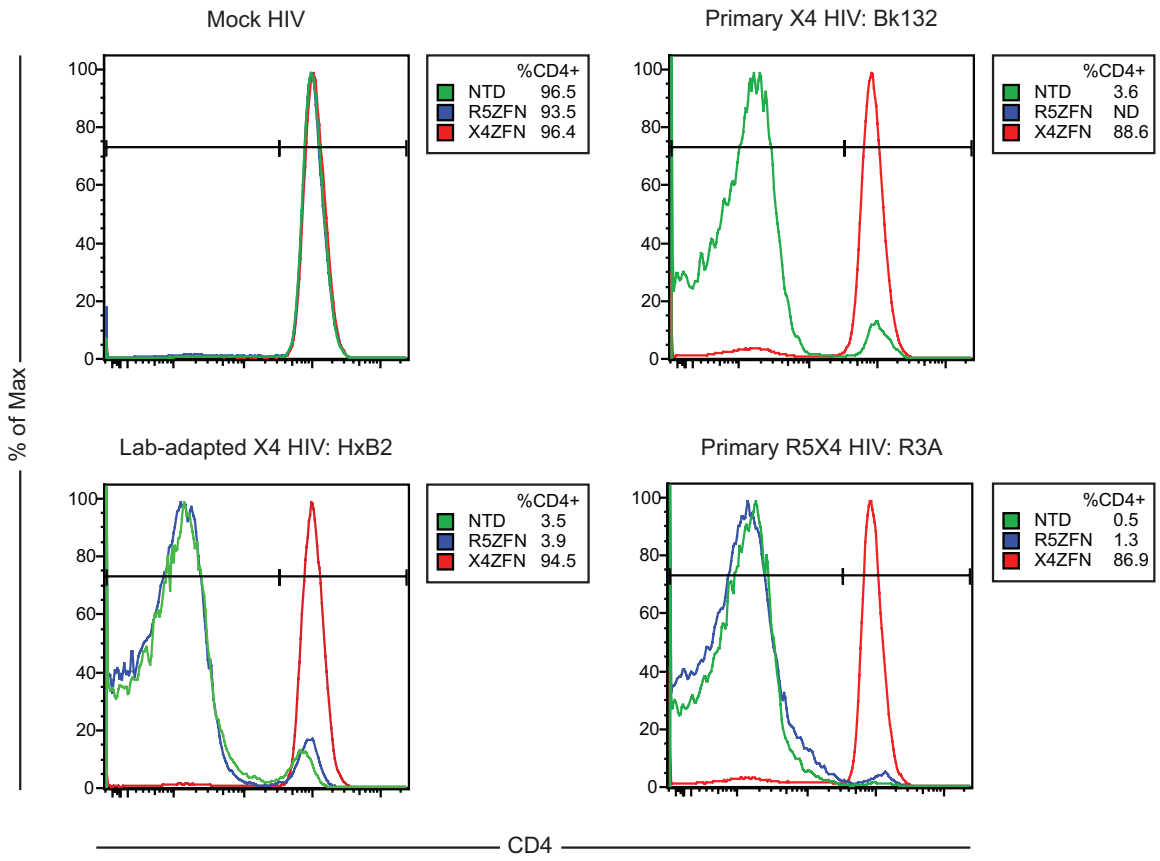


Figure 3-6. Treatment with X4-ZFNs prevents CD4 downregulation by HIV-1. CD4 is profoundly downregulated on live CD3+ cells HIV-1 infected cultures that were NTD or treated with R5-ZFNs but not X4-ZFNs. Thus, the limited cell growth remaining by 19 days post infection in NTD cultures and those treated with R5-ZFNs is due to HIV-1 induced CD4 downregulation, and thus the protective effect on cell growth for CD3+CD4+ cells is underestimated by the growth curves in Figure 4A. Cells are from same experiment as Figure 3-4 [6].

sequencing remained remarkably stable between 32-33% from day 5 to day 26 post-transduction in the absence of HIV, which suggests that simultaneous disruption of *ccr5* and *cxcr4* does not adversely affect cell growth. However, in the presence of Bk132, HxB2, and R3A, *cxcr4* disruption increased after 21 days of HIV challenge to 89%, 83%, and 90%, respectively (Figure 3-7B), and was associated with markedly diminished virus replication (data not shown), again consistent with significant protection conferred by *cxcr4* disruption. Thus, treatment with X4-ZFNs of both wild-type and *ccr5*Δ32 CD4+ T cells confers stable *cxcr4* disruption and a marked survival advantage in the presence of R5X4-HIV and X4-HIV *in vitro* without any detectable effect on cell growth or viability in the absence of HIV. This suggests that both *ccr5* and *cxcr4* can be genetically targeted simultaneously for the treatment of HIV infection, while preserving the replicative capacity of the CD4+ T cells.

X4-ZFNs confer partial protection in NSG humanized mouse model. As a first step in evaluating the safety and efficacy of the X4-ZFNs *in vivo*, we employed a NSG humanized mouse model. Briefly, human CD4+ T cells were stimulated with anti-CD3/anti-CD28 beads and transduced with either AdX4-ZFNs or an AdR5-ZFNs control at an MOI of 600. Cells were then expanded *in vitro* for ten days after which 10^7 CD4+ T cells treated with X4-ZFNs (n=23) or R5-ZFNs (n=22) were injected intravenously into each mouse. Engraftment was assessed by peripheral blood CD4+ T cell counts 27 days post-injection. All 45 animals successfully engrafted; however, one animal that received cells treated with the X4-ZFNs had a significantly higher but stable CD4+ T cell count and was thus excluded as an outlier from the remainder of the study. On day 28 post-

engraftment, mice were intravenously injected with 10^5 autologous CD4+ T cells that were previously infected with the highly cytopathic X4 HIV-1 strain Bk132 or a mock control. CD4 counts, viral load, and CXCR4 disruption were then monitored to determine the effect of treatment with X4-ZFNs.

To determine if X4-ZFNs impacted cell growth or viability in the absence of HIV, we first compared CD4 counts over time between the uninfected X4-ZFN and R5-ZFN control mice. There was no significant difference in CD4 counts between the two groups over the course of the 61 day experiment as determined by a generalized estimating equation (GEE) method (p=.88) (Figure 3-8A). Next, we examined the frequency of CXCR4 DNA disruption over time with the surveyor nuclease assay. At the time of injection the percentage of *cxcr4* alleles disrupted was 24.3%. This remained constant inboth the blood (p=0.32) and spleen (p = .70) over the course of the experiment suggesting that CXCR4 disruption did not significantly impact trafficking between these two compartments (Figure 3-8B). Next, we characterized CXCR4 cell surface expression over time by FACS. In the R5-ZFN control group, with intact *cxcr4* genes, 88% of CD4+ T cells expressed CXCR4 protein at day 27 post engraftment, compared to 84% of cells in the X4-ZFN mice (~24% *cxcr4* gene disruption) as determined by a fluorescence minus-one (FMO) control. This difference persisted over time in the absence of HIV-1 infection ($p < 0.001$) (data not shown). Together the stable disruption of CXCR4 as determined by both the surveyor nuclease assay and flow cytometry suggests that CXCR4 disruption did not negatively impact cell viability or growth in humanized NSG mice over a two-month period. As expected, xenogeneic graft versus host disease (GVHD), assessed clinically by dermatitis and hair loss, was observed in mice receiving

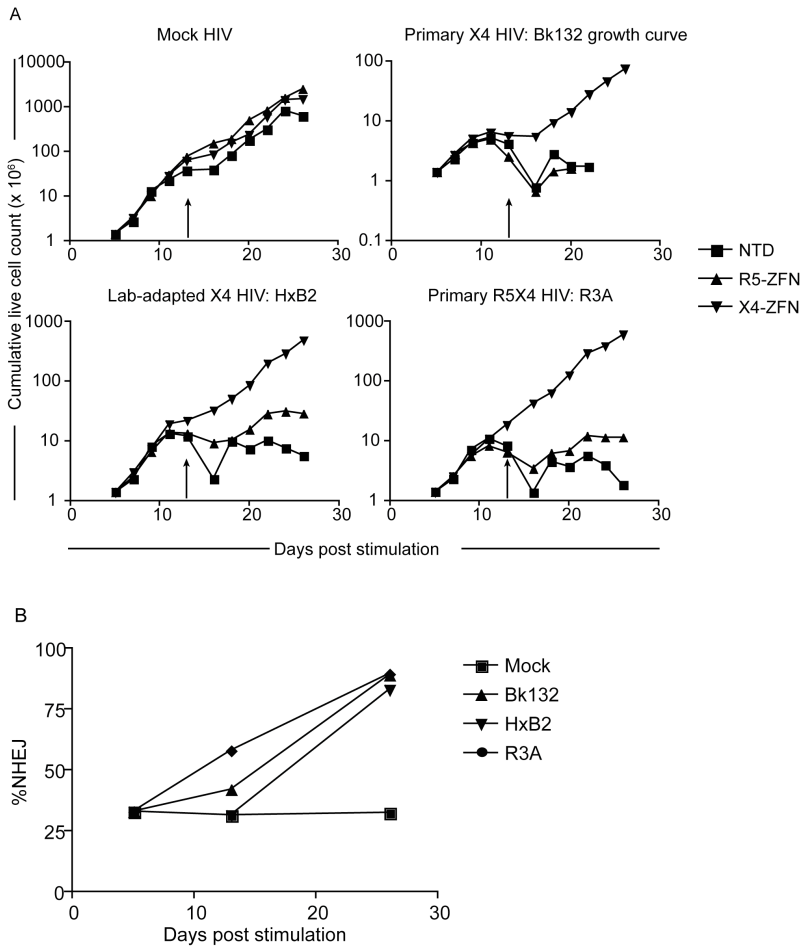


Figure 3-7. Treatment with X4-ZFNs is effective in *ccr5Δ32* homozgyous human CD4+ T cells. (A) *Ccr5Δ32* CD4+ T cells were stimulated on day 0 and transduced on day 1 with an Ad5/F35 vector expressing the X4-ZFNs, R5-ZFNs, or an untransduced control. On day 5, cells were HIV-infected with a mock, primary X4 HIV-1 (Bk132), lab-adapted X4 HIV-1 (HxB2), or a primary R5X4 HIV-1 (R3A). Live cells were counted approximately every two days. Cells were restimulated on day 13 (arrows). (B) *Cxcr4* disruption frequency was assessed at various times by 454 deep sequencing. Disruption remained stable in the absence of HIV-1 infection, but profoundly increased in the presence of the three HIV-1 strains examined. Data shown is from one of two representative experiments.

cells treated with both R5-ZFNs and X4-ZFNs in the absence of HIV challenge. The development of GVHD was equivalent between the two groups (data not shown), suggesting that treatment with X4-ZFNs did not affect CD4+ T cell effector functionality.

In response to X4 HIV challenge with HIV-1 Bk132, CD4 counts decreased in both X4-ZFN and R5-ZFN mice. However, this rate of decline was slower in the X4-ZFN mice. The X4-ZFN group exhibited a mean 1.1 log CD4 count protection by day 14 post infection ($p=.05$ for a parametric t-test). However, this protective effect waned over time and there was no significant difference in CD4 counts by day 33 post infection ($p=.88$) suggesting that treatment with X4-ZFNs conferred only transient protection (Figure 3-8A).

One mechanism that could account for this would be if mutations arose in the viral Env protein to enable it to use CCR5. To explore this possibility, we bulk cloned and sequenced the V3 loop of Env, the main determinant of coreceptor tropism [48], from plasma isolated from three R5-ZFN mice and three X4-ZFN mice at the time of sacrifice. We identified a single amino acid substitution (Y302N) present in Env isolated from X4-ZFN mice but not R5-ZFN mice or the viral innoculum. Next, we cloned six distinct, functional Envs from the X4-ZFN mice and three distinct, functional Envs from the viral innoculum. As full length Bk132 Env would not pseudotype on an NL43 HIV core we truncated the cytoplasmic tail of the Envs [49, 50], and conducted tropism testing on NP2 cell lines expressing CD4 with either CCR5 or CXCR4. Of the six functional Envs from X4-ZFN mice, four contained the Y302N mutation. Interestingly, these four Envs were able to utilize CCR5 and CXCR4 equivalently, similar to the R5X4-tropic control R3A. All clones with the wild type Tyr302, including the Envs from the viral innoculum and

two Envs from X4-ZFN mice utilized CXCR4 approximately 1000-fold more efficiently than CCR5 and comparably to the X4-tropic control TYBE (Figure 3-8C). Thus, in an NSG humanized mouse model of HIV infection, the cells treated with X4-ZFNs engrafted, trafficked, and persisted comparably to control cells. In addition, treatment with X4-ZFNs resulted in significant transient protection of CD4+ T cell counts in response to X4-tropic HIV challenge, and HIV challenge provided *cxcr4* disrupted cells with a survival advantage as determined by increase of *cxcr4* disruption in the presence but not the absence of HIV. However, the extent of the protection conferred by the X4-ZFNs was mitigated by evolution or outgrowth of preexisting R5X4-tropic HIV.

ZFN-mediated coreceptor disruption is feasible in rhesus macaque CD4+ T cells. While humanized mouse models for HIV infection have utility, the model is limited due to incomplete immune reconstitution, development of xenogeneic graft versus host disease (GVHD), and the absence of normal T cell homeostasis. For these reasons and others, the NSG model is suboptimal compared to non-human primate models to further elucidate the safety and efficacy of treatment with X4-ZFNs and R5-ZFNs. As a proof of concept for future clinical adoptive therapy studies, we attempted to disrupt the *ccr5* and *cxcr4* genes with ZFNs in rhesus macaque CD4+ T cells. Briefly, rhesus CD4+ T cells were isolated from whole blood, purified by magnetic bead negative selection, and then stimulated with anti-CD3/anti-CD28 coated beads as previously described [35, 36]. As the 24bp X4-ZFPs' binding site is identical between rhesus and humans, we were able to utilize the same ZFN pair. However, in order to target rhesus

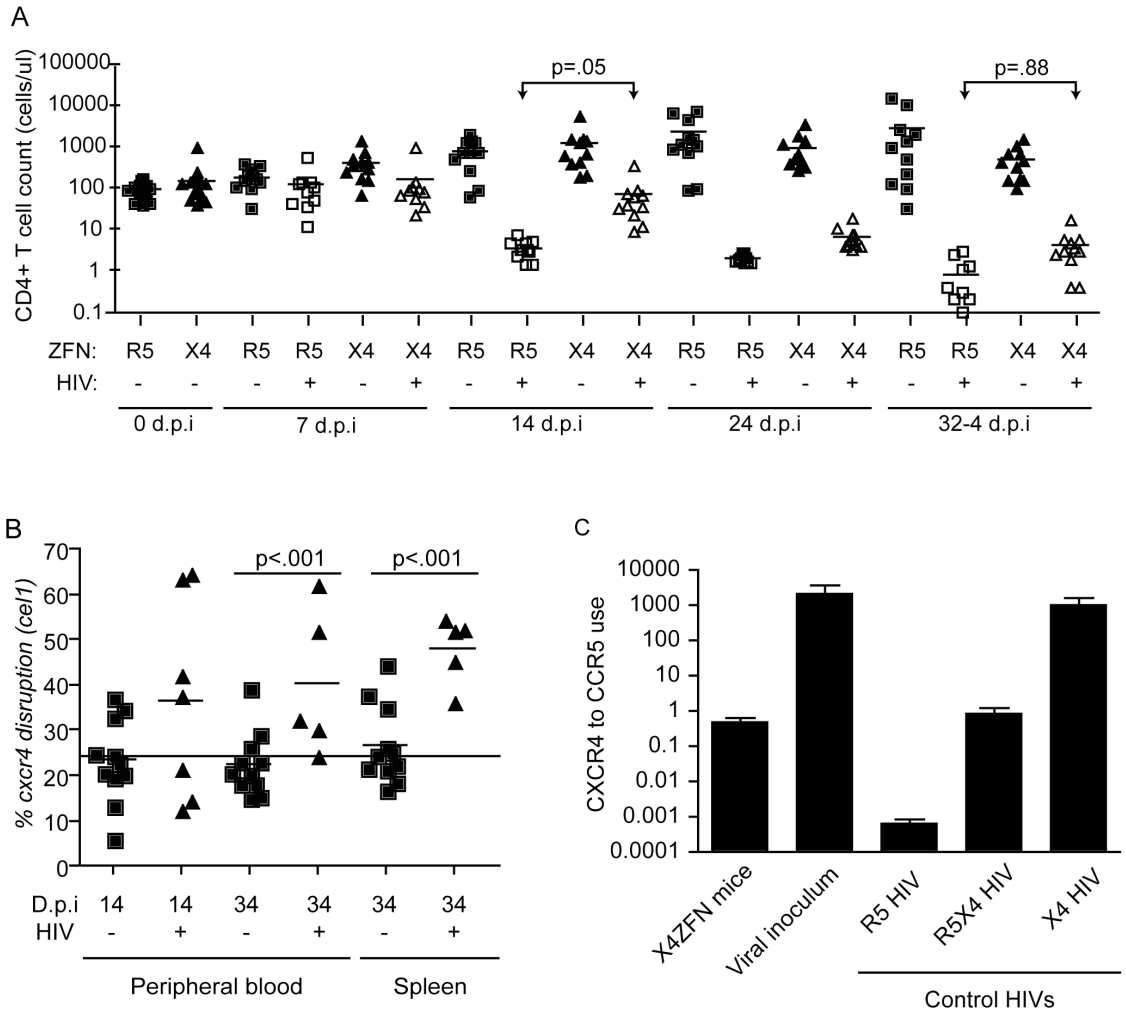

A
CD4+ T cell count (cells/ul)
100000
10000
1000
100
10
1
0.1
p=.05
p=.88
ZFN: R5 X4 R5 R5 X4 X4 R5 R5 X4 X4 R5 R5 X4 X4 R5 R5 X4 X4
HIV: - - - + - + - + - + - + - + - + - + - +
0 d.p.i
7 d.p.i
14 d.p.i
24 d.p.i
32-4 d.p.i
B
% cxcr4 disruption (cel1)
70
60
50
40
30
20
10
0
p<.001
p<.001
D.p.i 14 14 34 34 34 34
HIV - + - + - +
Peripheral blood
Spleen
C
CXCR4 to CCR5 use
10000
1000
100
10
1
0.1
0.01
0.001
0.0001
X4ZFN mice
Viral inoculum
R5 HIV
R5X4 HIV
X4 HIV
Control HIVs

Figure 3-8. Treatment with X4-ZFNs confers partial protection to HIV-1 in humanized mice *in vivo*. NSG mice were injected with human CD4+ T cells treated with X4-ZFNs or R5-ZFNs. 28 days post injection, mice were infected with primary X4 HIV-1 (Bk132) or were mock-infected. (A) CD4+ T cell counts were measured every 7-10 days post infection. In the presence of Bk132, treatment with X4-ZFNs conferred protection at 14 d.p.i ($p=.05$); however, this protection wanes by 34 d.p.i. ($p=.88$) (B) *Cxcr4* disruption frequency was assessed by the surveyor nuclease assay in both peripheral blood ($p<.001$) and spleen ($p<.001$). At day 34 post infection, human CD4+ T cells were purified by positive selection prior to analysis to reduce any bias from low frequency contaminating human cells. Only samples with a detectable PCR signal are shown. Disruption frequency did not deviate significantly from the cell innoculum in either the blood or spleen. Data in (A) and (B) were analyzed by a general estimating equation (GEE). (C) HIV-1 Env from X4-ZFN mouse plasma was sequenced revealing a consensus Y302N mutation. To evaluate coreceptor tropism, a representative Env from the X4-ZFN mice and the viral innoculum were pseudotyped and used to infect NP2 cell lines expressing CD4 and either CCR5 or CXCR4. R5 HIV-1 (JRFL), R5X4 HIV-1 (R3A), and X4 HIV-1 (TYBE) controls are shown. Infectivity on NP2/CD4/CXCR4 cells was divided by that on NP2/CD4/CCR5 cells to determine relative coreceptor use. Data is an average of three independent experiments each done in triplicate. Error bars represent standard error.

CCR5, rhesus specific R5-ZFNs were developed. As for human cells, the ZFNs were delivered with an Ad5/F35 vector and disruption was assessed by the surveyor nuclease assay. Utilizing a range of MOIs of 600, 1000, and 2000 we observed mean *ccr5* and *cxcr4* disruption levels of 19.6% and 14.0%, respectively (Figure 3-9), which suggests that adoptive therapy of cells modified with ZFNs is feasible to model in rhesus macaques.

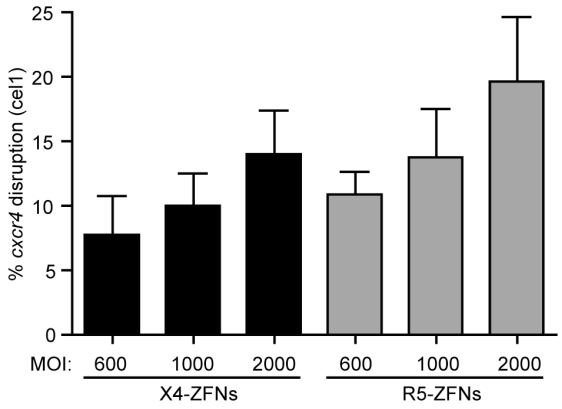


Figure 3-9. ZFNs can efficiently disrupt *ccr5* and *cxcr4* in rhesus macaque CD4+ T cells. The X4-ZFN pair's 24bp binding site is conserved between humans and rhesus macaques. However, the human and rhesus R5-ZFNs have different binding sites; thus, a novel CCR5-ZFN pair was generated targeting rhesus *ccr5*. The rhesus R5-ZFNs and X4-ZFNs were delivered by Ad5/F35 vector at MOIs from 600-2000 into rhesus CD4+ T cells. Disruption frequency was measured by the surveyor nuclease assay. Data shown is an average of three independent experiments in cells from two different animals. Error bars represent standard error.

Discussion

The apparent eradication of HIV resulting from a *ccr5Δ32* homozygous allogeneic bone marrow transplant into an HIV-infected patient represents the first reported "cure" of HIV [6]. While an important proof-of-principle, few individuals could benefit from allogeneic *ccr5Δ32* homozygous transplants due to toxicities of allogeneic rejection and limitations of finding sufficient HLA-matched *ccr5Δ32* homozygous donors. However, coreceptor-specific ZFNs represent a novel therapeutic approach to recapitulate this success via autologous transplantation of gene-modified hematopoietic stem cells and mature CD4+ T cells. *Ccr5* can be efficiently disrupted in both human CD4+ T cells and hematopoietic stem cells, conferring protection to HIV challenge *in vitro* and in humanized mice [23, 28]. In addition, transgenic autologous hematopoietic stem cells can be successfully transplanted in HIV-infected individuals [18] and several phase I adoptive transfer trials of CD4+ T cells treated with R5-ZFNs in HIV infected individuals are currently underway. By design, this strategy addresses only viruses that require CCR5 to infect cells. Our long-term goal, therefore, is to explore the potential to genetically disrupt both *ccr5* and *cxcr4* for cell replacement therapies in HIV infected individuals, and in the case of *cxcr4* do so in a way that specifically targets CXCR4 on T cells and not the many other cell types on which it is expressed. We hope this could lead to long-term virologic control in the absence of continued ART, a critical goal of the HIV field as discussed in chapter one.

Unlike for *ccr5,* there are no known humans with loss of function *cxcr4* mutations that would provide insight into the safety and viability of *cxcr4* disruption in mature CD4+ T cells. A concern associated with targeting CXCR4 is that it is broadly expressed,

while CCR5 expression is largely limited to hematopoietic cells. CXCR4, along with its natural ligand CXCL12, plays a critical role in normal B cell, cardiovascular, and cerebellar development, though T lymphocytes appear to develop normally in *cxcr4-/-* mice [51]. Thus, it is possible that the selective disruption of *cxcr4* in mature post-thymic CD4+ T cells may be tolerable. In addition to its role in development, the CXCR4-CXCL12 axis is a potent CD4+ T cell chemoattractant, and the broad expression of both proteins suggests that this axis may play a fundamental role in basal chemotaxis as opposed to a response to inflammation [52]. Indeed, inhibiting CXCR4 function systemically with the small molecule antagonist plerixafor results in the peripheral mobilization of hematopoetic stem cells, thus mitigating the potential of such therapy for long-term anti-retroviral therapy. However, plerixafor, which has not been reported to have adverse immunologic consequences resulting from inhibiting CXCR4 function in mature CD4+ T cells, provides proof of principle that inhibiting CXCR4 in mature CD4+ T cells may prove to be safe and viable [10, 53]. This suggests that this essential gene can be targeted in a cell-type specific manner with CXCR4-specific ZFNs that limits the toxicities of systemic disruption. While we have demonstrated that CXCR4 is not essential for CD4+ T cell viability and function *in vitro* and in humanized mice *in vivo,* the redundancy of lymphocyte chemokine receptors and their ligands makes predicting the *in vivo* consequences of *cxcr4* disruption in a normal host on CD4+ T cell function and trafficking difficult. We conclude that a logical next step will be to study the consequences of *cxcr4* disruption in a non-human primate model of HIV infection, which will simultaneously permit the assessment of the consequences of this approach on T cell function and trafficking.

A significant advantage of ZFN gene modification, compared to retrovirus based approaches, is that only transient transgene expression is required to permanently engineer an HIV resistant cell. As a result, adenovirus or other delivery mechanisms such as RNA transfection can be employed that avoid toxicities that can be associated with retroviral integration, such as cellular expansion or transformation. This "hit-and-run" approach limits the requirement of chronic transgene expression and the potential leakiness of other approaches including siRNA [21, 22], intrabodies [19], and ribozymes [17]. However, like most gene transfer approaches a major concern with ZFN technology is the potential for oncogenesis due to off-target effects. Efforts have been made to reduce off-target effects by using modified *Fok1* catalytic domains, which act as obligate heterodimers, and future work will examine the effects of modulating the DNA binding affinity of ZFN pairs on DNA specificity. While additional study is clearly needed, our current studies have clearly identified off-target disruption in two of the top 15 putative off-target sites: an extragenic site on chromosome 12 and in the metalloprotease ADAMTS17, which is not expressed in CD4+ T cells. In addition, mature CD4+ T cells appear to be resistant to malignant transformation [54], thus mitigating the potential concerns of off-target disruption. Consistent with this, more than 200 people have safely undergone adoptive transfer of genetically engineered lymphocytes with no reported cases of therapy-induced oncogenesis [55]. Reasons for resistance to transformation of mature lymphocytes are unclear, but may involve an unknown mechanism that ensures the diversity of the TCR repertoire and thus limits clonal outgrowth [54]. In contrast, the safety record of hematopoietic stem cell gene therapy is less clear, with a significant frequency of gene-therapy induced oncogenesis or clonal outgrowth reported in several

hematopoietic stem cell trials [56, 57]. However, given the continued production of CD4+ T cells, a ZFN-based approach in CD4+ T cells may require intermittent lifelong treatment.

One unexpected finding reported here is the predominance of in-frame mutations, particularly in-frame deletions, resulting from ZFN mediated cleavage of *cxcr4*. This has not been observed in other ZFN studies reported thus far. The deep-sequencing approach we have taken makes it possible to comprehensively and accurately assess the types and frequencies of mutations that result from ZFN cleavage followed by DNA repair. The striking preponderance of in-frame deletions may have resulted from toxicities of frameshift mutations shortly after treatment with X4-ZFNs leading to decreased survival relative to in-frame mutants. However, this is unlikely given that the frequency of in-frame mutations remained stable over nearly four weeks in culture, that there was no significant increase in cell death between control cultures and those treated with X4-ZFNs, and that the most common in-frame mutant was not expressed on the cell surface and thus cannot maintain functionality. Rather, the preference for in-frame deletions is likely due to preferential in-frame DNA repair. The deletion in the most common X4-ZFN-induced lesion, *cxcr4Δ18*, is flanked by a GTCA microhomology domain at the 5' and 3' ends consistent with a repair mechanism of microhomology-mediated NHEJ [58]. Similar microhomology sites are present in other common ZFN-induced *cxcr4* mutants that we identified. Thus, it appears that the nucleotide sequence of the X4-ZFN binding site directs a preference for an in-frame repair mechanism.

Our studies provide a fundamental demonstration that inactivation of *cxcr4* by treatment with X4-ZFNs rendered human CD4+ T cells resistant to infection by X4 virus

strains, while CXCR4 inactivation in the context of a *ccr5Δ32* homozygous background rendered cells resistant to infection by both R5 and R5X4 strains. Genetic ablation of both CCR5 and CXCR4 will likely make CD4+ T cells entirely resistant to HIV-1. Dual-disruption of CCR5 and CXCR4 will be needed for maximal therapeutic benefit since 46% of treatment-experienced individuals harbor R5X4 strains of HIV compared to 4% with only X4-HIV strains [59]. While virus strains have been identified that can infect cells in the absence of CD4 (reviewed in [60]), none have been identified that can infect cells in the absence of a suitable coreceptor. In addition, virus strains that can use coreceptors other than CCR5 or CXCR4 to infect primary human cells are exceedingly rare. However, targeting CXCR4 alone could provide a selective advantage to CCR5-tropic virus strains. Suppression of CXCR4 by plerixafor *in vitro* can lead to the emergence of CCR5-tropic virus strains [61], and highly active antiretroviral therapy can sometimes result in enhanced prevalence of R5 relative to R5/X4 virus strains in infected patients [62]. In the humanized mouse model under the conditions studied here, partial loss of *cxcr4* in human T cells due to treatment with X4-ZFNs provided selective pressure for either the evolution or emergence of a pre-existing single amino acid mutation in the V3 loop of the infecting X4 HIV-1 strain that enabled it to use CCR5 as efficiently as CXCR4. Thus, just as either genetic or therapeutic suppression of CCR5 can provide an advantage to virus strains that use CXCR4, deletion of CXCR4 is expected to provide an advantage to CCR5-tropic viruses. However, this could provide a clinical benefit given the increased *in vitro* pathogenicity and correlation with progression to AIDS of X4-tropic HIV.

While humanized mouse models provided a logical first approach to examine *in vivo* efficacy of CXCR4 disruption, this system does not make it possible to fully assess the functional impact of CXCR4 loss on CD4+ T cell function. To study this in the most rigorous way possible, we have explored the possibility of targeting CCR5 and CXCR4 in CD4+ T cells derived from rhesus macaques. Following re-design of the R5-ZFNs to account for sequence differences between the human and macaque alleles, we found that ZFNs could disrupt both alleles with reasonable efficiency in macaque CD4+ T cells. By inactivating CXCR4 singly and in combination with CCR5, it will be possible to study the effects of CXCR4 loss on T cell function as well as virus infection in a more relevant animal model.

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Chapter 4

Summary and future directions

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Introduction

Acquired immunodeficiency syndrome (AIDS) is a devastating disease caused by human immunodeficiency virus (HIV) mediated destruction of CD4+ T lymphocytes [1,2]. Since its emergence over 25 years ago, HIV/AIDS has killed more than 25 million people, and another 33 million are currently infected [3]. The profound effect of this pandemic has led to tremendous research efforts to elucidate the mechanisms of the HIV life cycle and identify susceptible targets for therapeutic intervention. Two critical goals of the field are development of a vaccine to prevent new HIV-1 infections and effective treatment enabling HIV-1 control in the absence of long-term anti-retroviral therapy, termed here a "functional cure."

Future directions

Understanding the HIV-1 transmission bottleneck. In chapter two, we examine phenotypic properties of T/F Env glycoproteins in an effort to elucidate Env properties that can be targeted in future vaccine and microbicide efforts. Probing for differences

between clade B T/F and chronic Env glycoproteins, we employed a variety of phenotypic assays in an attempt to reveal the cause of the genetic bottleneck during HIV-1 transmission. We demonstrated that clade B T/F Env glycoproteins are more sensitive to CD4 binding site MAbs b12 and VRC01 and that this differential neutralization sensitivity correlates with differential binding of these MAbs. This suggests that there are structural differences in the CD4 binding site between T/F and chronic Envs; however, this did not manifest itself as a phenotypic difference in a variety of *in vitro* functional assays assessing CCR5 utilization efficiency, entry inhibitor sensitivity, CD4+ T cell subset infection, DC *trans*-infection, or fusion kinetics. The significance of a more exposed CD4 binding site in clade B T/F Envs remains to be determined. However, it is possible that it may confer a subtle advantage during HIV-1 transmission that is magnified over multiple rounds of replication, and that the single-round infection assays we employed may be insufficient to detect such a modest difference.

There are several areas of future direction for the study of T/F viruses. First, while Env is a likely viral candidate responsible for the transmission bottleneck, a more rigorous approach would utilize full-length infectious molecular HIV-1 clones. This may reveal roles of other key HIV genes as well as interactions between these gene products and Env that may play a role in transmission. Second, our study has highlighted that the selection of well-matched chronic control viruses is critical. For instance, compared to the T/F Envs our test panel of chronic Envs was significantly more resistant to VRC01; however, our validation panel of chronic Envs was similar to the T/F Envs. The test panel was geographically matched to the T/F Envs while the validation panel was from a geographically distinct region; thus, geographical or other factors may have a significant

confounding effect. We propose that future studies should employ T/F and chronic control viruses from serodiscordant couples or less optimally, longitudinal samples from infected individuals. Third, transmission of clade B HIV-1 represents a fraction of new HIV-1 infections [4]. As it is possible that the cause of the transmission bottleneck may be different among HIV-1 clades, future studies should expand our work to examine other clades, most notably clade C, the predominant subtype globally [4]. Fourth, while the *in vitro* assays used in chapter two are well validated and have previously revealed subtle differences between different Envs [5,6,7,8], it is possible that they are not sufficiently replicating events impacting the genetic bottleneck *in vivo*. Thus, more sensitive and/or sophisticated assays involving human tissue explants and replication competent virus should be utilized, which may reveal more subtle functional differences between T/F and chronic HIV-1. In summary, future studies should use infectious molecular T/F and chronic HIV-1 clones from serodiscordant couples and examine functionality in tissue explants or other replication competent *in vitro* assays.

Towards a functional cure of HIV-1: the role of ZFNs. The recent report of the 'Berlin patient' suggests that heterologous transplants of HIV-resistant hematopoetic cells may be of clinical benefit [9,10]. However, this is not feasible on a large scale due to the morbidity and mortality of heterologous transplants and the dearth of *ccr5Δ32* donors. Coreceptor specific ZFNs may overcome this limitation by allowing gene modification of one's own cells for autologous transplant. Previously, CCR5-ZFNs have been developed [11] and several clinical trials are currently ongoing to assess preliminary safety and efficacy (clinicaltrials.gov identifiers NCT00842634, NCT01252641, NCT01044654). While CCR5-based therapies represent a viable approach to control

HIV-1, efficacy of such therapy may be limited due to evolution or outgrowth of pre-existing X4 HIV [12,13,14,15].

In chapter three, we generated X4-ZFNs to genetically engineer X4 HIV-resistant CD4+ T cells for autologous transplant in HIV-infected individuals. The X4-ZFNs efficiently and stably disrupt *cxcr4*, have no adverse affect on cell growth or stability, and provide protection to X4 HIV challenge *in vitro* and in a humanized mouse model. However, several areas of future work are needed prior to using X4-ZFNs in humans for the treatment of HIV-1 infection.

First, while infection with X4 HIV occurs in about 50% of late stage individuals in the developed world [16,17], infection with a pure population of X4 HIV is relatively uncommon since most individuals also harbor either R5 or R5X4 HIV [18]. Thus, genetic disruption of both *ccr5* and *cxcr4* may be needed for maximal therapeutic benefit. By using X4-ZFNs in *ccr5Δ32* cells, we have shown that genetic disruption of both coreceptors is viable *in vitro*, but future work is needed to optimize delivery of both R5- and X4-ZFNs to disrupt both copies of both genes in the same cell. One concern with simultaneous delivery of two ZFN pairs is increased off-target activity resulting from trans-heterodimerization of the two different ZFN pairs. Recent modifications in the *Fok1* catalytic domains have been made creating ZFN pairs that act as obligate heterodimers [19]. This should be applied to the R5- and X4-ZFNs to minimize off-target activity resulting from simultaneous delivery of two ZFN pairs.

Second, while *cxcr4* disruption does not impact T cell development or function in mice [20,21] and seems well tolerated in human CD4+ T cells *in vitro,* further work is needed to assess the safety and viability of *cxcr4* disruption *in vivo* because unlike for

ccr5 there are no known humans with *cxcr4* loss of function mutations due to CXCR4's critical role in embryonic development [21]. Thus, adoptive therapy studies of X4-ZFN modified CD4+ T cells should be conducted in rhesus macaques to evaluate the effect of *cxcr4* disruption on cell viability, trafficking, and function *in vivo*. If *cxcr4* disruption is well tolerated *in vivo* then *an* X4-SHIV challenge should be performed to assess efficacy prior to using X4-ZFNs in humans. Unlike for the R5-ZFNs, the 24bp binding site of the X4-ZFNs is identical between rhesus macaques and humans which would allow the use of the same ZFN pair and increase the validity of extrapolating safety, off-target, and efficacy data from macaques to humans.

It is unlikely that any gene therapy approach will achieve 100% gene modification as seen in the 'Berlin patient,' and thus important questions moving forward are can we increase *ccr5* and *cxcr4* disruption efficacy and what frequency of gene disruption is necessary for clinical benefit. Gene disruption efficiency may be increased through a combination of alternate ZFN delivery methods, hypothermic shock [22], optimization of cell stimulation, and administration of small molecules that alter chromatin structure, ZFN activity, or host DNA repair pathways. The minimal frequency of coreceptor disruption necessary for therapeutic benefit should be evaluated in humanized mice and rhesus macaques.

Next, in addition to receiving *ccr5Δ32* cells, total body irradiation, graft versus host disease, chemotherapy, and other immunosuppressants may have played a role in "curing" the 'Berlin patient' [10,23]. While we feel coreceptor ablation is critical, it may not be sufficient to recapitulate the 'Berlin patient,' and thus the role of immunosuppressants such as anti-thymocyte globulin and cyclophosphamide should be

explored to reduce the latent HIV-1 reservoir and improve engraftment of gene-modified CD4+ T cells and HSCs. In summary, genome editing of the HIV-1 coreceptors with ZFNs represent a novel therapeutic strategy that may lead to long-term control of HIV-1 in the absence of ART.

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