Adaptation of HIV-1 Envelope to Macaque Cells and Implications for the Design of Improved Models of HIV/AIDS

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Abstract

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Research in the area of Human Immunodeficiency Virus type 1 (HIV-1) vaccine development is hindered by the limitations of the Simian/Human Immunodeficiency Viruses (SHIVs) used in macaque studies. The relevance of SHIV infection of macaques to HIV-1 infection in humans depends on how closely SHIVs mimic aspects of HIV-1 transmission, pathogenesis, and diversity – properties partly determined by the HIV-1 envelope (Env). The inclusion of relevant Envs in SHIVs has proven to be difficult as many HIV-1 Envs are unable to mediate efficient infection of macaque cells.

To better understand barriers to HIV-1 Env in macaque cells, we adapted a subtype A based SHIV (SHIV-A) for replication in pig-tailed macaque (Pt) lymphocytes, and identified two mutations in gp120, A204E and G312V, that increased replication by >100-fold. Introduction of these changes into multiple subtype A Envs also greatly increased entry into Pt cells. A204E and G312V Env variants continued to require CCR5 for entry, displayed sensitivity to neutralization by soluble CD4 (sCD4), and all showed a greatly increased ability to use Pt CD4. These findings hinted at the inefficient use of CD4 as an unappreciated restriction to HIV-1 replication in macaque cells and identified changes to subtype A Envs that allowed for increased infection of macaque cells, which has direct implications for the development of a successful SHIV-A. In an effort to develop a SHIV-A for *in vivo* use, the A204E and G312V Env variants were introduced to SHIVs, which were further assessed for their ability to mediate spreading infection in macaque cells, as well as their neutralization properties. Two SHIV-As were identified that warrant further evaluation *in vivo*.

To establish whether CD4 is a determinant for limited infection of macaque cells by other globally relevant HIV-1 subtypes, HIV-1 Envs representative of diverse circulating strains from subtypes A to D were assayed for their ability to infect cells expressing Pt CD4 or Rhesus (Rh) CD4. Most of the 39 Envs tested (>74%) showed a >10-fold decrease in their ability to mediate infection using macaque CD4 as compared to human CD4. Infectivity using macaque CD4 was highly associated with sensitivity to sCD4 and the ability to mediate infection utilizing low levels of CD4 ($p < 0.0001$), thus identifying properties of HIV-1 Envs that allow for the increased ability to use macaque CD4. Additionally, the determinants of CD4 sufficient for increased infection by HIV-1 were mapped to a single amino acid difference in the D1 domain. This demonstrates that the inefficient use of macaque CD4 acts as a potent barrier to replication mediated by Envs from circulating strains of HIV-1 and identifies characteristics of HIV-1 Envs that may allow for the successful creation of relevant SHIV models.

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Chapter 1

Introduction

Human Immunodeficiency Virus type 1 (HIV-1), the major causative agent of Acquired Immune Deficiency Syndrome (AIDS), is an enormous public health challenge. Since the initial discovery of HIV-1 (8, 64, 142), advances in the understanding of HIV-1 biology have led to much improved treatment options. Nonetheless, HIV-1 infection remains a global problem; with an estimated 33 million people living with HIV-1 worldwide, and the most recent census estimating a further 2.6 million new infections in 2009 (www.unaids.org). Moreover, it is developing countries with limited access to expensive antiretroviral therapy that experience the greatest HIV/AIDS burden. For these reasons, the development of strategies to prevent HIV-1 transmission remains an important goal. However, research in this area has been hindered, in part, by the lack of an ideal model to test the efficacy of potential vaccine strategies and to understand the basic biological processes underlying HIV-1 transmission and pathogenesis.

The most commonly used animal models of HIV-1/AIDS are non-human primates, such as rhesus (Rh) and pig-tailed (Pt) macaques. Because HIV-1 alone is unable to establish infection in macaques, challenge viruses that act as surrogates for HIV-1 must be used to infect macaques. Infection of macaques with challenge viruses have been used to study aspects of HIV-1 pathogenesis, correlates of protection against HIV-1 infection, vaccine strategies, and other prophylaxes. Ideally these animal models should act as a testing ground to understand the host immune responses required to prevent HIV-1 acquisition or protect against disease progression, and to evaluate vaccine and prophylaxis strategies prior to testing in humans. Thus, it is imperative that these models provide some measure of predictive power for HIV-1 infection and disease progression in humans.

One of the major limitations of the currently used macaque models of HIV-1 for modeling human infection and disease is the lack of challenge viruses that adequately capture the diversity and phenotypes of strains of HIV-1 circulating in humans (56, 194). Unfortunately, there has been much difficulty in developing challenge viruses that capture the basic biology of circulating HIV-1, partly due to the fact that the envelopes (Envs) expressed by many circulating strains of HIV-1 do not effectively mediate infection of macaque cells. Work described in this thesis details the development of novel viruses that replicate in macaque cells by adaptive evolution of HIV-1 Env variants that

better mediate entry into macaque cells. Characterization of the Envs in the adapted viruses identified inefficient Env-host interactions that are acting to limit the replication of relevant HIV-1 strains and identified properties of HIV-1 Envs that are required to successfully mediate infection of macaque cells. These findings have direct implications for the construction of challenge viruses that represent HIV-1 strains that are most relevant to human infection.

The HIV-1 genome and HIV-1 proteins

HIV-1 is a member of the family *Retroviridae* in the genus *Lentivirus*, and is an enveloped virus encoding a single-stranded RNA genome. The HIV-1 genome contains the *gag* (group specific antigen), *pol* (polymerase), and *env* (envelope) genes, which encode for structural and enzymatic proteins. Additionally, the HIV-1 genome includes the *tat* (trans-activator of transcription) and *rev* (regulator of virion) genes, which encode for regulatory proteins; and the *vif* (viral infectivity factor), *vpr* (viral protein R), *vpu* (viral protein U), and *nef* (negative factor) genes, which encode for accessory proteins (62). The relative organization of the HIV-1 genes in the HIV-1 genome, and the proteins they encode is pictured in Figure 1.1a.

The Gag precursor polyprotein encodes the major structural proteins including the matrix (MA), capsid (CA), nucleocapsid (NC), and p6. The GagPol polyprotein, which is produced by ribosomal frame-shifting at the overlapping regions of the *gag* and *pol* portions of the viral transcript, encodes additional enzymatic proteins protease (PR), reverse transcriptase (RT), and integrase (IN). The Gag and GagPol polyproteins are cleaved into their component peptides by the PR protein. The *env* gene also encodes a polyprotein, gp160, which is cleaved by the cellular enzyme furin into two subunits: the surface unit, gp120, and the transmembrane domain, gp41 (201). The gp120 and gp41 components associate non-covalently on the exterior of the virion in an oligo-trimeric form, which has been termed the viral envelope (Env) (69). The Env is a particularly important component of the virus because it mediates viral entry into host target cells.

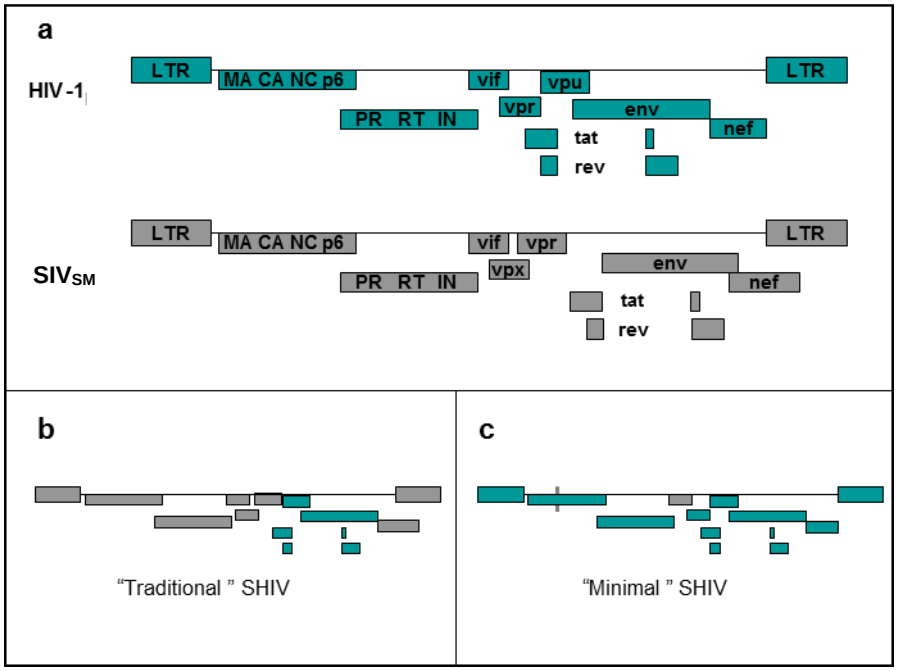

Fig. 1.1 Genomic architecture of HIV-1, SIV_{SM}, 'traditional' and 'minimal' SHIVs. *(a)* HIV-1 and SIV_{SM}, *(b)* a "traditional" SHIV containing *vpu*, *tat*, *rev* and *env* from HIV-1 in an SIV backbone, and *(c)* a "minimal" SHIV containing SIV *vif* and the CypA binding loop from the SIV capsid in an HIV-1 backbone (88).

The HIV-1 Life-cycle: The Env and Viral Entry

The entry of HIV-1 into target cells, such as CD4+ T cells and macrophages, requires the interaction of specific subdomains of gp120 with host cell proteins expressed on the surface of target cells. The gp120 protein is partitioned into domains known as the variable loops (V1 to V5) - which are demarcated structurally by disulphide bonds between cysteine residues - and the intervening constant regions (C1 to C5) (207). Variability in the form of recombination, point mutations, insertions, and deletions within the variable loops accounts for most of the Env diversity observed between different HIV-1 strains. The V1/V2 domain is the most variable in loop length, ranging from 50 to 90 amino acids in length, while the length variation of the V4 and V5 loops ranges from 19 to 44 aa and from 14 to 36 aa, respectively (28). The V3 loop is relatively well-conserved, displaying little variation in length and containing highly conserved motifs (69). Thus, despite the high level of sequence diversity between Envs, there are highly conserved features within all Envs that reflect their necessity in mediating viral entry.

The first step of HIV-1 entry involves the interaction of gp120 with the cluster of differentiation 4 (CD4) transmembrane glycoprotein (41, 92, 114) cellular receptor. CD4 is comprised of four immunoglobulin-like domains (D1-D4), and the major determinants of interaction with gp120 are located in the D1 and D2 domains (98). The CD4-binding site of gp120, is formed by conserved residues in discontinuous portions of the C1, C3, and C4 domains of gp120 that are folded into close proximity in the Env tertiary structure (95, 100, 134). The CD4-binding site is recessed within the core of the Env trimer and masked by the variable loops (164), thus protecting the highly conserved residues of the CD4-binding site from recognition by the host immune system.

Binding to CD4 results in conformational changes to gp120 that expose sites on gp120 required to engage a second surface receptor, termed the *coreceptor*. These CD4-induced conformational changes result in the formation of the coreceptor binding surface, termed the *bridging sheet*, which is made up of highly conserved residues that are spatially distinct prior to CD4 binding (29, 151). The coreceptors used by HIV-1 that are most important to infection in humans are the CCR5 (34, 46, 48, 49) and CXCR4 (58) chemokine receptors. CCR5 and CXCR4 are integral membrane proteins comprising seven transmembrane helices, an extracellular N-terminus, and extracellular loops (ECL1-3). Sulfated tyrosine residues in the N-terminus and residues within ECL2 of the coreceptor are critical for gp120 binding (6, 55, 155). Based on evidence garnered from mutagenesis studies and crystal structures, it is believed that the N-terminus of the coreceptor interacts with the HIV-1 Env at points in the bridging sheet and base of the V3 loop, and that the

ECL2 of the coreceptor interacts with the highly conserved tip of the V3 loop of gp120 (10, 38, 39, 81, 84).

Binding of gp120 with the coreceptor induces additional conformational changes to Env that stimulate the insertion of a hydrophobic domain of gp41 into the membrane of the host cell. Further conformational changes in the gp41 domain result in the transient formation of the *6-helix bundle* structure between conserved domains of gp41, which brings the viral and host membranes closer together, leading to membrane fusion and viral invasion of the host cell (102).

The HIV-1 Life-cycle: Post-Entry Steps

Fusion between the viral and host cell membranes results in entry of the nucleocapsid into the host cytosol. An uncoating step, in which the viral capsid proteins (CA) dissociate from the viral genome occurs prior to, or subsequent with, the start of reverse transcription of the viral RNA genome into double-stranded DNA (4). Reverse transcription is mediated by the RT enzyme, which includes an RNAse-H domain that degrades the original RNA genome, thus facilitating priming for the synthesis of the second strand (plus-strand) of DNA. The newly synthesized viral DNA is translocated across the nuclear pore as part of a structure known as the preintegration complex (185). In the nucleus, the double-stranded viral DNA is integrated into the host cell's genome; this integration process is catalyzed by the viral IN enzyme, in conjunction with other host factors (109).

The integrated viral DNA is known as the *proviral* DNA and includes all of the HIV-1 genes under the transcriptional control of promoter elements in the long-terminal repeat (LTR). Notably, the LTR contains enhancer elements, such as binding sites for the NF-κB transcription factor, that increase the transcription of the proviral elements (127). High-level transcription also requires binding of the viral Tat protein and subsequent recruitment of cyclin T1 and other host factors making up the positive transcription elongation factor b (P-TEF-b) (197). Rev, Tat, and Nef are the first viral proteins to be translated because they originate from multiply-spliced transcripts which can be exported from the nucleus to the cytosol in the same manner as host cell transcripts. Singly-spliced transcripts, encoding the Vif, Vpr, Vpu, and gp160 proteins, and unspliced viral transcripts, encoding the genomic RNA, and the Gag and GagPol precursor proteins, must rely on a different mechanism for export from the nucleus. The unspliced and singly-spliced RNA transcripts are transported to the cytoplasm only after the Rev protein binds the highly structured Rev responsive element (RRE) encoded in the *env* open reading frame, and mediates interaction with the host cellular nuclear export machinery, including the CRM1 nuclear export receptor (63).

Translation of the viral proteins originating from spliced and unspliced transcripts takes place in the cytosol. The newly synthesized viral proteins, together with two single-stranded copies of full-length (unspliced) viral RNA, assemble into a new generation of viral particles. In this process, the Gag and GagPol polyproteins are targeted to the plasma membrane, which initiates virion particle assembly and release. In addition to the Gag and GagPol polyproteins, the gp120, gp41 and the Vpr proteins are actively incorporated into the nascent virion (28, 36). At the same time virus particles are being released from the infected cell, the PR enzyme cleaves the Gag and GagPol polyprotein precursors, which converts the immature particle into a mature virion (62). These mature virus particles can now initiate a new cycle of infection.

Restriction Factors Block HIV-1 Infection of Macaque Cells

Ideally one would be able to simply infect macaques with HIV-1 strains of interest as a model for human disease, however this is not possible due to the species-specific tropism of HIV-1. There are intrinsic factors expressed by host cells that block post-entry steps of HIV-1 replication. These proteins are referred to as *restriction factors*, and at present there are three restriction factors that are suspected to be important in blocking the replication of HIV-1 in macaque cells.

The first of these restriction factors, TRIM5α, was identified as the cause of a block to HIV-1 replication in Rh cells that occurs early in replication, prior to the initiation of reverse transcription (179). The TRIM5α protein is a member of the *tripartite motif* (TRIM) family of proteins. The C terminus of TRIM5α encodes a B30.2 domain, and it is this domain that recognizes the capsid (CA) proteins of incoming HIV-1 particles, thereby leading to restriction (180, 181). Surpisingly, the proteins encoded by TRIM5 isoforms of Pts are either truncated before the B30.2 domain, or contain amino acid differences in the domain that prevent them from restricting HIV-1 replication (23). A restriction factor similar to TRIM5α, termed TRIMCyp, has also been identified in some populations of both Rh and Pts (22, 23, 107, 193). TRIMCyp arose as a result of a retrotransposition event that placed DNA encoding cyclophilin A (CypA) in the context of the TRIM5α locus. The resulting fusion protein encoded by the locus contains the CypA domain in place of the B30.2 domain, however the resulting fusion proteins are unable to restrict HIV-1 replication (22, 23, 107, 193). Pts have been known for some time to be more susceptible than Rhs to infection by HIV-1 (1, 7), and the lack of a restriction factor against the HIV-1 CA may provide an explanation for why this is.

A second restriction factor known to block HIV-1 infection of macaque cells is called *apolipoprotein B mRNA-editing catalytic 3G* (APOBEC3G), and was identified as a factor that restricts the replication of *vif*-deficient HIV-1 in certain non-permissive human cell lines (168). APOBEC3G has cytidine deaminase activity, meaning it can convert cytosines to uracil, and it operates on the minus strand DNA during reverse transcription. The result is a high frequency of G-to-A mutations in the plus-strand of integrated proviruses of *vif*-deficient HIV-1 (68, 115, 203), leading to the failure to produce infectious progeny virus due to the high level of hypermutation. It has also been shown that other members of the APOBEC3 family (A, B, C, F, D/E, and some APOBEC3H haplotypes), which are also cytidine deaminases, have a range in abilities to restrict *vif*-deficient HIV-1 (14, 20, 42, 108, 133, 153), though APOBEC3G and F are thought to be most important for blocking infection due to their expression in HIV-1 target cells (152). In the infection of human cells, the APOBEC3 proteins are targeted for degradation by the HIV-1 Vif protein, however, in macaque cells, the HIV-1 Vif protein fails to recognize and eliminate macaque APOBEC3 proteins, thus leading to non-productive infection of macaque cells by HIV-1 (169).

More recently, *tetherin* was identified as an interferon-inducible factor that acts to restrict the replication of *vpu*-deficient HIV-1 (129, 191). Production of *vpu*-deficient HIV-1 virions in cells expressing tetherin results in the 'tethering' of mature viral particles to the exterior of the plasma membrane, thus preventing their subsequent spread to other target cells. In the infection of human cells, the HIV-1 Vpu protein acts to downregulate tetherin expression on the cell surface (191), however the HIV-1 Vpu protein is unable to downregulate macaque tetherin (86). Taken together, the TRIM5α, APOBEC3, and tetherin restriction factors expressed by macaque cells prevents the direct use of HIV-1 as a challenge virus in macaque studies, and so, surrogate viruses that are able to evade these restriction factors must be used in place of HIV-1.

Simian Immunodeficiency Virus: A Surrogate for HIV-1

Because HIV-1 is unable to replicate in macaque cells, infection of rhesus and pig-tailed macaques with Simian Immunodeficiency Virus (SIV) is often used as a surrogate model for HIV-1 infection. The SIV_{MAC} lineage of lentiviruses was isolated in the early 1980's from Rhs that had developed AIDS-like symptoms (43, 126). It is now known that SIV_{MAC} is not a natural pathogen of Rhs, but is derived from SIV_{SM}, a lentivirus that is endemic and non-pathogenic in sooty mangabeys. SIV_{MAC} was generated serendipitously by inoculating Rhs with blood products from naturally infected sooty mangabeys (2, 3). The SIV_{MNE} lineage of viruses was generated in a similar fashion by

inoculating Pts with SIV_{MAC} (12). Throughout the rest of this chapter, SIV_{MAC} and SIV_{MNE} will be referred to collectively as SIVs.

The SIV genome and the relative organization of the genes it encodes is pictured in Figure 1.1a. Notably, SIV differs from HIV-1 in that it does not contain the *vpu* gene, however it does include an additional accessory gene termed *vpx* (viral protein X), and all other proteins share approximately 40-50% homology (167). It is now appreciated that the differences in the SIV CA and SIV Vif proteins compared to the HIV-1 CA and HIV-1 Vif proteins allow SIV to circumvent the macaque TRIM5α and APOBEC3 restriction factors. Unlike the HIV-1 CA, the SIV CA is not recognized by Rh TRIM5α, and so SIVs are not subject to restriction by TRIM5α in Rh cells. The SIV Vif protein is able to recognize and degrade macaque APOBEC3 proteins, thus allowing SIV to evade the APOBEC3 restriction of macaque cells (116, 192). Moreover, although SIV does not encode a Vpu protein, the virus is nonetheless able to circumvent the block imposed by macaque tetherin. Interestingly, unlike in HIV-1, it is SIV's Nef protein that acts to down-regulate tetherin expression in macaque cells (86). Thus, SIV expresses viral determinant that allow the virus to escape the macaque intrinsic restriction factors and mediate infection of macaque cells.

Despite differences between the HIV-1 and SIV genomes, the course of SIV infection in macaques has been useful in providing insight into basic aspects of HIV-1 biology and pathogenesis *in vivo*. One such insight was the discovery that *nef*, which is dispensable for the replication of HIV-1 and SIV *in vitro*, is required for pathogenic *in vivo* infection (89). Moreover, SIVs are typically CCR5-tropic (30, 90, 91, 204), and so the cell types infected by SIV infection of macaques closely mirrors the pathogenesis of HIV-1 in humans. This has provided insight into the initial cellular targets of HIV-1 infection in the gut-associated lymphoid tissue (GALT) and subsequent spread of transmitted virus (1). Nonetheless, the divergence between the SIV and HIV-1 proteins renders SIV an inadequate model for the study of certain prophylaxis and vaccine strategies in macaques. For example, differences between the reverse transciptase (RT) proteins of SIV and HIV-1 limits traditional SIVs as a model for some RT inhibitors used in prophylactic gels as they are effective against the SIV RT only at high concentrations (189). Additionally, the antigenic differences between the SIV and HIV-1 Envs make the SIV Env a poor model for the neutralization determinants of HIV-1, thus limiting its use in evaluating vaccine approaches targeting the HIV-1 Env (137). In recognition of the need for challenge viruses that express HIV-1 proteins, chimeric viruses that encode portions of the HIV-1 genome in the context of the SIV genome have been constructed.

SIV/HIV-1 Chimeric Viruses: Surrogates For HIV-1

The construction of SIV/HIV-1 chimeras (SHIVs) that include HIV-1 genetic material in the context of the SIV genome has resulted in challenge viruses that incorporate HIV-1 proteins. These 'traditional' SHIVs comprise the *vpu, tat, rev,* and *env* genes of HIV-1 in an SIV backbone (Fig. 1.1b). These first attempts at SHIVs were able to establish spreading infection *in vitro,* however they were unable to establish pathogenic infection *in vivo* (103, 150, 159). Ultimately, adaptation of these non-pathogenic viruses has resulted in SHIVs that are highly pathogenic *in vivo*, and result in AIDS-like symptoms (87, 149). These pathogenic 'traditional' SHIVs have been the most widely used in studying vaccine strategies seeking to elicit neutralizing antibodies targeting the HIV-1 Env.

Traditional SHIVs were generated prior to the discovery of the restriction factors responsible for blocking HIV-1 replication in macaque cells. The identification and characterization of macaque restriction factors blocking HIV-1 replication has informed the construction of SHIVs that include an even greater amount of HIV-1 genetic material. These 'minimal' SHIVs comprise the SIV *vif* and a portion of the SIV CA in an HIV-1 backbone (Fig 1.1c), and have been shown to infect macaque cells *in vitro* (71, 88), presumably by skirting the APOBEC3 and TRIM5α restriction factors. Given that the TRIM5 and TRIMCyp isoforms expressed by Pts are not active against the HIV-1 CA, additional minimal SHIVs that differ from HIV-1 only in the *vif* gene have been constructed, and have been shown to establish infection in Pt cells, and, to a limited extent, *in vivo* (70, 188). These 'minimal' SHIVs hold promise for studying vaccine strategies seeking to elicit a cellular immune response to HIV-1 proteins other than Env, such as Gag (1).

There is still much room for improvement to SHIVs, particularly in regards to the HIV-1 strains on which they are based. There is an acknowledged need for SHIVs that represent the diversity, tropism and neutralization phenotypes of circulating HIV-1 strains in humans in order to provide models of HIV-1 for use in macaques that act as better predictors of the efficacy of vaccine approaches in humans (56, 194). The properties of transmitted circulating strains of HIV-1 are primarily defined by the nature of their Envs, thus construction of SHIVs expressing HIV-1 Envs that encompass biological properties of transmitted globally circulating HIV-1 variants remains an important goal.(194)(193)

Improving SHIVs by Changing the Env: Inclusion of CCR5-using Envs obtained from Early in Infection

The most basic biological property of Envs from transmitted HIV-1 strains is the identity of the coreceptor they use to mediate entry. CCR5-tropic viruses account for the majority HIV-1 transmissions and are dominant in the early and chronic phases of HIV-1 infection. The importance of CCR5 in the initiation of infection is perhaps best illustrated by the fact that individuals homozygous for the deleterious CCR5Δ32 allele are protected from HIV-1 infection (161). CXCR4-tropic viral isolates arise predominantly during the later stages of infection, with initial studies estimated the emergence of CXCR4-tropism in 40–50% of individuals in the late stages of infection (32, 163, 186). Thus for SHIVs to be most informative as surrogates of those transmitted HIV-1 strains that vaccine strategies hope to neutralize, they should include CCR5-using Envs.

The first iterations of pathogenic SHIVs expressed CXCR4-using HIV-1 Envs. These SHIVs induce a rapid loss of peripheral naïve CD4+ T cells upon primary infection (111, 149). This contrasts with human disease progression in which primary HIV-1 infection results in much more gradual decline in peripheral CD4+ T-cells (118). Furthermore, CXCR4-tropic SHIVs do not mimic the steep decline in memory CD4+ T cells seen in the GALT, which is a hallmark of acute infection in humans (21, 67, 119). In order to develop viruses that are more relevant to HIV-1 transmission and pathogenesis, SHIVs expressing R5-tropic HIV-1 Envs have been developed. The $SHIV_{162}$ lineage of viruses are the most commonly used R5-tropic SHIVs (33, 112). *In vivo* passaging of $SHIV_{162}$ in Rhs resulted in a pathogenic viral population, termed $SHIV_{162P3}$ that was found to better mimic peripheral and GALT CD4+ T-cell loss observed in human infection (67). Moreover, this virus can be mucosally transmitted (66), which is how the vast majority of HIV-1 transmission occurs in humans (80).

Unfortunately, these intial CCR5-tropic SHIVs have failed to capture other properties associated with HIV-1 Envs involved in viral transmission. In particular, $SHIV_{162P3}$ expresses the Env from the neurovirulent SF162 strain of HIV-1, which was isolated from cerebrospinal fluid at late stages of infection (33). SF162 is exquisitely sensitive to neutralization by monoclonal antibodies (52) and plasma (165), which does not reflect the neutralization sensitivity of transmitted strains of HIV-1. HIV-1 Envs obtained from acute or early stages of infection, which are highly similar in sequence and phenotype to transmitted strains, typically display only low or moderate sensitivity to neutralization by monoclonal antibodies and plasma (104, 105, 117), and so these initial CCR5-tropic

SHIVs do not provide an adequate benchmark for the levels of neutralization that an Env-directed vaccine must achieve in humans.

Improving SHIVs by Changing the Env: Inclusion of HIV-1 Envs from Different Subtypes

An additional consideration in developing SHIVs that better capture aspects circulating strains of HIV-1 is the inclusion of Envs that represent the global diversity of HIV-1. HIV-1 arose in humans as a result of multiple cross-species transmission events, resulting in four genetically distinct groups: M (major), O (outlier), N (non-M, non-O), and more recently, group P (140). The transmission of an SIV that naturally infects chimpanzees (SIV_{CPZ}) to humans is believed to be the source of HIV-1 group M, which is responsible for most of the world-wide pandemic [as reviewed in (166)]. Within HIV-1 Group M, the high error rate of the HIV-1 encoded RT coupled with its high rates of replication has resulted in the development of an array genetically distinct virus strains that predominate in different regions of the world. Thus, Group M has been further divided into 9 genetically distinct clades or subtypes (A-D, F-H, J and K) and a number of circulating recombinant forms (CRFs). These CRFs are thought to have arisen due to RT-mediated recombination between genomes from distinct subtypes within the same host cell, resulting in the creation of new HIV-1 strains. Subtypes have been genetically defined by their *env* sequences which can differ by 17%-35% between viruses of different subtypes, and 8-17% between viruses within a subtype (94). This extreme sequence diversity in Env provides an additional hurdle to the development of a vaccine targeting the HIV-1 Env.

Subtype C is the most prevalent subtype, accounting for an estimated 48% of worldwide infection. It is followed in worldwide prevalence by subtype A (estimated 12% prevalence), subtype B (11%), CRF02_AG (8%), CRF01_AE (5%), subtype G (5%) and subtype D (2%). Importantly the localized HIV-1 epidemics do not necessarily reflect this worldwide distribution. For example, subtype B infections account for most of the infections in North America and Western Europe (72). As a result subtype B is by far the best studied subtype, and most SHIVs constructed to date have made use of subtype B Envs. However, in sub-Saharan Africa, where the majority of new cases of HIV-1 infection occur, it is subtypes C, A, and D that predominate (72). Thus there is a need for SHIVs that include diverse Envs from globally circulating subtypes other than subtype B.

In recognition of the need for SHIVs expressing non-subtype B CCR5-using Envs obtained from early in infection, pathogenic SHIVs expressing HIV-1 Envs from subtype C (31, 172, 175), and CRF_AE (79, 90) have been developed. However, despite these successes, incorporating Envs with

ideal biological characteristics into SHIVs has largely proven to be a difficult task. This is highlighted by failed attempts to construct subtype A-based SHIVs (78), which were unable to establish infection in Rh cells even at the *in vitro* level. Mixed results with Envs from the circulating recombinant form AE (CRF_AE) indicate that it is the choice of Env that mediates success in macaque cells (78, 79, 93), however attempts to characterize the differences between those CRF_AE Env that function in macaque cells and those that do not have thus far been lacking (78).

Goals of this thesis

Despite the importance of constructing SHIVs expressing Envs that accurately convey aspects of transmission, diversity, and neutralization the viruses faced in human infection, there is a dearth of information regarding what distinguishes those HIV-1 Envs that are able to successfully mediate the infection of macaque cells from those that cannot. Thus, the goals of this thesis are to identify distinguishing properties of HIV-1 Envs that allow them to successfully mediate the infection of macaque cells and further characterize factors in macaque cells that limit the tropism of HIV-1 Envs. An understanding of these Env properties and host factors will allow for the identification of Envs that may form the basis of SHIVs for use in relevant *in vivo* models of HIV/AIDS.

Chapter 2

Adaptation of subtype A HIV-1 envelopes to pig-tailed macaque cells

Introduction

For Simian/Human Immunodeficiency Viruses (SHIVs) to be effective as predictors of human disease and vaccine efficacy, they should closely mimic the transmitted strains in human infection. The Envs from most circulating strains of HIV-1 require CCR5 as a co-receptor for entry, whereas many of the current SHIVs make use of envelopes from CXCR4-tropic or dual-tropic clones, such as NL4-3, HXB2, HIV_{SF33} and $HIV_{89.6}$ (103, 112, 149, 170). Moreover, the Envs encoded by these SHIVs are highly sensitive to neutralization as compared to circulating HIV-1 variants (17, 18, 123, 183, 184). Among subtype B CCR5-tropic SHIVs (66, 130, 136), $SHIV_{SF162}$ passaged isolates are the most commonly used, however the SF162 Env encoded by these SHIVs is also extremely sensitive to neutralization (165, 195). Thus, current SHIVs do not provide a realistic benchmark for neutralizing antibody protection from circulating strains of HIV-1, and many also do not model the dominant CCR5-mediated mode of transmission.

The world-wide epidemic is comprised of very diverse HIV-1 genotypes, termed clades or subtypes. In sub-Saharan Africa, which carries the highest burden of new HIV-1 infections and HIV-1 related deaths, it is subtypes C and A that predominate (72). SHIVs that are infectious to macaques have been generated using subtype C *env* sequences (31, 172, 175), as well as *env* sequences from the circulating recombinant CRF_AE, which is the most common HIV-1 subtype in Southeast Asia, (79, 90). Despite the relative prominence of subtype A strains in the most afflicted regions of the world, attempts to make subtype A-based SHIVs (SHIV-As) have thus far been unsuccessful as the SHIV-As tested to date failed to replicate in macaque cells (78).

In order to gain further insight into barriers to SHIV-A replication in macaque cells, we created $HIVA_{Q23/}SIV_{vif}$, a minimal SHIV encoding the *vif* gene from $SIV_{mac}239$ in the context of the Q23-17 provirus, which is a CCR5-tropic subtype A HIV-1 molecular clone obtained soon after seroconversion (143). This minimal SHIV approach takes advantage of the fact that the APOBEC3-mediated restriction to HIV-1 replication in Pt cells can be countered by SIV Vif (70, 188), and, the fact that, in contrast to Rh TRIM5α, the Pt TRIM5 isoforms and TRIMCyp do not antagonize HIV-1 infection (22, 23, 107, 193). In this chapter, the replicative properties and adaptation of $HIVA_{Q23/}SIV_{vif}$ to Pt cells were explored. Two adaptive mutations were identified that, when

introduced into different subtype A Envs, permit much more efficient usage of Pt CD4, resulting in a dramatic increase in the infectivity of Pt cells. These findings identify the inefficient use of Pt CD4 as a previously uncharacterized barrier to subtype A HIV-1 replication in Pt cells, and provide approaches to increase SHIV-A infection in macaque cells.

Materials and Methods

Cells

HEK 293T cells (referred to throughout as 293Ts) and the HeLa-derived TZM-bl reporter cell line (NIH AIDS Reference and Reagent Program), were maintained in DMEM medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine.

Human peripheral blood mononuclear cells (PBMCs) from HIV-negative donors were isolated by the Ficoll gradient method, activated for 72 h with 10 U of phytohemagglutinin M/mL (Roche), and maintained in RPMI 1640 medium (Invitrogen) with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U of penicillin/ml, 100 µg of streptomycin/ml, and 10 U/mL of interleukin-2 (Roche) for 48 h prior to infection and thereafter.

Pt PBMCs were isolated using a 95% Ficoll gradient, activated for 40 h in with 4 U of phytohemagglutinin M/mL, and maintained in RPMI 1640 medium with 25 mM HEPES, 20 % heat-inactivated FCS, 2 mM L-glutamine, 100 U of penicillin/ml, 100 µg of streptomycin/ml, and 100 U/mL of recombinant human interleukin-2 (Roche).

The immortalized Pt lymphocytes used in this chapter have been described previously [(125); a gift from Drs. Nina Munoz and Hans-Peter Kiem]. Pt lymphocytes were maintained in IMDM medium (Invitrogen) with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U of penicillin/ml, 100 µg of streptomycin/mL, and 100 U of interleukin-2/mL (Chiron Corporation).

Construction of $HIVA_{Q23}/SIV_{vif}$

A proviral clone defective for *vif* expression (Q23Δ*vif*) was constructed from a full-length, CCR5-tropic, subtype A clone, Q23-17, which was originally isolated from a Kenyan individual early in infection (143). Q23Δ*vif* was created according to methods described by Sakurai et al. (160). A SalI site was introduced immediately 3′ of the pol stop codon resulting in a frameshift mutation in *vif* that prevented *vif* expression. To eliminate alternate initiation codons in the *pol*/*vif* overlapping

region, four point mutations were introduced. Three of these were synonymous in the pol reading frame, and the other resulted in a methionine-to-valine change but did not affect viral replication (data not shown). In addition to the SalI restriction site, an MluI restriction site was introduced immediately 5′ of the *vpr* initiation codon so that *vif* variants of interest could be inserted into this clone (see below). All mutations were made by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

$HIVA_{Q23}/SIV_{vif,}$ a full-length replication-competent clone expressing *vif* from $SIV_{mac}239$, was created from Q23Δ*vif*. To make $HIVA_{Q23}/SIV_{vif}$, the entire $SIV_{mac}239$ *vif* open reading frame was amplified from $SIV_{mac}239\Delta env$ (a gift from Dr. David Evans) using forward primer 5'-GAAGGTCGACATGGAGGAGGAAAAGA-3' and reverse primer 5'-AGTGACGCGTTCATGCCAGTATTCCCAA-3' (restriction sites underlined). The PCR product was then digested with the SalI and MluI restriction enzymes, ligated into Q23Δ*vif*, and verified by sequencing.

Env clones and mutagenesis

In addition to Q23ENV.17 [(146); referred to throughout as Q23-17], the following plasmids expressing subtype A Envs were used in the study: QF495.23M.ENV.A3 [(17); referred to throughout as QF495.A3], BG505.W6M.ENV.B1 and MG505.W0M.ENV.H3 [(199); referred to throughout as BG505.B1 and MG505.H3, respectively], and Q259.D2.26 and Q259.D2.17 (110). Additionally, the SF162P3 clone, constructed by inserting the predominant V1-V5 sequence from the $SHIV_{SF162P3}$ isolate into the $HIV\text{-}1_{SF162}$ Env clone, [(83); a gift from Dr. Cecilia Cheng-Mayer] and the SIV Mne CL8 Env clone (139) were used.

Mutations were introduced to the subtype A Env clones by site-directed mutagenesis using primers designed according to the Quik-Change site-directed mutagenesis kit (Stratagene) to amplify 25 ng of plasmid with Pfu Turbo (Invitrogen) under the following reaction conditions: 95°C for 5 min, followed by 18 cycles of 95°C for 30s, 55°C for 1 min, and 68°C for 16 min. The Env mutants were sequenced through the entirety of the *env* open reading frame to verify that no undesired nucleotide changes had occurred. The mutagenesis primers used are listed in Table 2.1.

Construction of other full-length molecular clones

Chimeric full-length molecular clones were constructed by digesting the subtype B pNL-DT5R [(88); a gift from Dr. Malcolm Martin] and $HIVA_{Q23}/SIV_{vif}$ with EcoRI (restriction site located

in *vpr*) and XhoI (restriction site located in *nef*) and ligating the heterologous fragments. The resulting chimeras were named $Q/N_{vpr\text{-}nef}$ and $N/Q_{vpr\text{-}nef}$ to indicate the origin of the *vpr* to *nef* portion in each clone (Fig. 1b).

The $HIVA_{Q23}/SIV_{vif}$ provirus was engineered to express different *env* genes of interest using a previously described method (144). In short, $HIVA_{Q23}/SIV_{vif}$ was digested with SmaI (restriction site located in *vpr*) and XhoI to excise an ~3 kb fragment encompassing the Q23-17 *env* gene. Heterologous *env* genes were then introduced into $HIVA_{Q23}/SIV_{vif}$ by digesting the *env* clones of interest with SmaI and XhoI and ligating the fragment into $HIVA_{Q23}/SIV_{vif}$.

Virus production and titration

Full-length replication-competent viruses were produced by transfecting 293T cells using polyethyleneimine (PEI; Polysciences). Briefly, 2×10^6 293Ts were plated 24 hours prior to transfection in a T75 tissue culture flask. The next day 6 µg of DNA was incubated with 60 µg of PEI in 600 µL of serum-free DMEM for 10 minutes before adding to cells. Viral supernatants were collected 72 hours after transfection, filtered through a 0.22 µm filter (Millipore Corporation) and stored at -80°C until use.

The following *env*-deficient HIV-1 proviruses were used to generate pseudoviruses: Q23Δ*env* and pLai3Δ*env*Luc2 (a gift from Dr. Michael Emerman), both of which have been described previously (110, 202); and Q23Δ*env*-GFP, which was constructed by sub-cloning eGFP from pEGFPN1 (Clontech) into Q23Δ*env*. For this purpose, BamHI and NotI sites that were introduced at nucleotides 31 and 63 in the *nef* open reading frame of Q23Δ*env* and the eGFP sequence was introduced using these same restriction sites.

Pseudoviruses were produced by co-transfecting 293T cells with one of the plasmids encoding *env*-deficient proviruses described above and plasmids encoding *env* clones at a 2:1 mass ratio. To do this, 2.5×10^5 293T cells were plated in each well of a 6-well dish 24 hours prior to transfection. For each well, 1 µg of total DNA was mixed with 10 µg of PEI in 100 µL of serum-free DMEM. In some cases, 3 µL of Fugene 6 (Roche) was used in place of PEI per the manufacturer's instructions. Pseudoviruses lacking Env (Env(-)) were used to determine the background levels of

Table 2.1 Primers used for site-directed mutagenesis of subtype A Envs

Envelope clone	Residue Change	Primer Sequence[a]
BG505.B1	A204E	GCCATTACACAGGAGTGTCCAAAGGTATCC[b]
	G312V	GGAAGAGTATACGTATAGTACCAGGACAAGCATTC
MG505.H3	A204E	GCCATTACACAGGAGTGTCCAAAGGTATCC
	G312V	GGAAGAGTATACGTATAGTACCAGGACAAGCATTC
QF495.A3	A204E	GCCATTACACAGGAGTGCCCAAAGGTATC
	G312V	CAAGAAAAAGTGTGCGTATAGTTCCAGGACAAGTATTCTATGCAAC
Q259.D2.26	A204E	GCCATTACACAGGAGTGTCCAAAGGTAACC[c]
	G312V	GAAGAAGTGTACATATAGTACCAGGACAAGCATTCTATG
Q259.D2.17	A204E	GCCATTACCCAGGAGTGTCCAAAGGTAACC
	G312V	GAAAAAGTGTACGTATAGTACCAGGACAAGCATTCTATG

[a]The sequence of the primer in 5' to 3' orientation. The underlined nucleotide(s) represent the changes being introduced. Note that the reverse complement primer was also used to introduce the mutations of interest, as described in the text.
[b]The same primers were used to introduce the G312V change to MG505.H3 and BG505.B1
[c]The same primers were used to introduce the G312V and A204E changes to Q259.D2.26 and Q259.D2.17

some assays, and were generated by co-transfecting the empty pCI-neo (Promega) mammalian expression vector in place of an *env* expressing plasmid. Viral supernatants were harvested 48-72 hours post-transfection and cleared of cellular debris by centrifuging at 1300 rpm for 5 min. In some cases, cleared supernatants were concentrated ~20-50-fold using Amicon Ultra 10K filters (Millipore Corporation). All viral preparations were frozen at -80°C until use.

Viral titers were determined by infecting TZM-bl cells with thawed cell-free virus in the presence of 10 µg/mL of DEAE-dextran. Forty-eight hours later, the cells were fixed and stained for beta-galactosidase activation, and blue foci were counted to obtain infectious titers. Infectious titers were reported as infectious particles (IP)/mL (18).

Virus replication assays

Viral stocks were mixed with either 3×10^6 stimulated donor human PBMCs, 2×10^6 Pt PBMCS, or 1×10^6 immortalized Pt lymphocytes at a multiplicity of infection (MOI) of 0.02 or 0.2 in a final volume of 250 µL. After spinoculation (132) at room temperature for 2 to 3 hours at 1200×*g*, cells were washed three times in 1.5 mL of the appropriate media and resuspended into duplicate 600 µL cultures in a 48-well dish. Cultures were maintained for 15 days, with approximately two-thirds of the medium being replaced every 3 days. The $p24^{gag}$ levels were determined by measuring cleared culture supernatants with a $p24^{gag}$ Antigen ELISA kit (ZeptoMetrix, Buffalo, NY).

Luciferase assays

Infections of 8×10^4 immortalized Pt lymphocytes were performed in triplicate by spinoculation with luciferase reporter viruses for 2 hours at an MOI of 0.02 in a final volume of 100 µL. Infections were allowed to proceed for 72 hours before lysis with Brite-Glo reagent (Invitrogen) according to the manufacturer's guidelines. Luciferase activity was immediately read on the Fluoroskan Ascent FL luminometer (Thermo LabSystems) with a 1000 ms integration period. Infections with Env(-) pseudoviruses were used to determine background levels for the assay.

Long-term culturing of $HIVA_{Q23}/SIV_{vif}$ and sequencing of outgrowth variants

Cultures of 1×10^6 immortalized Pt lymphocytes were infected with $HIVA_{Q23}/SIV_{vif}$ at an MOI of 0.2 in an initial volume of 1 mL in 12-well dishes. Cells were maintained at a concentration of 0.8 to 3×10^6 cells/mL without discarding cells and with at least two-thirds of the media being replenished every 4-5 days. Supernatant was assessed for the presence of virus every 4-5 days by infecting TZM-bl cells.

Using the QIAgen Blood DNA Kit (QIAgen), total DNA was extracted from cells in cultures in which viral outgrowth was identified. The number of integrated proviral copies was determined using a previously described real-time PCR assay (11). To sequence viral variants, 500 copies of the integrated provirus were amplified with 4 nested PCRs, resulting in overlapping amplicons spanning the entirety of the HIV-1 genome. The primers and conditions used for each set of nested PCRs are listed in Table 2.2. The nested PCRs were performed in triplicate with TaqPlus Precision (Agilent), under the following thermocycling conditions: 95 °C for 5 min, followed by 25 cycles of 95 °C for 30s, 55 °C for 30s, 68 °C for 4 min 30s; followed by 68 °C for 10 min. The PCR products were detected as single prominent bands by gel electrophoresis. All reactions were treated with ExoSap (Amersham Biosciences) and the amplicons were sequenced directly without gel purification.

Infection of Pt lymphocytes in the presence of TAK779

TAK779 treated immortalized Pt lymphocytes were pre-incubated with 1 μM of TAK779 (NIH AIDS Reference and Reagent Program) for 2 hours at 37°C and maintained thereafter in 1 μM of TAK779. Triplicate infections of 8×10^4 TAK779-treated or untreated cells were then performed by spinoculation for 2 hours with pseudotyped luciferase reporter viruses at an MOI of 0.02 in a final volume of 100 uL. Infected cultures were maintained for 72 hours before lysis and subsequent measurement of luciferase activity. Percent inhibition by TAK779 was determined by comparing relative luciferase levels in TAK779-treated cells versus untreated cells.

Neutralization assays.

Neutralization assays were performed using the TZM-bl neutralization assay as described previously (18). Approximately 500 IPs of Q23Δ*env*-derived pseudoviruses were incubated with 6 serial three-fold dilutions of IgG1b12 [(25); referred to throughout as b12] or soluble CD4 (sCD4) in a 96-well plate. One hour later, 1×10^4 TZM-bl cells were added to each well with DEAE-dextran to a final concentration of 10 μg/mL. The relative levels of infection were determined by assessing β-galactosidase activity in triplicate wells after 48 h. Median inhibitory concentrations (IC_{50}s) were defined as the concentration of b12 or sCD4 that resulted in 50% inhibition of β-galactosidase, and were calculated using the linear fit model.

Table 2.2 Primers used to amplify and sequence integrated provirus from adapted $HIVA_{Q23}/SIV_{vif}$

Genomic Fragment[a] (Start nt, End nt)[b]	Primer Name[c]	Sequence[d]	Genome Location (Start nt, End nt) [Gene Start nt, Gene End nt][e]	Usage[f]
5'LTR - pol (535, 4807)	LTR_328-354_RD1_F	GCTGACACAGAAGTTGTTGACTGGGAC	5' LTR (328,354), 3' LTR (9413, 9439)	forward primer round 1
	Pol4	CTGCCCCTTCACCTTTCC	*pol* (4957,4974) [2890,2873]	reverse primer round 1
	LTR1	TTGCCTTGAGTGCTTCAAGTAGTGTGTG	5' LTR (535, 562), 3' LTR (9620, 9647)	forward primer round 2, sequencing
	Pol2	CCCCCAATCCCCCCTTTTC	*pol* (4807,4789) [2723, 2705]	reverse primer round 2, sequencing
	Gag17	GAGGAGCTCTCTCGACGCAGG	non-coding region (675, 695)	sequencing
	Pol103wt-1	CCAGCGGGTCTAAAAAAGAAA	*pol* (2838, 2858) [754, 774]	sequencing
	Pol14	GTCTACTTGTCCATGCATGGC	*pol* (4394, 4377) [2310, 2293]	sequencing
	Pol18	GATCCTACATACAAATCATCC	*pol* (3121, 3104) [1037, 1020]	sequencing
	Pol22	GCTCCTGTATCTAATAGAGC	*pol* (2335, 2319) [251, 235]	sequencing
	Pol23	AGCTGGACTGTCAATGATATAC	*pol* (3300, 3321) [1216, 1237]	sequencing
	Pol53	GGATGATTTGTATGTAGGATC	*pol* (3101, 3121) [1017, 1037]	sequencing
pol - env (4534, 7224)	Pol11	GAAGCCATGCATGGACAAGTAGAC	*pol* (4371, 4394) [2287, 2310]	forward primer round 1
	Env70	TTGCAATAGAAAAATTCTCCTC	*env* (7381, 7363) [1157,1139]	reverse primer round 1
	Pol19	TAAAATTAGCAGGAAGATGGCCAG	*pol* (4534, 4557) [2450, 2473]	forward primer round 2, sequencing
	Env68	CATTACAATGTGCTTGTC	*env* (7224, 7204) [1000, 980]	reverse primer round 2, sequencing
	Pol15	TACAGTGCAGGGGAAAGAATA	*pol* (4809, 4829) [2725, 2745]	sequencing
	Vpu2	GCCACTGTCTTCTGCTCTTT	*vpu* (6223, 6207) [162, 146]	sequencing
	Env18	CAATAATGTATGGGAATTGG	*env* (6871, 6861) [653, 637]	sequencing

Table 2.2 continued

env - nef (6576, 8813)	Env17	AACATGGTAGAGCAGATGCA	*env* (6519, 6538) [295, 314]	forward primer round 1
	Nef54a	CTAAATCTCGAGATACTGCTCC	*nef* (8909, 8890) [112, 94]	reverse primer round 1
	Env15	CCATGTGTAAAGTTAACCCC	*env* (6576, 6595) [352, 371]	forward primer round 2, sequencing[g]
	Nef48	GACCACTTGCGGCCGCTGTTATAGCAAAG CCCTTTCTAAGCC	*env/nef* (8813, 8775) [2571, 2551]/[17, 1]	reverse primer round 2, sequencing[h]
	Env28	TAATCGAATCGATCTGCCTCTGCCTTGCT CTCC	*env* (8468, 8439) [2244, 2215]	sequencing
	Env67	GATATAAGACAAGCACATTG	*env* (7197, 7216) [973, 992]	sequencing
env - 3'LTR (7895, 9583)	Env39	GTCAATAACGCTGACGGTACAGG	*env* (7823, 7845) [1599, 1621]	forward primer round 1[i]
	LTR3	TTATTGAGGCTTAAGCAGTGGGTTCCC	5' LTR (531, 508), 3' LTR (9616, 9593)	reverse primer round 1
	Env41	GGCTATAGAGGCTCAACAAC	*env* (7895, 7914) [1671, 1690]	forward primer round 2, sequencing
	R4	GCCAGAGAGCTCCCAGGCTC	5' LTR (498, 482), 3' LTR (9583, 9567)	reverse primer round 2, sequencing
	Env45	GCTACCACCGCTTGAGAGACTTC	*env* (8524, 8546) [2300, 2322]	sequencing
	Nef11	CTGACTGTGCCTGGCTAGAA	*nef* (8954, 8973) [158, 177]	sequencing

[a] Proviral genomic fragment amplified for sequencing.

[b] Start and end nucleotide (based on HXB2 numbering) of the amplified fragment.

[c] Name of the primer being used (as named by Overbaugh Lab conventions)

[d] Sequence of the primer in a 5' to 3' direction

[e] Start and end nucleotide of the primer as it binds to the proviral genome relative to the HIV-1 ORF in which it is located (based on HXB2). Instances where the end nucleotide is greater than the start nucleotide indicates a reverse primer

[f] What the primer was used for, whether to amplify the first round product, amplify the second round product and/or for sequencing

[g] This primer was also used to sequence the pol - env fragment

[h] This primer was also used to sequence the env - 3'LTR fragment

[i] This primer was also used to sequence the env - nef fragment

Construction and transient transfection of CD4 and CCR5 expression plasmids

Human CCR5 and Pt CCR5 (referred to throughout as huCCR5 and ptCCR5, respectively) in the pBABE-puro vector were described previously (46, 88). To clone human and Pt CD4 (referred to throughout as huCD4 and ptCD4, respectively), total RNA was isolated from human PBMCs and Pt lymphocytes using the Qiagen RNeasy Mini Kit. Total cDNA was obtained by reverse-transcribing 2 µg of RNA with SuperScript II RT (Invitrogen) using oligo-dT primers. The CD4 open-reading frame was PCR-amplified using forward primer 5'-GATGGATCCATGAACCGGGGAGTCCC-3' with reverse primer 5'-GGTGTCGACTCAAATGGGGCTACATG-3' for huCD4 and forward primer 5'- GATGGATCCATGAACCGGGGAATCCC-3' with the same reverse primer for PtCD4. The PCR products were digested with BamHI and SalI (restriction sites underlined in the primers), cloned into the pBABE-puro plasmid (124), and verified by sequencing.

CD4 and CCR5 expression plasmids were co-transfected in equal amounts into 293T cells using Fugene 6 at a ratio of 3 µL of transfection reagent to 1 µg of DNA as per the manufacturer's protocol. In some cases, transfections were performed with 6 µg of DNA in T75 tissue culture flasks with 18 µL of Fugene in a total volume of 200 µL of serum-free DMEM.

Analysis of receptor expression levels by flow cytometry

CD4 and CCR5 expression levels were determined by flow cytometry using APC conjugated mouse anti-human CD4 antibody (cat. No. 551980, BD Biosciences) and PE conjugated mouse anti-human CCR5 (cat. no. 550632, BD Biosciences). Briefly, 1-2×10^5 cells were washed in PBS/2% FBS, and incubated in ~100 µL of PBS/2% FBS with 2 uL of the anti-CD4 antibody and/or 5 µL of the anti-CCR5 antibody at room temperature for 30 mins. The cells were then washed once in 1 mL of PBS/2% FBS and resuspended in 200 µL PBS/2% FBS for analysis by flow cytometry.

Results

Replication of $HIVA_{Q23}/SIV_{vif}$ in immortalized Pt lymphocytes

$HIVA_{Q23}/SIV_{vif}$, a minimal SHIV derived from the subtype A CCR5-tropic Q23-17 provirus, was tested for its ability to replicate in human PBMCs and immortalized Pt lymphocytes. NL-DT5R, a CXCR4-tropic minimal SHIV that is derived from NL4-3 and encodes the *vif* gene and the cyclophilin A (CypA)-binding loop from $SIV_{mac}239$, was used as a positive control for replication in Pt cells [(88); Fig. 2.1a]. In human PBMCs, $HIVA_{Q23}/SIV_{vif}$ achieved peak levels of ~300 ng $p24^{gag}$/mL, slightly higher than NL-DT5R, which reached peak levels of ~100 ng $p24^{gag}$/mL. In Pt

cells, however, while NL-DT5R reached levels of ~300 ng $p24^{gag}$/mL, $HIVA_{Q23}/SIV_{vif}$ reached peak levels that were ~300-fold lower at ~1 ng $p24^{gag}$/mL, despite the fact that infections with $HIVA_{Q23}/SIV_{vif}$ were performed at a 10-fold greater MOI (Fig. 2.1b).

To determine which regions of the $HIVA_{Q23}/SIV_{vif}$ genome were responsible for its impaired infection of Pt lymphocytes, reciprocal chimeras between $HIVA_{Q23}/SIV_{vif}$ and NL-DT5R were constructed and evaluated for their ability to replicate in human and Pt cells (Fig. 2.1a). In human PBMCs, both chimeric viruses replicated to levels of $p24^{gag}$ that were within the same range as $HIVA_{Q23}/SIV_{vif}$ and NL-DT5R (Fig. 2.1b). In Pt lymphocytes, $Q/N_{vpr\text{-}nef}$ replicated to similar levels as NL-DT5R, indicating that the 5'LTR and *gag-pol* region of $HIVA_{Q23}/SIV_{vif}$ functioned for virus replication in Pt cells. Conversely, $N/Q_{vpr\text{-}nef}$ reached a peak level of $p24^{gag}$ that was >2 logs lower than NL-DT5R, even with infections performed at a 10-fold greater MOI (Fig. 2.1b). This suggested that the 3' region of $HIVA_{Q23}/SIV_{vif}$, encompassing the entirety of *tat*, *rev*, *vpu* and *env*, as well as parts of *vpr* and *nef*, was responsible for the low levels of replication in Pt cells.

Entry of viruses bearing subtype A Envs into immortalized Pt lymphocytes

Given that the viral determinant for impaired replication of $HIVA_{Q23}/SIV_{vif}$ in Pt cells included the *env* gene, the Q23-17 Env was examined for its ability to mediate entry into Pt cells. The Q23-17 Env was compared to the SF162P3 Env, which is derived from $SHIV_{SF162P3}$, a CCR5-tropic isolate known to establish persistent spreading infection in Pts (83). Pseudotyped luciferase reporter viruses, which were normalized based on MOI, infected Pt lymphocytes ~30-fold less efficiently when carrying the Q23-17 Env as compared to those pseudotyped with the SF162P3 Env (Fig. 2.2).

To determine whether the Q23-17 Env was typical of subtype A Envs, 5 additional CCR5-tropic subtype A Envs, obtained from recently infected individuals, were tested for their ability to infect immortalized Pt lymphocytes. Viruses carrying the BG505.B1, Q259.d2.26 and Q259.d2.17 Envs mediated entry into Pt cells to levels that were comparable to Q23-17, while the infectivity of viruses carrying the QF495.A3 and MG505.H3 Envs was below the background levels of the assay (Fig. 2.2). Overall, these results suggested that inefficient entry into Pt cells is a common characteristic of subtype A Envs.

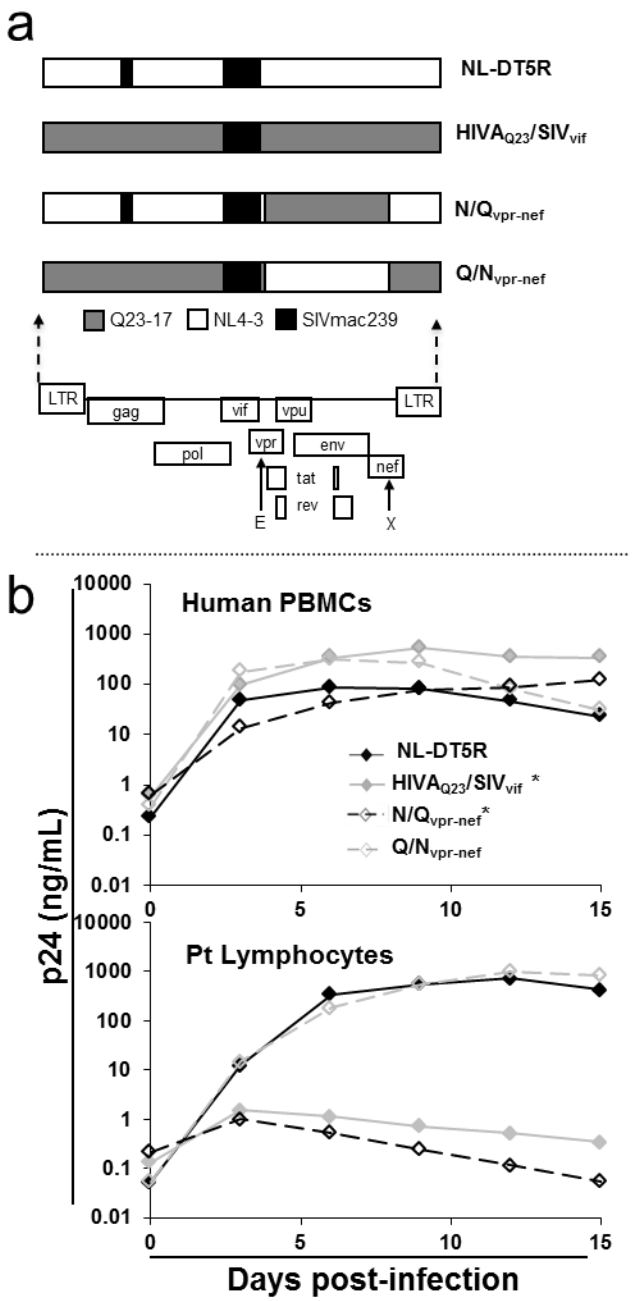

Fig. 2.1 Infection of human PBMCs and Pt lymphocytes with minimal SHIVs. (*a*) Schematic representation of the contribution of Q23-17, NL4-3 and SIVmac239 sequences to the minimal SHIVs used for replication experiments. E and X represent the EcoRI and XhoI sites used to make N/Q$_{vpr-nef}$ and Q/N$_{vpr-nef}$. (*b*) p24 levels are shown as a function of time post-infection in human PBMCs and Pt lymphocytes. The data points represent the average measurement from duplicate infected cultures. The figure legend is shown in the top plot, and viruses with an asterisk were used at a 10-fold greater MOI for infection of Pt cells. The results are representative of at least three independent experiments.

Adaptation of $HIVA_{Q23}/SIV_{vif}$ to immortalized Pt lymphocytes and identification of adaptive amino acid changes

To determine if $HIVA_{Q23}/SIV_{vif}$ could be adapted to replicate in immortalized Pt lymphocytes, the virus was maintained in multiple long-term cultures in two independent experiments. In the first experiment, viral outgrowth was seen in 1 of 9 cultures after approximately 35 days in culture, with viral supernatant reaching titers of $>10^6$ IP/mL in TZM-bl cells (data not shown). Sequencing of the integrated proviral genome in triplicate from cells in this culture revealed only a single G to T mutation that was clearly present in all sequences, which encoded a glycine to valine change at position 312 of the Env surface unit protein, gp120 (G312V, HXB2 numbering). Introduction of the G to T mutation to $HIVA_{Q23}/SIV_{vif}$ resulted in virus replication that achieved a > 2 log increase in $p24^{gag}$ levels in immortalized Pt lymphocytes as compared to wild-type (Fig. 2.3a). However, the $HIVA_{Q23}/SIV_{vif}$ G312V molecular clone showed intermediate levels of replication compared to the viral quasispecies, with the peak $p24^{gag}$ level of the Pt cell-adapted viral quasispecies being ~2 log higher than the G312V molecular clone. This indicated that the G312V amino acid change may only confer a portion of the full replication potential of the adapted viral quasispecies in Pt cells. While no other dominant mutation was identified in the genome of the adapted virus that could readily explain these differences, the possibility that other compensatory mutations arose in the viral quasispecies over the 15-day period of the replication assays cannot be ruled out.

In a second experiment, replicating virus was found in 2 out of 30 cultures after approximately 32 days of culturing, with titers of $>10^5$ IP/mL. Sequencing of the integrated proviral genome from cells in each of the two independent cultures harboring outgrowth virus revealed an identical adaptive mutation; a single C to A nucleotide change resulting in a predicted alanine to aspartic acid change at position 204 of gp120 (A204E, HXB2 numbering). Introduction of this same C to A nucleotide change into $HIVA_{Q23}/SIV_{vif}$ resulted in a > 4 log increase in $p24^{gag}$ levels as compared to the wild-type virus (Fig. 2.3b), which was similar to the levels of replication observed with the adapted viral quasispecies, thus indicating that the A204E amino acid change is responsible for the increased ability of the second adapted virus to infect Pt cells.

Viruses bearing the envelope protein with the G312V and A204E amino acid changes were also tested for replication in primary Pt PBMCs from two different donor animals. Peak replication was achieved at day 3 or 6 for the wild-type $HIVA_{Q23}/SIV_{vif}$ and at day 6 for the G312V and A204E variants. The G312V change increased peak $p24^{gag}$ levels by approximately 4-fold compared to wild-type and the A204E $p24^{gag}$ levels increased by approximately 10-fold as compared to wild-type (Fig.

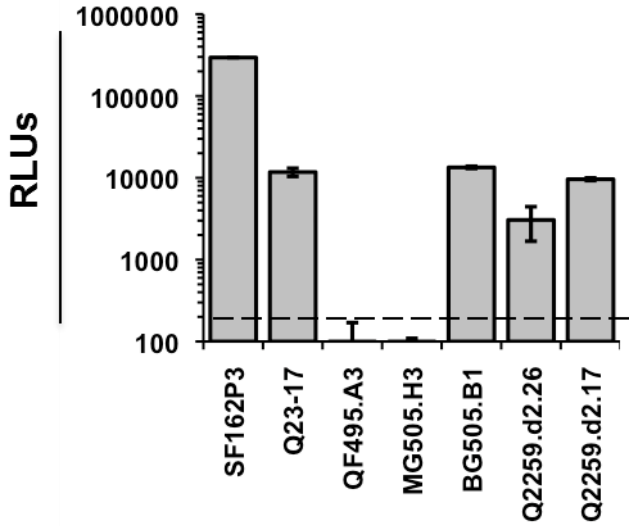

Fig 2.2 Single-cycle infection of Pt lymphocytes with luciferase pseudoviruses bearing subtype A Envs. The y-axis shows relative light units (RLUs) in cells infected with the virus indicated on the x-axis. The dashed line represents background RLU levels for the experiment observed in cells infected with Env(-) pseudovirus. Error bars represent the standard deviation obtained from triplicate wells. The results are representative of at least three independent experiments.

2.3c). The levels of replication of these variants were considerably lower (~10-100-fold) than the replication levels of the CXCR4-tropic minimal SHIV, NL-DT5R (not shown).

Effect of amino acid changes at G312 and A204 on the ability of the Q23-17 Env to mediate entry into immortalized Pt lymphocytes

To examine how the G312V and A204E changes were affecting entry into Pt cells, reporter pseudoviruses carrying the Q23-17 Env with the G312V or A204E change were used to infect Pt lymphocytes. Levels of entry were ~100-fold higher than wild-type for the Q23-17 G312V variant, and ~50-fold higher for the Q23-17 A204E variant. These levels were generally slightly higher than those of the SF162P3 Env, in the case of the G312V substitution; and similar to the SF162P3 Env, in the case of the A204E substitution (Fig. 2.4). Additionally, a double mutant was generated to determine whether there were any synergistic effects between the G312V and A204E substitutions. However, pseudoviruses bearing the Q23-17 double-mutant Env were not infectious in TZM-bl cells and so were not examined for entry into Pt cells.

The adaptive A204E and G312V changes that were observed in culture are rare among HIV-1 sequences, with only three examples of the G312V substitution and seven examples of the A204E substitution observed in the >10,000 HIV-1/SIVcpz Env sequences surveyed (http://www.hiv.lanl.gov). Furthermore, changes to G312 and A204 have not been reported in any infectious SHIVs constructed to date (data not shown). The G312 residue, which is the first amino acid of the GPG(R/Q) motif located at the tip of the V3 loop (99), is conserved in >92% of Env

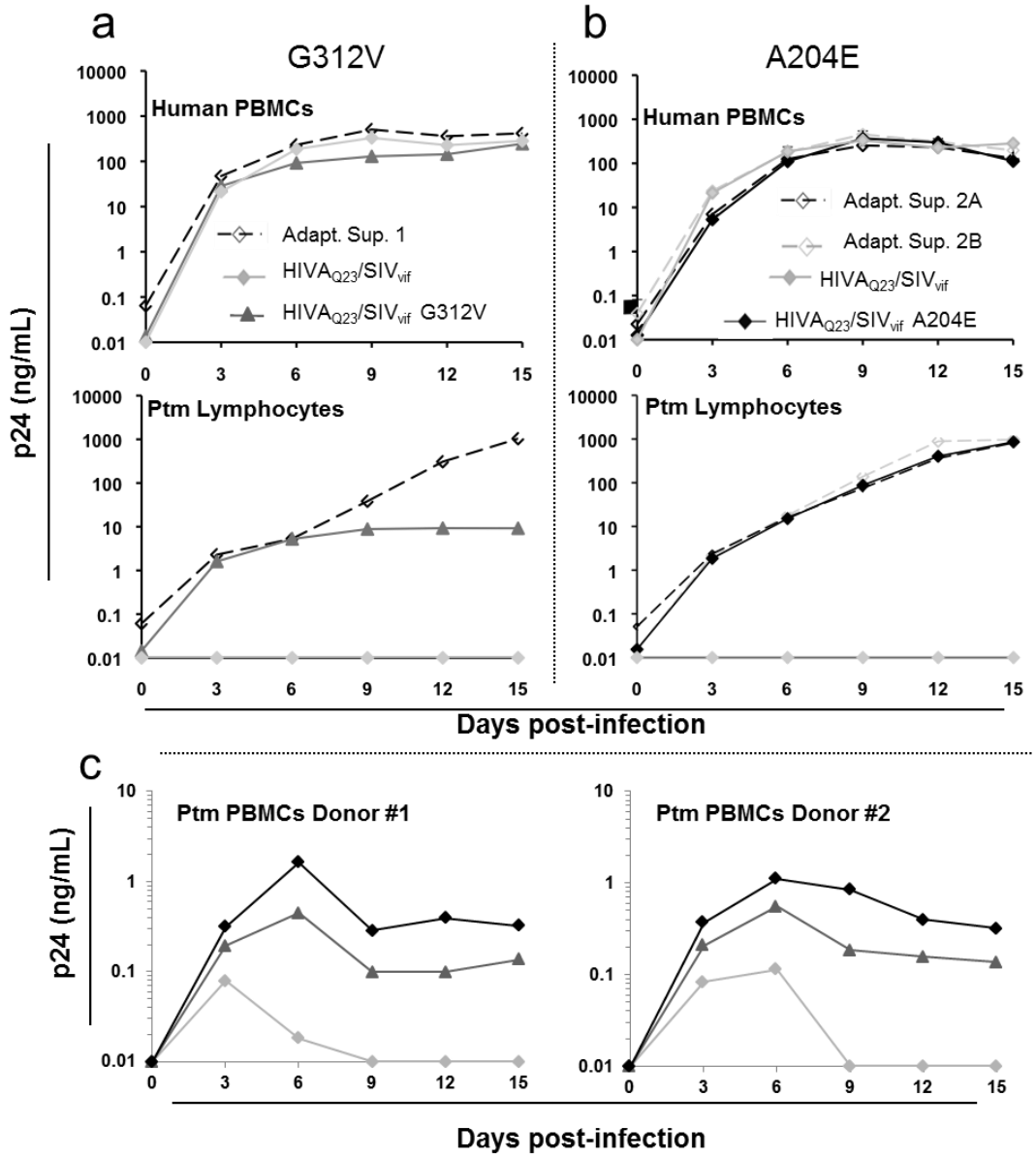

Fig 2.3. Infection of human PBMCS, immortalized Pt lymphocytes, and primary Pt lymphocytes with HIVA$_{Q23}$/SIV$_{vif}$ carrying the G312V or A204E change. Infection of human PBMCs and immortalized Pt lymphocytes with HIVA$_{Q23}$/SIV$_{vif}$ carrying the G312V change *(a)* or the A204E change *(b)*. The p24 levels are shown as a function of time post-infection in human PBMCs (top graphs) and Pt lymphocytes (bottom graphs). The data points represent the average measurement from duplicate cultures. The figure key is shown in the top plots, and 'Adapt. Sup.' refers to the uncloned adapted viral quasispecies obtained by long-term culturing from which the mutations were isolated. The results are representative of at least three independent experiments. *(c)* Infection of primary Pt PBMCs from two different donors with HIVA$_{Q23}$/SIV$_{vif}$ (black), HIVA$_{Q23}$/SIV$_{vif}$ G312V (grey) , or HIVA$_{Q23}$/SIV$_{vif}$ A204E (open diamonds, dashed line). The p24 levels are shown as a function of time post-infection.

sequences from all HIV-1 subtypes and >96% in subtype A Envs. The most common amino acid substitutions at G312 are alanine, arginine, and, specific to subtype A, histidine. The A204 residue, which is located adjacent to the β3 strand of the bridging sheet in the C2 region of gp120 (151), is conserved in >98% of all HIV-1 subtypes and is invariant in subtype A. The most common substitutions for A204 are serine and threonine.

A panel of mutants encoding the most common amino acid substitutions at G312 and A204 was made to examine how these substitutions compared to the adaptive G312V and A204E substitutions for their ability to mediate entry into Pt cells. The titers of luciferase reporter pseudoviruses carrying the mutant Q23-17 Envs were comparable to those carrying the wild-type Q23-17 in HeLa-derived TZM-bl cells, with the exception of the G312R variant, whose limited infectivity precluded it from further study (data not shown). In Pt cells, the level of entry of the G312A mutant was comparable to wild-type whereas the G312H mutant had a >15-fold increased level of entry. Substitutions of serine and threonine at A204 had more modest outcomes; the A204S and A204T mutants mediated entry into Pt lymphocytes at levels that were only 3 to 5-fold greater than wild-type Q23-17 (Fig. 2.4).

To see if the A204E change was eliciting its effect due to the introduction of a negative charge, A204 was substituted with aspartic acid (A204D), which also bears a negative charge. Notably, the viruses carrying the A204D Env variants typically showed an approximately 100-fold decrease in infectivity on TZM-bl cells as compared to the wild-type Q23-17 Env (data not shown). However, when equal MOIs of the A204D virus were used to infect Pt cells, luciferase levels were comparable to the A204E variant that was adapted in culture (Fig. 2.4). All together these results indicate that it is not the presence of a specific amino acid at position 204 (E) or G312 (V) that confer increased entry into Pt lymphocytes; other amino acid changes at these positions can also impact infectivity in Pt cells.

Effects of G312V and A204E amino acid changes in other subtype A Envs on entry and replication in Pt cells

To examine whether the effects of the G312V and A204E changes were context-specific, the changes were introduced individually to each of the subtype A Envs tested in Fig. 2.2 and the mutated variants were assayed for their ability to mediate entry into Pt lymphocytes. Most of the reporter viruses carrying the G312V and A204E Envs had infectious titers that were comparable to the parental wild-type in TZM-bl cells. This was not true, however for the Q259.d2.26 G312V and

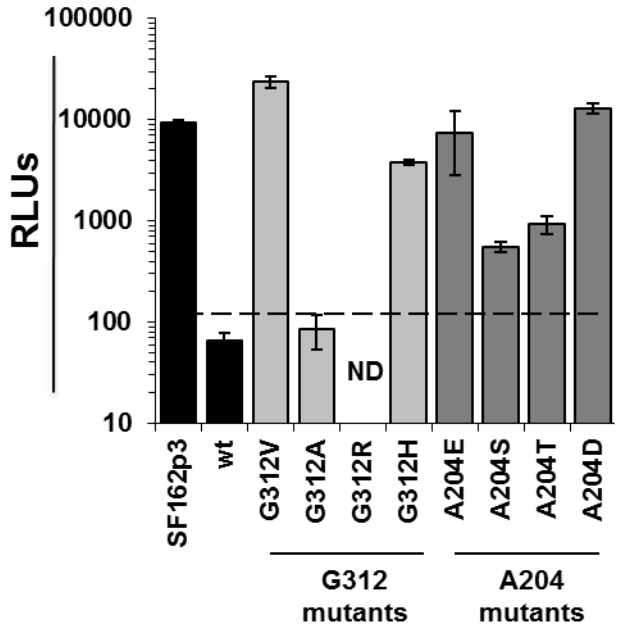

Fig 2.4 Single-cycle infection of Pt lymphocytes with luciferase pseudoviruses bearing Q23-17 Env mutants. The y-axis shows relative light units (RLUs) in cells infected with the virus indicated on the x-axis. The dashed line represents background RLU levels for the experiment observed in cells infected with Env(-) pseudovirus. Error bars represent the standard deviation obtained from triplicate wells. 'wt' refers to the wild-type Q23-17 envelope, and 'ND' is not determined due to insufficient titer. The results are representative of at least two independent experiments.

A204E variants, which were 10 to 20-fold less infectious than wild-type, and the Q259.d2.17 G312V and A204E variants, whose low titers precluded them from use in the assay (data not shown). In all cases where the mutants retained infectivity in human cells, introduction of the G312V and A204E changes increased entry into Pt cells compared to the wild-type Envs. These increases in entry ranged from a 10-fold increase, in the case of the Q259.d2.26 A204E variant, to a >100-fold increase, in the case of the MG505.H3 A204E variant (Fig. 2.5a). Despite the increase in entry, the G312V and A204E variants did not all mediate entry to the same degree as the SF162P3 Env, with levels of entry that were as much as 10-fold less, in the case of Q259.d2.26 (Fig. 2.5a).

To determine whether increases in entry predicted increased replication in Pt lymphocytes, full-length molecular clones of $HIVA_{Q23}/SIV_{vif}$ expressing the BG505.B1 or Q259.d2.26 Envs and their associated A204E and G312V variants were tested. Replication-competent viruses harboring the BG505.B1 Env and its variants were able to establish levels of infection reaching ~300 ng $p24^{gag}$/mL in human PBMCs, similar to those seen previously with $HIVA_{Q23}/SIV_{vif}$. The results in Pt cells were also reminiscent of $HIVA_{Q23}/SIV_{vif}$, with viruses harboring the BG505.B1 G312V and A204E variants reaching peak $p24^{gag}$ levels that were more than 3 logs greater than wild-type (Fig. 2.5b, left panel). However, the results for Q259.d2.26 and its G312V and A204E variants did not follow this trend. The G312V and A204E amino acid changes resulted in a 1.5 to 2 log reduction in $p24^{gag}$ levels in human PBMCs as compared to the virus expressing the wild-type Q259.d2.d26 Env (Fig. 2.5b, right panel). These results were mirrored in Pt lymphocytes, where the G312V and A204E changes did not appreciably increase spreading infection compared to the wild-type virus, reaching maximum $p24^{gag}$ levels of only 1 ng/mL.

Influence of the G312V and A204E amino acid changes on co-receptor usage

To establish if the G312V and A204E amino acid changes were exerting their effects by causing a change in co-receptor usage, Pt lymphocytes were treated with saturating amounts of the CCR5 antagonist TAK779, and infected with luciferase pseudoviruses carrying the G312V and A204E Env variants. Infection mediated by the CCR5-tropic positive control SF162P3 was 100% inhibited by TAK779, and, as expected for a CXCR4-tropic Env, treatment with TAK779 had relatively little effect on infection mediated by the NL-DT5R Env (Table 2.3). Much like SF162P3, entry by the G312V and A204E variants was greatly inhibited (98 – 100%) by TAK779 (Table 2.3), thus indicating that Envs with the G312V and A204E changes continued to require CCR5 as a co-receptor for entry into Pt lymphocytes.

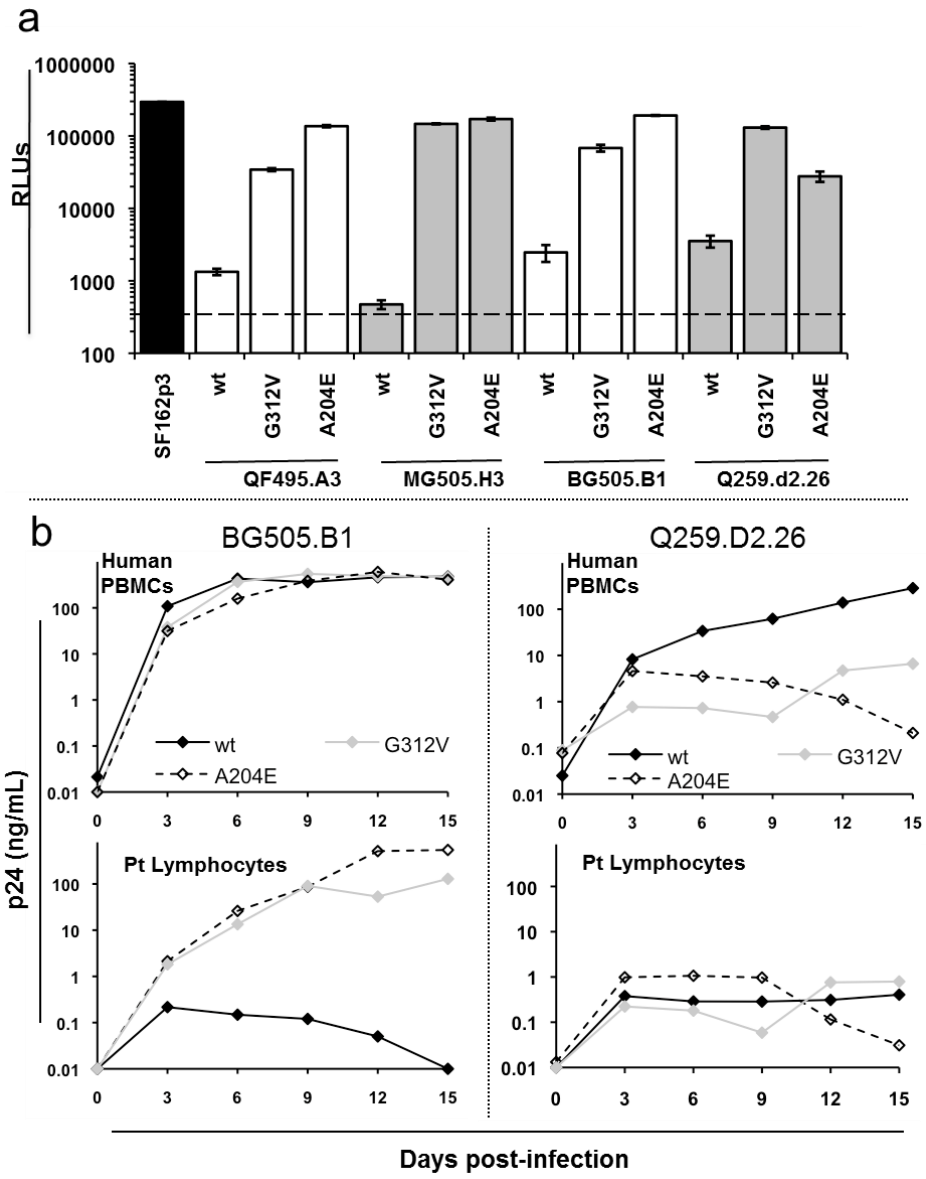

Fig 2.5. Single-cycle and spreading infection of Pt lymphocytes with G312V and A204E Env variants. (*a*) Single-cycle infection of Pt lymphocytes with luciferase pseudoviruses bearing G312V and A204E Env variants. The y-axis shows relative light units (RLUs) in cells infected with the virus indicated on the x-axis. The dashed line represents background RLU levels for the experiment observed in cells infected with Env(-) pseudovirus. Error bars represent the standard deviation obtained from triplicate wells. (*b*) Infection of human PBMCs and Pt lymphocytes with $HIVA_{Q23}/SIV_{vif}$ expressing BG505.B1 (*left panel*) and Q259.D2.26 (*right panel*) Env variants. The p24gag levels are shown as a function of time post-infection in human PBMCs (top graphs) and Pt lymphocytes (bottom graphs). The data points represent the average measurement from duplicate cultures. The figure key is shown in the top plots. 'wt' refers to the wild-type Env to which the G312V or A204E change was introduced. Both a) and b) are representative of three independent experiments.

Table 2.3. Inhibition of G312V and A204E variants by TAK779

Clone	Residue Change	Percent Inhibition[a]
Q23-17	G312V	100 ± 0
	A204E	98 ± 1
QF495.A3	G312V	99 ± 1
	A204E	100 ± 0
MG505.H3	G312V	100 ± 0
	A204E	100 ± 0
BG505.B1	G312V	100 ± 0
	A204E	99 ± 0
Q259.D2.26	G312V	100 ± 0
	A204E	100 ± 0
SF162P3	--	100 ± 0
NL-DT5R	--	15 ± 21

[a]Percent inhibition of luciferase activity in cells treated with 1uM TAK779 compared to untreated cells (avg ± SD for two independent experiments).

Neutralization of the G312V and A204E variants by b12 and sCD4

The b12 monoclonal antibody, which targets an epitope overlapping the CD4-binding site (25, 206), and sCD4 were used to probe differences that the G312V and A204E variants may be causing in Env conformation and interaction with CD4. To do this, IC_{50} of pseudoviruses bearing the wild-type, G312V, and A204E Env variants to b12 and sCD4 was assessed using the TZM-bl cell assay (18). None of the viruses bearing the wild-type Envs were sensitive to neutralization by b12 (IC_{50} > 50 μg/mL), as has been observed previously (199). The G312V and A204E amino acid changes had variable effects on neutralization by b12, depending on the viral context (Table 2.4). For example, the QF495.A3, Q259.d2.26, and MG505.H3 G312V and A204E Env variants were susceptible to neutralization, with IC_{50} values ranging from ~0.6 μg/mL for the QF495.A3 A204E variant to ~8.6 μg/mL for the MG505.H3 G312V variant. Conversely, much like their wild-type counterparts, the G312V and A204E variants of Q23-17 and BG505.B1 were resistant to b12 neutralization. Notably, even those A204E and G312V variants that did display some sensitivity to b12 continue to be less sensitive than SF162P3 which had an IC_{50} of ~0.41 μg/mL (Table 2.4).

The effects of the amino acid changes on sensitivity to sCD4 were more dramatic and consistent. The wild-type Envs were all relatively insensitive to sCD4, with IC_{50}s > 40 μg/mL; compared to SF162P3, which had an IC_{50} of approximately 3.7 μg/mL. Introduction of the G312V and A204E changes rendered each Env highly susceptible to neutralization by sCD4, with IC_{50} values ranging from ~8 μg/mL to < 0.2 μg/mL, the latter representing a greater than 200-fold increase in susceptibility compared to wild-type (Table 2.4). There did not appear to be a correlation between the sensitivities of the G312V and A204E variants to b12 and to sCD4. This was exemplified by the Q23-17 and BG505.B1 variants, which were insensitive to b12, but exquisitely sensitive to sCD4.

Use of Pt CD4 and CCR5 by G312V and A204E variants

The G312V and A204E Env variants were next examined to see if they showed any differences in their ability to mediate entry using Pt CD4 or CCR5. To do this, 293T cells were transiently transfected with CD4 and CCR5 expression plasmids in all possible combinations: huCD4/huCCR5, huCD4/ptCCR5, ptCD4/ptCCR5, and ptCD4/huCCR5 (Fig. 2.6a). In a given experiment, 20-50% of the cells were found to be double positive for CD4 and CCR5 expression, and the levels of expression for the receptors were similar across the four receptor combinations (Fig. 2.6a). The cells were infected with GFP reporter pseudoviruses carrying different Envs, and GFP-positive cells were counted in triplicate wells, with the resulting titers being normalized to the

Table 2.4. Neutralization of G312V and A204E variants by b12 and sCD4

Envelope Clone	Residue Change	IC_{50} (μg/mL) [a]	
		b12	sCD4
Q23-17	--	>50[b]	>50[b]
	G312V	>50[b]	0.8 ± 0.7
	A204E	>50[b]	0.3 ± 0.2[c]
QF495.A3	--	>50[b]	48 ± 2.8[d]
	G312V	1.4 ± 0.1	<0.2[e]
	A204E	0.6 ± 0.0	<0.2[e]
MG505.H3	--	>50[b]	>50[b]
	G312V	8.6 ± 0.4	6.9 ± 2.0
	A204E	1.5 ± 0.4	0.7 ± 0.0
BG505.B1	--	>50[b]	>50[b]
	G312V	>50[b]	<0.2[e]
	A204E	>50[b]	0.3 ± 0.2[c]
Q259.D2.26	--	>50[b]	47 ± 4.5[d]
	G312V	2.0 ± 0.6	0.2 ± 0.1[c]
	A204E	1.8 ± 0.5	<0.2[e]
SF162P3	--	0.4 ± 0.4	3.7±2.1

[a]Expressed as avg ± SD for two experiments unless otherwise noted
[b]IC50s from duplicate experiments were both >50
[c]IC50 value of <0.2 from one experiment was set to 0.1 and avg ± SD was reported
[d]IC50 value of >50 from one experiment was set to 50 and avg ± SD was reported
[e]IC50s from duplicate experiments were both <0.2.

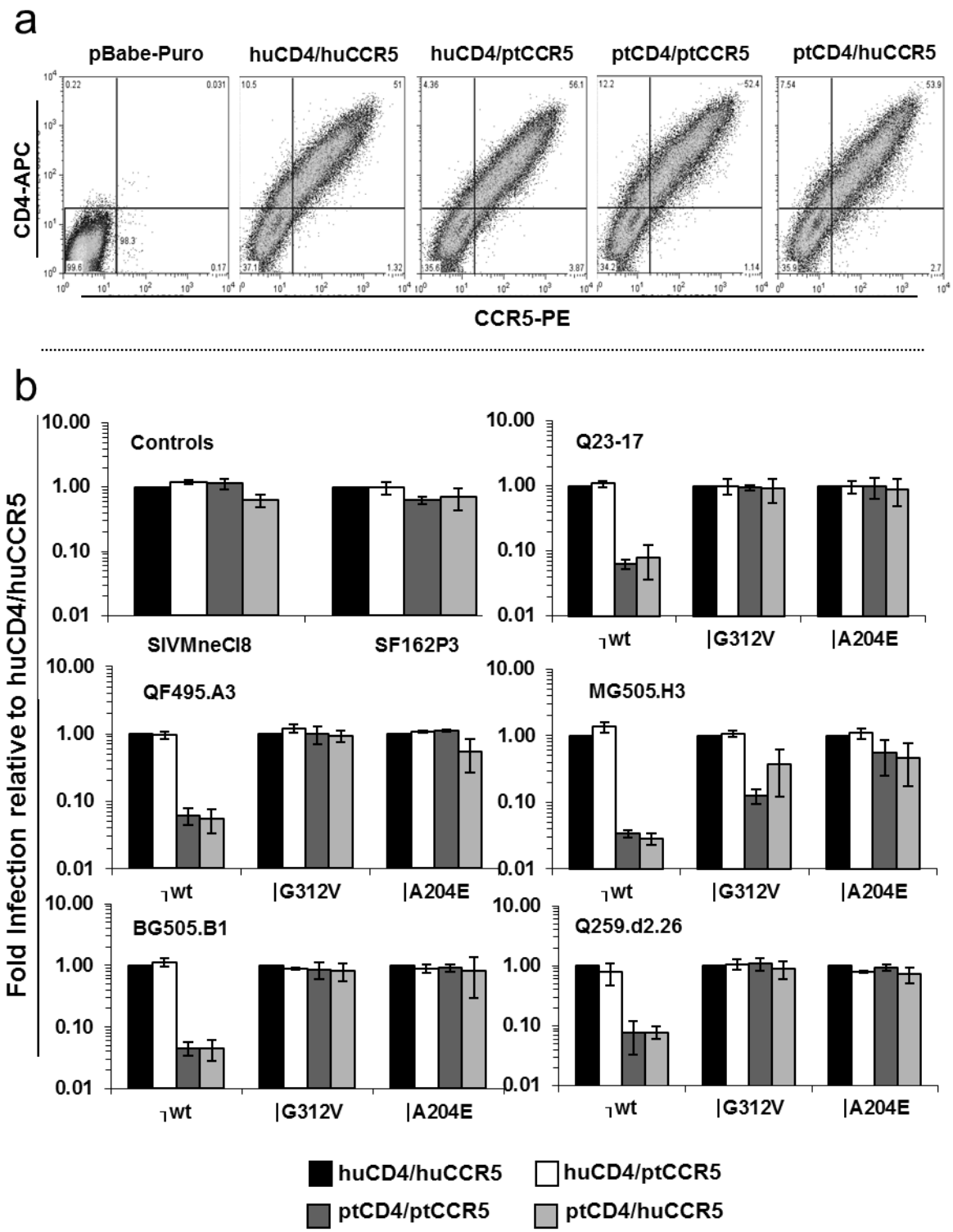
a
pBabe-Puro
huCD4/huCCR5
huCD4/ptCCR5
ptCD4/ptCCR5
ptCD4/huCCR5
CD4-APC
CCR5-PE
b
Fold Infection relative to huCD4/huCCR5
Controls
Q23-17
SIVMneCl8
SF162P3
QF495.A3
MG505.H3
BG505.B1
Q259.d2.26
wt
G312V
A204E
huCD4/huCCR5
huCD4/ptCCR5
ptCD4/ptCCR5
ptCD4/huCCR5

Fig 2.6. Infection of 293T cells expressing hu and pt CD4 and CCR5 with viruses expressing G312V and A204E variants. *(a)* Transient expression of human and Pt CD4 and CCR5 in 293T cells. The y-axis represent CD4 expression and the x-axis represents CCR5 expression as determined by flow cytometry. The CD4/CCR5 combination is denoted at the top of each plot and 'pBabe-puro' refers to cells transfected with the empty pBabe-puro. These plots are representative of three replicate experiments. *(b)* Single-cycle infection of 293T cells expressing human versus Pt CD4 and CCR5 with GFP reporter pseudoviruses bearing wild-type subtype A Envs and G312V and A204E variants. The y-axis represents viral infection relative to the huCD4/huCCR5 combination, which is set to 1 for reference. The name of the parental virus is indicated at the top of each plot, and the amino acid change is indicated on the x-axis ('wt' refers to the wild-type Env variant). In general 20-300 GFP-positive cells were enumerated per well, depending on the dilution, the virus and the CD4/CCR5 pair. No GFP-positive cells were observed when cells were infected with Env(-) pseudoviruses (not shown). Three replicates were performed, and each replicate included all of the receptor pairings with the exception of one in which the ptCD4/huCCR5 pair was not included. The final replicate for the ptCD4/huCCR5 pair was later performed in a separate experiment. The error bars represent the standard deviation of the mean from all of the data amassed in the replicate experiments.

huCD4/huCCR5 combination. Viruses bearing the SF162P3 Env, as well as those bearing the Env from SIV Mne CL8, a molecular clone that establishes persistent infection in Pts (135), were used as positive controls. These positive control viruses were able to infect cells expressing any of the four combinations of CD4 and CCR5 to comparable levels (Fig. 2.6b).

Viruses carrying each of the 5 wild-type Envs infected cells expressing PtCCR5 to similar levels as those expressing huCCR5, but showed a 10 to 30-fold decrease in infection in cells expressing ptCD4 versus cells expressing huCD4 (Fig. 2.6b). The G312V and A204E Env variants were also unchanged in their ability to mediate infection using ptCCR5 compared to huCCR5, but mediated increased levels of infection via ptCD4 by as much as 50-fold, typically reaching the levels of infection that were comparable to those in cells expressing huCD4 (Fig. 2.6b). An exception to this was the MG505.H3 G312V variant, which increased PtCD4-mediated entry by as little as 5-fold, still ~10-fold lower than entry mediated by huCD4. Overall, however, these findings suggested that the subtype A Envs used in this study were limited in their capacity to infect cells expressing ptCD4 and that the G312V and A204E increased the efficiency with which the Envs are able to utilize ptCD4.

Expression of CD4 on immortalized Pt lymphocytes

To determine whether differences in the level of CD4 expression may contribute to differences in infectivity of the adapted $HIVA_{Q23}/SIV_{vif}$ variants in Pt versus human lymphocytes, the expression of CD4 in the immortalized Pt lymphocytes was compared to the expression of CD4 in primary Pt PBMCs and in human PBMCs. Expression of CD4 was equivalent between all of the cells types (Fig 2.7a), suggesting that differences in infectivity cannot be explained by differences in cell surface CD4 levels. There are differences in amino acid sequence, between the human and Pt CD4 molecules, including in regions that play a role in envelope binding, as shown in Fig. 2.7b.

Discussion

In this chapter, the limited ability of subtype A Envs to use PtCD4 for entry was identified as a major barrier to the replication of SHIV-As in macaque cells. A minimal SHIV-A, $HIVA_{Q23}/SIV_{vif}$, replicated poorly in Pt lymphocytes, but variants displaying increased replication in Pt lymphocytes were selected after long-term culturing. Two independent adaptive amino acid changes in Env, G312V and A204E, conferred more efficient entry into Pt cells when introduced into the Q23-17 parental Env. These same changes conferred high levels of replication in Pt lymphocytes when

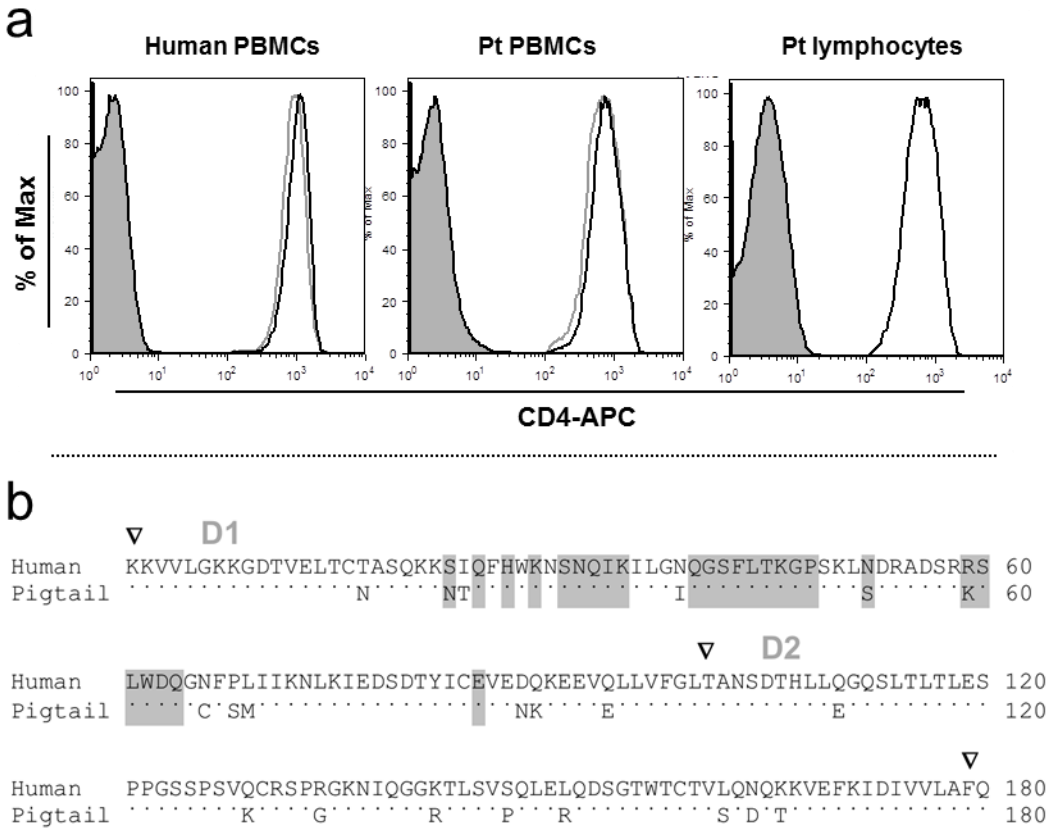

Fig. 2.7 Comparison of huCD4 and ptCD4 expression in lymphocytes and sequence alignments. *(a)* CD4 expression on human PBMCs, Pt PBMCs and immortalized Pt lymphocytes. Live cells were gated on CD4 expression and CD4-positive cells are shown in the histogram. Shaded curves represent unstained cells and, in the PBMC expression profiles, the black and grey curves represent profiles from two different donors. *(b)* Amino acid alignment of the D1 and D2 domains of human and Pt CD4. Vertical lines above the alignment denote the beginning and end of the D1 and D2 domains. Dots indicate conserved residues, and positions where the Pt and human sequence differ are shown as letters. The residues that are highlighted in grey have previously been found to be implicated in gp120 binding (5).

introduced into the parental $HIVA_{Q23}/SIV_{vif}$ proviral clone. Importantly, increased entry was also observed when either of these changes was independently introduced into multiple subtype A Envs, suggesting that these amino acid positions have a general impact on the interactions between subtype A Env and PtCD4. Env variants encoding G312V and A204E maintained CCR5-tropism, but were much more sensitive to neutralization by sCD4 and mediated more efficient entry by PtCD4. These findings implicate Env/CD4 interactions in the restriction of SHIV-A replication in macaque cells, and provide insight into specific amino acid positions in gp120 that can enhance these interactions.

Prior studies with subtype B minimal SHIVs suggested that the inclusion of the *vif* gene from $SIV_{mac}239$ in $HIVA_{Q23}/SIV_{vif}$ would be sufficient for the virus to evade APOBEC3-mediated restriction, thus allowing for replication in Pt cells (70, 188). However, $HIVA_{Q23}/SIV_{vif}$ replicated poorly in immortalized Pt lymphocytes, in contrast to NL-DT5R, a CXCR4-tropic minimal SHIV that encodes both the *vif* gene and the CypA-binding loop of $SIV_{mac}239$ (88), which achieved high levels of replication. Similar results were observed when these SHIVs were used to infect primary Pt lymphocytes (data not shown). The SIV CypA binding loop is required to evade Rh TRIM5α, but should not be necessary for replication in Pt lymphocytes because the capsid-directed TRIM5 isoforms and TRIMCyp expressed by Pts do not restrict HIV-1 (70, 107, 188, 193). Indeed, the lack of restriction to the HIV-1 capsid in Pt cells was further verified here using chimeras between NL-DT5R and $HIVA_{Q23}/SIV_{vif}$, which showed that the $HIVA_{Q23}/SIV_{vif}$ capsid supported replication in Pt lymphocytes. Instead, the chimeras demonstrated that it was the 3‘ portion of $HIVA_{Q23}/SIV_{vif}$, including *tat*, *rev*, *vpu*, and *env*, that limited the replication of the SHIV-A in Pt lymphocytes. These findings were consistent with previous studies using traditional SHIVs encoding the 3’ elements of HIV-1, which implicated *env* as the main determinant for infection of macaque cells (78). The identification of the Q23-17 Env as the reason for reduced replication in Pt cells was definitively shown when mutations encoding the G312V or A204E amino acid changes to gp120, which were identified in viruses selected for increased replication in immortalized Pt lymphocytes, were introduced into the parental clone and the resulting viruses replicated in immortalized Pt lymphocytes to levels that were comparable to NL-DT5R. Viruses bearing the G312V or A204E mutations also showed increased replication compared to the parental virus in primary Pt PBMCs, but the differences were much more modest than those observed in immortalized CD4 lymphocytes, perhaps reflecting the fact that CD4/CCR5 positive lymphocytes comprise only a fraction of the PBMCs.

The increases in entry into Pt lymphocytes by the G312V and A204E variants in luciferase reporter assays were not necessarily directly correlated with increases in virus spread, as exemplified by the G312V change in the context of Q23-17 and BG505.B1. The G312V changes conferred similar increases in entry in the single-cycle reporter assay as the A204E mutants, yet in the viral replication assays, peak replication levels were much lower for a full-length clone encoding the Q23-17 G312V Env as compared to one encoding the BG505.B1 G312V Env. The same immortalized Pt lymphocytes were used for both the single cycle and replication assays, ruling out the possibility of differences in cell targets. This suggests that differences in infectivity may be due to differences in how the viruses were generated. Depending on the Env being expressed, pseudoviruses have been shown to express artificially high amounts of Env, both in the processed and unprocessed forms (73), and this can result in higher levels of infectivity compared to replication competent virus (144).

The G312 residue, located at the tip of the V3 loop, and the A204 residue, located adjacent to the β3 strand of the bridging sheet, are in regions of gp120 that are known to mediate interaction with the co-receptor (84, 151). However, the increased ability of the G312V and A204E variants to infect Pt cells was not due to a change in co-receptor use or an increased ability to mediate infection by PtCCR5. Surprisingly, subtype A Envs were deficient in their ability to mediate infection of cells expressing PtCD4, and the G312V and A204E amino acid changes rescued this deficiency. Flow cytometric analyses demonstrated that the differences in infectivity could not be explained by differences in cell-surface CD4 expression. These results argue that the barrier to $HIVA_{Q23}/SIV_{vif}$ replication in Pt lymphocytes was due to inefficient use of PtCD4, and that the G312V and A204E changes arose to circumvent this barrier.

A notable property of the G312V and A204E variants was their increased sensitivity to sCD4, with both mutations conferring increased sensitivity to sCD4 in all Envs tested, in most cases, by more than 100-fold. This finding suggests that sensitivity to sCD4 may predict how well a particular envelope uses PtCD4 for entry. If this is the case, then most circulating HIV-1 Env variants, which tend to be relatively insensitive to sCD4 [e.g. (18, 105)], may not promote efficient entry via PtCD4. However, these findings also raise the interesting possibility that sensitivity to sCD4 may provide a means to identify representative transmitted Env variants that would be the best candidates for a successful SHIV. In support of this hypothesis, it is significant that many of the existing CCR5-tropic SHIVs incorporate HIV-1 Envs that are relatively sensitive to soluble CD4, including the SF162 lineage (35, 93), ADA (183, 184), BaL (47) and the subtype C isolate HIV2873i (172).

One model that may explain how changes to G312 and A204 are eliciting their effect is by increasing exposure of the CD4-binding site, which could then allow for better usage of PtCD4. G312 is found in the V3 loop, which has been shown to be a determinant for increased sensitivity to sCD4 (85, 131, 182), implying that changes to the V3 loop may participate in quaternary interactions in Env that could lead to a more exposed CD4-binding site. A204 is adjacent to the highly conserved C205 residue, whose replacement has been shown to increase the susceptibility of the HIV-1 Env to sCD4, presumably by abrogating a highly conserved disulfide bond, resulting in a more open Env conformation (190). It is possible that changes to A204, particularly the introduction of a negative charge as in the A204E and A204D variants, may also be serving to modulate this disulfide bond or may be opening up the Env structure via other steric interactions. The increased exposure of the CD4-binding site in the G312V and A204E variants is further supported by the sensitivity that some of the variants display to the b12 monoclonal antibody, whose epitope overlaps the CD4-binding site (25, 206). The high degree of conservation of the G312 and A204 residues may indicate that they play some role in maintaining the structural integrity of the envelope trimer. This may explain why the G312V/A204E double mutant, and, in some contexts the single G312V or A204E changes (for example, in the case of the Q259.D2.17 Env) result in an envelope that does not support efficient entry or viral replication**.**

Little is known regarding how differences in macaque and human CD4 impact the ability of HIV-1 Envs to mediate infection of macaque cells, although one study has concluded that pt CD4 was not a barrier to HIV-1 infection (61). The lab-adapted LAI IIIb strain of HIV-1 was used in this previous study, and these results are consistent with observations that CXCR4-tropic subtype B SHIVs are infectious in macaque cells. The differences between this study and the data presented here most likely reflect differences in the biology of lab-adapted strains compared to CCR5-tropic variants cloned directly from infected individuals, and serves as a reminder of the difficulty of extrapolating findings with lab-adapted HIV variants to more relevant, circulating strains of HIV-1.

The primary determinants of HIV Env-CD4 interaction have been mapped to the D1 and D2 domains of human CD4, and human and PtCD4 differ at 17 amino acid positions in these domains. Structural studies indicate that F43 and R58 from CD4 form contacts with gp120 (97). There is an amino acid change at R58 (K in Pt) that could alter Pt CD4-Env interactions, but it is a conservative amino acid difference. There are other, non-conservative amino acid differences at S23 (N in Pt) and N52 (S in Pt). Although these residues have not been recognized as contact residues in structural analyses, they have been identified in mutagenesis studies as being critical for gp120 binding (5).

Thus, disruption of CD4-gp120 binding, either by modulation of direct amino acid interactions, or indirectly, through effects on binding affinity, may explain the differences in HIV-mediated entry between human and PtCD4 observed here.

Prior studies have shown that SHIV-As were not infectious in Rh cells (78), and our findings may provide an explanation for these results. The published sequence of Rh CD4 is identical to PtCD4 at each of the four residues noted above (data not shown). Further studies are needed to determine whether Rh CD4 presents a similar barrier to infection as PtCD4 and whether amino acid changes at positions A204 and G312, which are at highly conserved across all subtypes, can overcome this barrier. Such studies may also help define the precise changes that alter HIV Env/macaque CD4 interactions.

The usefulness of SHIV models, particularly as tools to examine the biology of HIV-1 transmission and strategies to prevent infection, will depend on how well they mimic HIV-1 transmission and early infection in humans. There are numerous barriers to HIV-1 infection in macaques, and SHIV proviruses tested to date have required further adaptation in the animal to increase replication. The improved understanding of host restriction factors has permitted more targeted approaches to developing infectious SHIVs although the initial chimeric viruses did not replicate to high levels in infected animals (70, 71, 88). Here, we identify differences in CD4 as a barrier to HIV-1 infection of pig-tailed macaque cells, and show that a single amino acid change in Env is sufficient to surmount this limitation, at least for subtype A Envs. These data indicate it may be possible to develop SHIVs that are derived from more relevant HIV-1 variants with only minor modifications. However, in using these findings to develop more relevant SHIV models, it will be important to consider how changes that permit efficient CD4 interaction impact other key biological properties of the envelope, such as sensitivity to neutralization. Identifying inefficient use of CD4 as a barrier to HIV-1 infection of macaque cells provides a critical first step in the process of designing SHIVs based on biologically relevant HIV-1 variants.

Chapter 3

Replication of Simian/Human Immunodeficiency Viruses expressing A204E and G312V subtype A envelope variants

Introduction

To date, attempts to construct simian/human immunodeficiency viruses (SHIVs) expressing subtype A HIV-1 envelopes (Envs) have been unsuccessful, as these SHIVs fail to establish infection in macaque cells (78). The finding that the introduction of the A204E and G312V amino acid changes to subtype A envelopes (Envs) increased their ability to mediate infection of pig-tailed macaque (Pt) cells raised the possibility that one or more of these Env variants may be used to form the basis for a subtype A-based SHIV (SHIV-A) for use in *in vivo* studies. In this chapter, the replication properties of SHIVs expressing subtype A A204E and G312V Env variants in human and macaque cells are described, with an aim of identifying SHIV-As that warrant further development *in vivo*.

All of the replication studies described in Chapter 2 were carried out in the context of the minimal SHIV $HIVA_{Q23}/SIV_{vif,}$ a virus derived from the CCR5-tropic subtype A Q23-17 HIV-1 strain and encoding the Vif protein from $SIV_{MAC}239$. Unfortunately, the minimal SHIVs studied to date are poorly infectious in Pts. Infection of Pts with minimal SHIVs is not pathogenic and results in an extremely low viral set-point ($10^2 – 10^3$ RNA copies/mL) and the emergence of pathogenic viruses has so far not been observed, thus limiting their usefulness in animal models (70, 188). In contrast, a number of traditional SHIVs, encoding the HIV-1 *tat*, *rev*, *vpu*, and *env* genes in the context of $SIV_{MAC}239$ have been established as pathogenic challenge viruses for use in Pts (66, 130, 136). Thus, a traditional SHIV expressing the subtype A A204E and G312V Env variants may result in the more rapid development of a pathogenic SHIV-A for use *in vivo*. Furthermore, the traditional SHIV architecture has the advantage that it encodes the SIV capsid, and so eludes recognition by rhesus macaque (Rh) TRIM5α, thereby expanding the potential tropism beyond Pts to Rhs, which are the most widely-used and defined macaque model for HIV/AIDS research (7).

Because of the perceived benefits of traditional SHIVs, the A204E and G312V Env variants were assessed for their ability to mediate replication in human and macaque cells when expressed in the context of a traditional SHIV. It should be noted that the Q259.d2.26 Env variants were excluded from this evaluation, given their poor performance at establishing spreading infection in the context

of minimal SHIVs. The CCR5-tropic SHIV AD8 was used in this chapter as a basis of comparison for replication in different cell types (130). SHIV AD8 has been used as the source for the generation of pathogenic SHIV lineages that result in sustained high levels of viremia (> 10^7 RNA copies/mL), depletion of CD4+ T lymphocytes and induction of AIDS (130), and so, provides a benchmark of replication to which the SHIV-As described herein can be compared.

The major goal of this chapter is to identify candidate SHIV-As whose further *in vivo* development might result in a pathogenic model whose neutralization properties are relevant to human disease. Thus, as an additional criterion, the A204E and G312V Env variants were further evaluated for their sensitivity to neutralization by polyclonal antibodies. Ideally this would allow for the identification of A204E and G312V Env variants that retain neutralization characteristics of Envs from circulating subtype A strains in addition to allowing for robust infection of macaque cells in the setting of a traditional SHIV.

Materials and Methods

Cells

HEK 293T cells, TZM-bl cells, immortalized Pt lymphocytes, and human peripheral blood mononuclear cells (PBMCs) were maintained as described in Chapter 2.

Primary Rh PBMCs were isolated by Ficoll gradient from heparinized whole blood obtained from the University of Washington Non-Human Primate center according to the Milltenyi Biotec sample preparation protocol (http://www.miltenyibiotec.com). Briefly, whole blood was diluted 4X in PBS (Gibco) and 35 mL of the diluted whole blood was carefully layered over 15 mL of 96% Ficoll (Invitrogen, diluted with PBS) and centrifuged without brakes at 400×*g* at 20°C for 40 minutes. The buffy layer was carefully removed into a new tube and washed 3 times in 50 mL of PBS with the cells pelleted at 300×*g* and 20°C for 10 minutes for the first wash, and 200×*g* for the final two washes to remove any contaminating platelets. The cells were then activated with 4 U of phytohemagglutinin M (PHA-M)/mL at a cell density of 3×10^6 cells/mL in RPMI 1640 medium with 25 mM HEPES, 20 % heat-inactivated FCS, 2 mM L-glutamine, 100 U of penicillin/ml, 100 µg of streptomycin/ml, and 100 U/mL of recombinant human interleukin-2 (Roche). After approximately 40 hours, the cells were washed 3 times in media and maintained without PHA-M at 2×10^6 cells/mL.

Cloning of Env variants into pEOmacUCK YE vector

The pEOmacUCK YE plasmid (kindly provided by Dr. Malcolm Martin) encodes a full-length 'traditional' SHIV genome encoding the *tat*, *rev*, *vpu*, and *env* genes from the R5-tropic AD8 (130), and the virus it encodes is referred to throughout as SHIV AD8. The viral genome has been further modified to allow easy insertion of the region spanning the *vpu* and *env* open reading-frames from different HIV-1 isolates by introduction of an EcoRI site 5' of the *vpu* start codon and a SalI restriction site immediately 3' of the *env* stop codon (Drs. Malcolm Martin and Masashita Shingai, personal communication). Furthermore, the *nef* gene contains the R17Y and Q18E polymorphisms which increase infectivity of SHIV AD8 in inactivated lymphocytes (50).

Expression plasmids encoding the Q23-17, BG505.B1, MG505.H3, and QF495.A3 A204E and G312V *env* variants were described in Chapter 2. The ~2.7 kb region encoding the *vpu* and *env* open-reading frames was amplified using 25 ng of each of the above plasmids as templates and the primers detailed in Table 3.1 under the following reaction conditions: 95°C for 5 min, followed by 25 cycles of 95°C for 30s, 55°C for 1 min, and 68°C for 3 min; and a clean-up step at 68°C for 7 min. The resulting amplicons were gel purified using the Qiagen QIAquick Gel Isolation Kit, digested with EcoRI and SalI and ligated into the pEOmacUCK YE SHIV plasmid.

The ligated products were transformed into One-Shot Stbl3 Chemically Competent *E. Coli* (Invitrogen) according to the manufacturer's protocol, and plated on LB-agar plates supplemented with 50 µg/mL carbenicillin. In order to reduce recombination of the full-length plasmid, the bacteria were plated and maintained at 30°C for 24 hours before further screening. Only smaller colonies were screened for the presence of the proper insert. Clones containing the proper insert were sequenced throughout the *vpu* and *env* open reading-frames to ensure that no undesired changes had occurred, and all further plasmid preparations were carried out with bacteria grown at 30°C. The resulting plasmids and the SHIVs they encode were named as described in Table 3.1.

Virus production

Viruses were produced by transfecting 293Ts seeded in T75 tissue culture flasks with Fugene 6 (Roche) as described in Chapter 2. Viral supernatants were harvested 72 hours post-transfection, filtered through a 0.22 µm filter (Millipore Corporation), concentrated 50 to 100-fold using Amicon Ultra 10K filters (Millipore Corporation), and titred on TZM-bl cells.

Table 3.1 Primers used to amplify subtype A *envs* for cloning into pEOmacUCK YE

Virus	Envelope clone	Residue Change[a]	Forward primer sequence[b]	Reverse primer sequence[c]
SHIV Q23	Q23-17	--	ATATATGAATTCGTAAATGTAATGTCTCCTTTGGAATCAGTGCAATAG	ATTTATGTCGACTTATAGCAAAGCCCTTTCTAAGCCCTG
SHIV Q23 A204E		A204E		
SHIV Q23 G312V		G312V		
SHIV BG505 A204E	BG505.B1	A204E	ATATATGAATTCGTAAATGTAATGTAATGCTTCCTTTGGAAATTTGGGGAATAG	AAAATAGTCGACTTATAGCAAAGCCCTTTCGAAGCCC
SHIV BG505 G312V		G312V		
SHIV MG505 A204E	MG505.H3	A204E	ATATAGAATTCGTAAATTCGTAAATGCTTCCTTTGGGAATTTGGGCAATAG	ATATTAGTCGACTTATAGCAAAGCCCTTTCGAAGCCTG
SHIV MG505 G312V		G312V		
SHIV QF495 A204E	QF495.A3	A204E	ATATATGAATTCGTAAATGTAATGTCTCCTTTAGAAATCTGGGCAATAG	ATTTTAGTCGACTTATAGCAAAGCCCTTTCAAAGCCCTG
SHIV QF495 G312V		G312V		

[a]'--' indicates that no residue change is present

[b]The sequence is in a 5' to 3' orientation, the underlined portion represents the EcoRI site used for cloning, note that line breaks were used for space reasons

[c]The sequence is in a 5' to 3' orientation, the underlined portion represents the SalI site used for cloning, note that line breaks were used for space reasons

Virus replication assays

Virus replication assays were performed as described in Chapter 2 with some exceptions. Firstly, due to the low titer of some viruses, infections were performed on either 3×10^6 stimulated donor human PBMCs, 2×10^6 Rh PBMCS, or 1×10^6 immortalized Pt lymphocytes at a reduced multiplicity of infection of 0.01 in a total volume of 1 mL in 24-well dishes. Infected cells were then washed 3 times in 1.5 mL of media and split into two 600 µL cultures, which were maintained in a 48-well dish. Secondly, unlike in Chapter 2, the levels of replication were reported as infectious particles (IP)/mL as determined by titration on TZM-bl cells as opposed to p24 ELISAs to allow for direct comparison between 'minimal' and 'traditional' SHIVs.

Virus Inhibition and Neutralization assays

Virus inhibition and neutralization assays were performed with TAK779, enfuvirtide (T20, obtained from the NIAID Reagents sharing program) and plasma from HIV-1 infected donors as described in Chapter 2, with some exceptions. In the case of TAK779, cells were pre-incubated with serial 3-fold dilutions of TAK779, beginning at 0.3 µM, for 1 hour prior to the addition of virus. For T20, viruses were pre-incubated for 1 hour with serial 3-fold dilutions of T20, beginning at 10 µg/mL, prior to the addition of TZM-bl cells. In the case of pooled (18) and autologous plasma, the plasma was first heat-inactivated at 56°C for 1 hour, pulse centrifuged at top speed (16 000 rpm) for 30 s and frozen at -80°C before use. Two-fold dilutions of heat inactivated plasma, starting from a 1:50 dilution, were incubated with virus for 1 hour prior to the addition of TZM-bl cells. For plasma, the median inhibitory concentrations (IC_{50}s) was defined as the reciprocal of the plasma dilution resulting in 50% inhibition of TZM-bl β-galactosidase activity as calculated using the linear fit model.

Results

Replication of SHIVs bearing subtype A A204E and G312V Env variants

To examine if the A204E and G312V changes continued to have the effect of increasing replication in Pt lymphocytes in the setting of traditional SHIVs, we first constructed traditional SHIVs expressing the wild-type Q23-17 Env, as well as the Q23-17 A204E and G312V Env variants. To determine if changing the context of Env expression from a minimal SHIV to a traditional SHIV architecture had an effect on viral replication, replication of these traditional SHIVs was compared to the minimal SHIV $HIVA_{Q23}/SIV_{vif}$ A204E, which replicates to high levels in immortalized Pt

lymphocytes and human PBMCs (Chapter 2). As a further benchmark for replication, these SHIVs were also compared to SHIV AD8, which expresses the CCR5-using AD8 Env in the same context as the subtype A Envs examined in this chapter, and has been used as the basis for a SHIV that establishes *in vivo* infection in rhesus macaques (130).

In immortalized Pt lymphocytes, traditional SHIVs expressing the Q23 A204E and Q23 G312V Env variants replicated to peak levels of infection of 4.4×10^6 and 4.8×10^6 IP/mL at day 15 post-infection, respectively. In comparison, SHIV Q23 replicated poorly in immortalized Pt lymphocytes, reaching peak levels of only $<1\times 10^2$ IP/mL, representing a $>4\times 10^4$-fold decrease in infection compared to SHIVs expressing the Q23-17 A204E and G312V Env variants. Thus, in the setting of traditional SHIVs, the A204E and G312V changes continue to have the effect of increasing infection in Pt lymphocytes. The levels of infection achieved by traditional SHIVs expressing the Q23 A204E and G312V Env variants were comparable to the minimal $HIVA_{Q23}/SIV_{vif}$ A204E, which reached levels of replication of 5.0×10^6 IP/mL at day 12 post-infection. This data suggests that the Q23 A204E and G312V Env variants continue to mediate high levels of infection in immortalize Pt lymphocytes when expressed in the context a traditional SHIV. Nonetheless, the levels of replication reached by the SHIV Q23 variants were approximately 13 to 15-fold lower than the levels of replication achieved by SHIV AD8, which reached levels of replication of 6.5×10^7 IP/mL at day 6 post-infection (Fig 3.1a and Table 3.2).

The levels of replication of traditional SHIVs expressing the Q23-17 Env variants in primary human PBMCs were much lower than in immortalized Pt cells. Traditional SHIVs expressing the Q23 A204E Env variant and the Q23 G312V Env variant reached levels of only 3.8×10^2 and 1.5×10^3 IP/mL, representing a $>10^4$-fold decrease in replication compared to $HIVA_{Q23}/SIV_{vif}$ A204E, which achieved levels of replication of 6.4×10^6 (Fig 3.1a,b and Table 3.2). However, SHIV Q23 replicated to levels of 5.3×10^4 IP/mL in human PBMCs, representing an increase in replication of 35 and 140-fold compared to the G312V and A204E variants, respectively, suggesting that this decrease in replication in primary human PBMCs was caused in part by the A204E and G312V changes. Thus, traditional SHIVs expressing the Q23-17 Env and the Q23-17 A204E and G312V Env variants maintained relatively high levels of replication in immortalized Pt lymphocytes, however there was a marked reduction in replication in human PBMCs.

In order to determine whether traditional SHIVs expressing other subtype A A204E and G312V Env variants would show increased ability to infect immortalized Pt lymphocytes and human

PBMCs compared to SHIVs expressing the Q23 A204E and G312V Env variants, traditional SHIVs expressing the BG505.B1, MG505.H3, and QF495.A3 G312V and A204E Env variants were constructed. Traditional SHIVs carrying these additional subtype A A204E and G312V Env variants also replicated to relatively high levels in immortalized Pt lymphocytes, ranging from 2.4×10^6 IP/mL, for SHIV BG505 G312V, to 1.2×10^7 IP/mL, for SHIV QF495.A3 A204E. Much like with the Q23-17 A204E and G312V Env variants, SHIVs expressing these additional Env variants reached peak levels of replication with slower kinetics than SHIV AD8, peaking on days 9-17 post-infection. The only exception was SHIV QF495 A204E, which was comparable to SHIV AD8, reaching peak levels of replication by day 6 post-infection (Fig 3.1e and Table 3.2).

Traditional SHIVs expressing the additional subtype A A204E and G312V Env variants displayed a range in abilities to replicate in human PBMCs (Fig 3.1c-e and Table 3.2). Much like traditional SHIVs expressing the Q23-17 Env variants, SHIVs QF495 G312V, BG505 A204E, and BG505 G312V reached relatively low levels of peak infection, ranging from only 1×10^4 IP/mL to 1.5×10^4 IP/mL. However, SHIVs MG505 G312V, MG505 A204E, and QF495 A204E were able to achieve relatively high levels of replication in human PBMCs, ranging from 2.5×10^6 IP/mL for MG505 G312V, to 4.9×10^6 IP/mL for QF495 A204E. These levels of replication compared favorably to SHIV AD8, which reached levels of replication of 9.0×10^6 IP/mL in human PBMCs, albeit with faster replication kinetics, reaching peak replication at day 6 post-infection, compared to SHIV MG505 G312V, SHIV MG505 A204E, and SHIV QF495 A204E, which peaked between days 15 and 18 post-infection. Thus, traditional SHIVs expressing some of the A204E and G312V subtype A Env variants are able to establish high levels of replication in both immortalized Pt lymphocytes and primary human PBMCs.

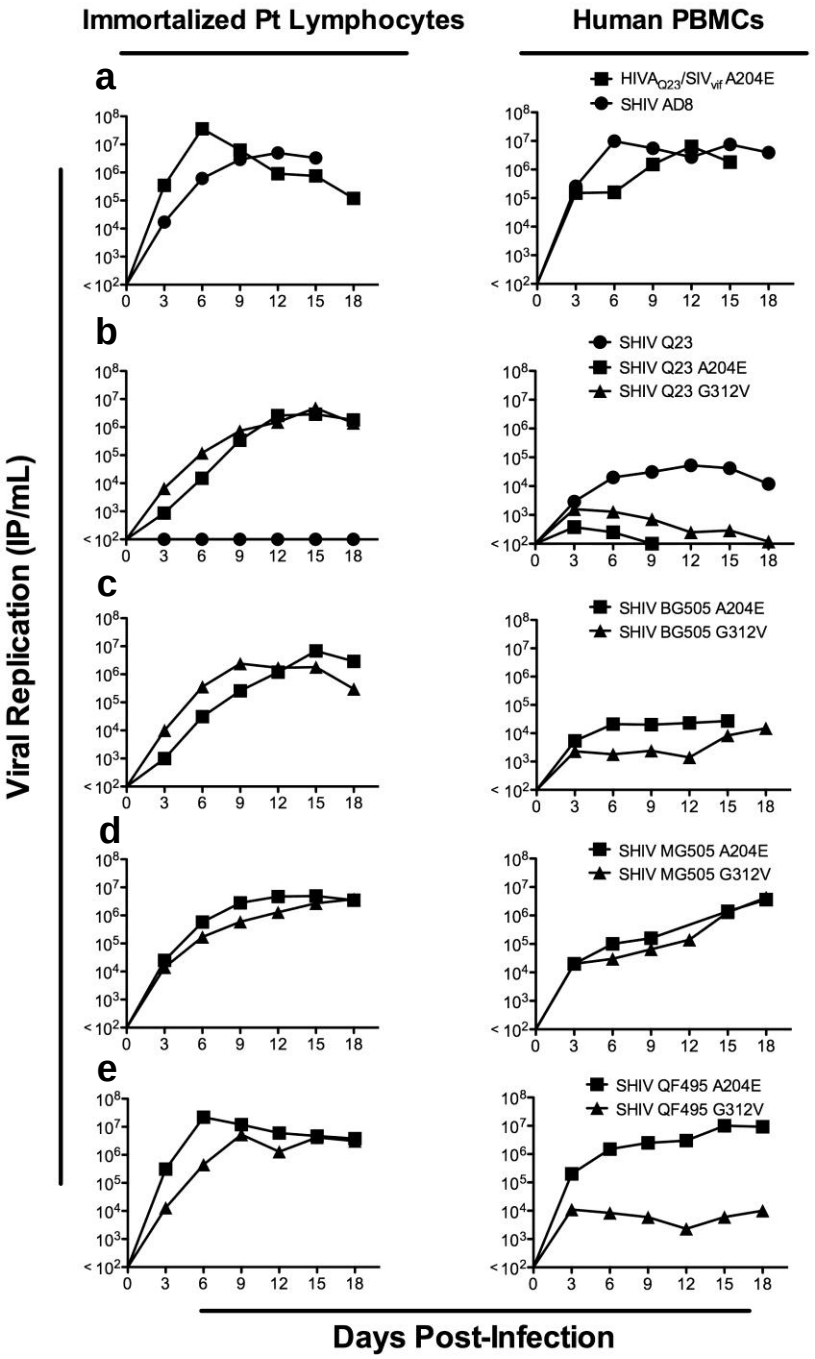

Fig. 3.1 Infection of human PBMCs and immortalized Pt lymphocytes with traditional SHIVs expressing A204E and G312V Env variants. Infectious particles (IP)/mL are shown as a function of time post-infection in immortalized Pt lymphocytes (left panels) and human PBMCs (right panels). The different growth curves have been separated into multiple graphs (*a* to *e*) to aid interpretation, and the legends for each set of graphs are located on the right-hand graphs of the figure. The data points represent the average measurement from duplicate infected cultures. The results are representative of two independent experiments.

Table 3.2 Replication of traditional SHIVs expressing A204E and G312V subtype A Env variants in human PBMCs and immortalized Pt lymphocytes

Virus	Human PBMCs		Immortalized Pt lymphocytes	
	Peak replication[a] (IP/mL)	Peak Day[b]	Peak replication[a] (IP/mL)	Peak Day[b]
$HIVA_{Q23}/SIV_{vif}$ A204E	6.4×10^6	12	5.0×10^6	12
SHIV AD8	9.0×10^6	6	6.5×10^7	6
SHIV Q23	5.3×10^4	6	$<1.0 \times 10^2$	--
SHIV Q23 A204E	1.9×10^2	3	4.4×10^6	15
SHIV Q23 G312V	1.5×10^3	3	4.8×10^6	15
SHIV BG505 A204E	1.3×10^4	15	6.7×10^6	15
SHIV BG505 G312V	1.5×10^4	18	2.4×10^6	9
SHIV MG505 A204E	3.5×10^6	18	4.9×10^6	15
SHIV MG505 G312V	2.5×10^6	17	6.3×10^6	17
SHIV QF495 A204E	4.9×10^6	15	1.2×10^7	6
SHIV QF495 G312V	1.0×10^4	17	3.7×10^6	12

[a] Average (from two independent experiments) peak replication of the virus.
[b] Average day on which the virus reached peak replication (from two independent experiments).
[c] n.d., not determined.

One advantage of expressing the A204E and G312V subtype A Envs in the context of traditional SHIVs is the potential use for these SHIVs in Rh models. Thus, we also examined the ability of the traditional SHIVs expressing the subtype A A204E and G312V Env variants to replicate in primary Rh PBMCs. In these experiments, viral replication was only measured for 3 days because, in our hands, primary Rh PBMCs did not maintain cell division or sustain viral replication beyond this time. Because of this limited time-course, the data in Table 3.3 compares virus levels at day 3 post-infection in human PBMCs, immortalized Pt lymphocytes, and Rh PBMCs. SHIV QF495 A204E achieved the highest levels of replication among SHIVs expressing the subtype A Env variants in Rh PBMCs, reaching 1.4×10^5 IP/mL. These levels of replication were comparable to SHIV AD8, which replicated to 1.1×10^5 IP/mL in Rh PBMCs. SHIVs expressing other subtype A Env variants displayed a lower ability to mediate infection of Rh PBMCs ranging from only 6.4×10^2 IP/mL, for SHIV Q23 A204E; to 9.1×10^3 IP/mL, for SHIV MG505 G312V. Thus, traditional SHIVs expressing subtype A Env variants displayed a range of abilities to infect Rh PBMCs.

Inhibition of viruses bearing A204E and G312V Env variants by T20 and TAK779

The differences in replication efficiency of some of the traditional SHIVs in immortalized Pt lymphocytes compared to primary human and Rh PBMCs was surprising. One possible explanation is that effects mediated by the A204E and G312V changes in CCR5 affinity or fusion kinetics that are masked in the immortalized Pt lymphocytes, which are comprised of a homogenous population of cells expressing relatively high levels of CCR5 compared to primary PBMCs (125). To test this hypothesis, differences in CCR5 affinity and fusion kinetics were examined indirectly by comparing neutralization properties of the wild-type, G312V and A204E Env variants to T20 and TAK779.

TAK779 binds directly to CCR5 and antagonizes viral entry, hence Envs with greater affinity for CCR5 are generally better able to compete with TAK779 and so these Envs will have greater IC_{50}s for inhibition by TAK779 (148). T20 is a fusion inhibitor, and acts by blocking the formation of the six-helix bundle structural intermediate of gp41 after engagement of CCR5, but prior to fusion (198). Thus, the relative sensitivity of a given Env variant to T20 is a measure of how quickly it undergoes fusion.

Table 3.3 Replication of traditional SHIVs expressing A204E and G312V subtype A Env variants in Rh PBMCs

Virus	Replication at Day 3 Post-infection (IP/mL)[a]		
	Human PBMCs	Immortalized Pt lymphocytes	Rh PBMCs
$HIVA_{Q23}/SIV_{vif}$ A204E	1.5×10^5	1.7×10^4	n.d[b]
SHIV AD8	2.2×10^5	3.5×10^5	1.1×10^5
SHIV Q23	2.9×10^3	$<1.0 \times 10^2$	4.0×10^2
SHIV Q23 A204E	1.9×10^2	9.8×10^2	6.4×10^2
SHIV Q23 G312V	1.6×10^3	6.6×10^3	1.2×10^3
SHIV BG505 A204E	3.2×10^3	2.0×10^4	5.2×10^3
SHIV BG505 G312V	2.3×10^3	1.0×10^4	1.7×10^3
SHIV MG505 A204E	2.0×10^4	2.5×10^4	7.4×10^3
SHIV MG505 G312V	2.5×10^4	2.0×10^4	9.1×10^3
SHIV QF495 A204E	2.0×10^5	3.1×10^5	1.4×10^5
SHIV QF495 G312V	6.5×10^3	1.1×10^4	3.1×10^3

[a] Average from two independent experiments
[b] n.d., not determined

The wild-type subtype A Envs displayed variable sensitivity to TAK779, with IC_{50}s ranging from approximately 119 μM, in the case of Q23-17, to approximately 4.5 μM, in the case of MG505.H3. Introduction of the G312V change greatly increased sensitivity to TAK779 for all Envs tested. The increases in sensitivity ranged from approximately 77-fold in the case of QF495.A3 G312V, to only 2.1-fold in the case of MG505.H3 G312V. Conversely, the A204E change had little effect on increasing sensitivity to TAK779, with all changes in sensitivity being less that 2-fold compared to wild-type (Table 3.4). This data suggests that while the G312V variants may have decreased affinity for CCR5, the A204E variants appear to be unchanged in their affinity for CCR5.

Decreases in sensitivity to T20 among the G312V Env variants were less marked than what was observed for TAK779. Of the 4 G312V Env variants, 3 displayed an increased sensitivity to T20, ranging from a >2-fold increase in sensitivity for the QF495.A3 G312V Env, to a >5-fold increase the case of the Q23-17 G312V Env. However, the MG505.H3 G312V variant was essentially unchanged in its sensitivity to T20. The A204E variants displayed a >9-fold and >3-fold decreased sensitivity to T20 in the cases of BG505.B1 and MG505.H3, respectively, or were unchanged sensitivity to T20, in the cases of Q23-17 and QF495.A3 (Table 3.4). Thus, while some of the G312V variants may also have decreased fusion kinetics that contribute to their inability to mediate infection of primary cells, decreased fusion kinetics does not appear to be responsible for the inability of some of the A204E Env variants to effectively mediate infection of primary human and Rh PBMCs.

Neutralization of viruses bearing A204E and G312V Env variants by HIV-1 positive plasma antibodies

To determine how the A204E and G312V amino acid changes affected overall neutralization sensitivity, viruses expressing wild-type, A204E, and G312V Env variants were examined for their sensitivity to neutralization by pooled HIV-1 positive plasma. With the exception of Q23-17, viruses carrying the wild-type Envs were relatively insensitive to the pooled plasma and were not neutralized even at the lowest dilution (Table 3.5). Introduction of the A204E amino acid change had a moderate effect on QF495.A3, increasing sensitivity by >4-fold; and a substantial effect in Q23-17, increasing the sensitivity by >60-fold, however the remainder of the Envs were relatively unaffected by introduction of the G312V and A204E changes (Table 3.5).

Table 3.4 Neutralization of pseudoviruses expressing A204E and G312V subtype A Env variants by T20 and TAK779

Envelope clone	Residue Change	IC_{50}[a]	
		T20 (μg/mL)	**TAK779 (μM)**
Q23-17	--	>10	119 ± 50
	A204E	>10	80 ± 27
	G312V	1.89 ± 1.7	4.1 ± 2.6
BG505.B1	--	0.08 ± 0.01	64 ± 19
	A204E	0.74 ± 0.01	36 ± 13
	G312V	<0.04	1.5 ± 0.7
MG505.H3	--	0.06 ± 0.01	4.5 ± 1
	A204E	0.18 ± 0.01	5.6 ± 1.5
	G312V	0.07 ± 0.01	2.1 ± 1.3
QF495.A3	--	0.39 ± 0.11	46 ± 4.1
	A204E	0.36 ± 0.03	34 ± 10
	G312V	0.12 ± 0.01	0.59 ± 0.26

[a]Expressed as avg ± SD for two experiments

Table 3.5 Neutralization of pseudoviruses expressing A204E and G312V subtype A Env variants by plasma antibodies

Envelope Clone	Residue Change	IC_{50}[a]		
		Pooled HIV+ Plasma	Contemporaneous Plasma	Plasma from < 6 mos. Post-isolation[b]
Q23	--	150 ± 25	<50	<50
	G312V	65 ± 16	<50	<50
	A204E	9528 ± 3708	>1600	>1600
BG505.B1	--	<50	<50	<50
	G312V	<50	<50	<50
	A204E	<50	139 ± 48	51 ± 1
MG505.H3	--	<50	221 ± 61	140 ± 67
	G312V	<50	120 ± 9	41 ± 22
	A204E	<50	170 ± 47	118 ± 60
QF495.A3	--	<50	nd[c]	nd
	G312V	<50	nd	nd
	A204E	211 ± 28	nd	nd

[a]Expressed as average ± standard deviation for two replicate experiments
[b]*Plasma from an infection time-point less than 6 months after the envelope was cloned*
[c]nd, not determined due to unavailability of the sample

Differences in overall neutralization sensitivity imparted by the A204E and G312V changes were further examined by testing the Env variants for neutralization by autologous plasma obtained from two different time-points. The A204E amino acid change had a variable effect on neutralization sensitivity depending on the Env to which it had been introduced. In the case of the Q23-17 Env, introduction of the A204E change increased sensitivity to autologous plasma by >32-fold, whereas in the case of MG505.H3 neutralization was essentially unchanged (Table 3.4). The G312V change typically had little effect on sensitivity to autologous plasma, although, in the case of the MG505.H3 variant, sensitivity to neutralization was actually decreased by ~1.5 to 3-fold. Unfortunately plasma samples for the QF495.A3 variants were unavailable, and so differences in neutralization sensitivity induced by the A204E and G312V amino acid changes could not be examined. This limited survey suggested that the A204E and G312V changes had a range of effects on increasing sensitivity to neutralization by plasma antibodies. Importantly, as in the case of the MG505.H3 A204E and G312V Envs, it allowed for the identification of a A204E and G312V Env variants with the ability to mediate replication in macaque cells whose overall neutralization sensitivity is not greatly changed from the original wild-type Env.

Discussion

In this chapter, the replication properties of traditional SHIVs expressing subtype A G312V and A204E Env variants were evaluated as a first test to determine their potential use in an *in vivo* setting. The major parameters under consideration for this evaluation were the capacity of the given Env to mediate replication in primary PBMCs when expressed in the setting of a traditional SHIV, and their sensitivity to polyclonal antibody neutralization. Based on these criteria, SHIV QF495 A204E and SHIV MG505 G312V appear to be the most ideal candidates for further evaluation *in vivo*.

SHIV QF495 A204E is a clear front-runner because of its high level of replication in all the macaque cell types tested, including primary PBMCS. The ability to replicate in PBMCs is an essential property for its ability to replicate in the host, as viruses that do not replicate well in PBMCs typically do not replicate well *in vivo* (for example, (31, 130, 174)). Encouragingly, the kinetics and levels of viral replication of SHIV QF495 A204E compared favorably to SHIV AD8, which has been used as the basis to generate highly pathogenic CCR5-tropic SHIV lineages (130). SHIV MG505 G312V should also be considered as a candidate for further development in an animal model. The MG505.H3 G312V Env retained its neutralization properties to pooled and autologous plasma, suggesting that the G312V change had little effect on changing the overall antigenicity of the Env.

Moreover, it was determined in Chapter 2 that the MG505.H3 G312V Env is only moderately sensitive to neutralization by the b12 monoclonal antibody, a significant improvement over Envs from existing CCR5-tropic SHIVs, such as SF162p3, and more reflective of circulating HIV-1 strains, which tend to be relatively insensitive to neutralization by b12 (13, 18, 158). Importantly, SHIV MG505 G312V replicated well in human PBMCs and immortalized Pt lymphocytes, albeit with slower kinetics than SHIV QF495 A204E and SHIV AD8. Although replication in Rh PBMCs was ~20-fold lower than compared to SHIV QF495 A204E, it should be noted that we were unable to achieve sustained viral replication in Rh PBMCs, possibly due to reactivation of latent foamy virus causing cell death (57). Thus, given its slower kinetics, it is plausible that SHIV MG505 G312V would have achieved comparable levels of infection if viral replication could have been sustained in culture for a longer period of time.

Unfortunately many of the traditional SHIVs carrying the A204E and G312V Env variants failed to establish robust replication in human and Rh PBMCs, despite being able to establish robust spreading infection in immortalized Pt lymphocytes. One clue as to why this is, specifically for the G312V variants, is their increased sensitivity to TAK779, and to a certain extent, T20. The inverse correlation between sensitivity to TAK779 and affinity for CCR5 is well-established (148). Interestingly, a similar inverse correlation between affinity for CCR5 and sensitivity to the T20 fusion inhibitor has been observed (148) although the mechanism for why this might be is not well-understood. Thus, one plausible model is that the G312V Env variants have less affinity for CCR5 and so may not be able to establish high levels of infection in primary cells expressing relatively low levels of CCR5. This is further supported by the fact that the MG505.H3 G312V Env, which was best among the G312V Env variants at mediating the infection of primary human and Rh PBMCs, was relatively unchanged in its sensitivity to both T20 and TAK779. Because the G312V amino acid change arose through adaptation to immortalized Pt cells, which express relatively high levels of CCR5 (125), it is possible that there was an absence of pressure on the Env to maintain its affinity for CCR5. However, this explanation does not cover the failed replication of some SHIVs carrying the A204E Env variants given that they showed only modest increases in sensitivity to T20 and TAK779, thus implying that other factors beyond affinity for CCR5 are at work.

The existence of other viral factors that govern the successful replication of traditional SHIVs is witnessed by the fact that $HIVA_{Q23}/SIV_{vif}$ A204E replicated 2×10^4 times higher in human PBMCs as compared to SHIV Q23 A204E. This suggests that the Q23 A204E Env functions poorly in the context of a traditional SHIV, and it is possible that this is the case for other envelopes that did not

function well in the setting of traditional SHIVs. One possible reason for this may be that some Envs are not properly incorporated and presented on the virion surface in traditional SHIVs, potentially due to faulty matrix-gp41 interactions (28). Additionally, it is possible that the A204E change may decrease Env processing in certain contexts, thus decreasing infectivity of the virus (15). Further delineation of potential mechanisms required for increased SHIV infectivity in primary cells, whether by determinant mapping or passaging of poorly replicating SHIVs on primary cells may provide additional insight into this surprising limitation to SHIV development.

Chapter 4

Inefficient usage of macaque CD4 limits infection mediated by envelopes from circulating HIV-1 strains

Introduction

In chapter 2, the limited ability of subtype A envelopes (Envs) to use pig-tailed macaque (Pt) CD4 for entry was identified as a major barrier to the replication of SHIVs carrying subtype A Envs (SHIV-As) in Pt cells. Adaptation of a minimal SHIV-A allowed for the identification of two independent adaptive amino acid changes in Env, A204E and G312V, that conferred more efficient entry via Pt CD4 for multiple subtype A Envs. These findings implicated Env/CD4 interactions in the restriction of SHIV-A replication in macaque cells.

Based on our findings with subtype A Envs, we hypothesize that inefficient use of CD4 may provide an explanation for the limited ability of SHIVs expressing diverse circulating HIV-1 Envs to replicate in macaque cells (78). One line of evidence in support of this hypothesis comes from the properties of the A204E and G312V Env variants. Introduction of the A204E or G312V changes to subtype A Envs increased their sensitivity to soluble CD4 (sCD4), and, in some cases, to the CD4-binding site directed IgG1b12 (b12) monoclonal antibody (25, 206). This suggests that the A204E and G312V Env variants have more accessible CD4 binding sites, a property inferred from increased sensitivity to neutralization by sCD4 and CD4 binding site-directed antibodies (51-54, 65, 154, 178). Thus, one model of how changes to A204 and G312 increase Pt CD4 usage is by increasing exposure of the CD4 binding site. More generally, this model implies that only those Envs with more accessible CD4 binding sites may be able to efficiently engage Pt CD4. In support of this idea, a number of the existing CCR5-tropic SHIVs incorporate HIV-1 Envs that are relatively sensitive to sCD4 (35, 47, 93, 172, 183). A prediction of this model is that circulating HIV-1 Env variants, which tend to be insensitive to neutralization by sCD4 and b12 (18, 104, 105), may not promote efficient entry via Pt CD4.

The potential requirement of a more exposed CD4 binding site in HIV-1 Envs to better use Pt CD4 may be explained by the need to accommodate amino acid differences between Pt CD4 and human CD4. The primary determinants of HIV-1 Env-CD4 interaction have been mapped to the D1

and D2 domains of human CD4 (5, 98), and there are a number of amino acid differences between Pt CD4 and human CD4 in these regions. The published sequence of CD4 from rhesus macaques (Rhs), another model species, is largely identical to Pt CD4. Thus, if amino acid differences in the D1 and D2 domains account for the inability of HIV-1 Envs to use Pt CD4, it suggests that inefficient usage of Rh CD4 may also impose a barrier on HIV-1 replication.

In this chapter we seek to address the hypothesis that inefficient usage of macaque CD4 presents a broad limitation to infection mediated by diverse circulating HIV-1 Envs, beyond the subtype A Envs in which the limitation was first identified. To test this hypothesis, we assayed 39 Envs, representing circulating subtypes A to D and obtained from viral isolates present during the acute/early stages of infection, for their ability to mediate infection of cells expressing Pt and Rh CD4 compared to cells expressing human CD4. Acute/early Envs were chosen for this study because panels of acute/early Envs have previously been assembled, and many of these Envs are well-characterized for a myriad of properties, including co-receptor usage, sensitivity to sCD4, and sensitivity to plasma and monoclonal antibodies (18, 104, 105, 117, 200), thus allowing for the identification of Env properties that might be associated with the ability to use macaque CD4. We have found that the majority (>74%) of the acute/early HIV-1 Envs tested had a greater than 10-fold decrease in their ability to mediate infection using Pt or Rh CD4 as compared to human CD4. The ability to use macaque CD4 showed a statistically significant association with sensitivity to sCD4 and the infection of cells expressing low levels of CD4 ($p < 0.0001$). Additionally, the major determinant in Pt CD4 responsible for limited usage by the diverse HIV-1 Envs was mapped to a single amino acid.

Materials and Methods

Cells

HEK 293T cells (referred to throughout as 293Ts), Cf2Th/syn CCR5 cells, JC.24 cells, and RC.49 cells were maintained in DMEM medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine. Cf2Th/syn CCR5 cells, which were obtained from the NIH AIDS Reagents program (contributed by Drs. Tajib Mirzabekov and Joseph Sodroski.), are dog thymocytes that have been engineered to express high levels of codon optimized human CCR5 (121). These cells were further supplemented with 400 μg/mL of geneticin (Gibco) to maintain CCR5 expression. The JC.24 and RC.49 cell lines, referred to throughout as $CD4_{HIGH}$ and $CD4_{LOW}$ cells, are HeLa-derived cells lines that express comparable levels of CCR5, but JC.24 cells

have a 40-fold higher expression of surface CD4 than RC.49 cells (141). These cells were kindly provided by Drs. Emily Platt and David Kabat.

Generation of stable cells expressing human, rhesus, and pig-tailed macaque CD4 variants

Expression plasmids encoding the open-reading frames (ORFs) from human CD4 (huCD4) and pig-tailed macaque CD4 (ptCD4) in pBabe-Puro, were both described in Chapter 3, and an expression plasmid encoding rhesus macaque CD4 (rhCD4) in the pcDNA3.1 +Zeo expression vector was a kind gift from Dr. Robert Doms (145). These plasmids were used as templates to amplify the CD4 ORFs using forward primer 5'-GATGTCGACATGAACCGGGGAGTCCC-3' with reverse primer 5'-GGTCTCGAGTCAAATGGGGCTACATG-3' for huCD4 and forward primer 5'-GATGTCGACATGAACCGGGGAATCCC-3' with the same reverse primer for ptCD4 and rhCD4. The PCR products were digested with SalI and XhoI (restriction sites underlined in the primers), cloned into pLXSH, an MLV-based retroviral vector encoding a hygromicin resistance gene [(120), a kind gift from Dr. Dusty Miller], and verified by sequencing.

Virus-like particles (VLPs) were generated by co-transfecting the pLXSH vector encoding the CD4 of interest, an MLV-based retroviral packaging vector [pJK3, (9), a kind gift from Dr. Michael Emerman](128)(128), and a plasmid expressing the vesicular stomatitis virus G protein (VSV-G) under the CMV promoter [pMD.G, (128)], at a ratio of 1:1:0.1. Supernatants were harvested twice at 48 and 72 hours post-transfection, filtered through a 0.22 µm filter, and used directly to infect cells.

Twenty-four hours prior to infection with VLPs, 1×10^5 Cf2Th/syn CCR5 cells were plated in T75 tissue culture flasks. The plated cells were incubated with 4-5 mL VLP supernatant harvested at 48 hours in the presence of 10 ug/mL of DEAE dextran at 37°C for 3 hours before bringing the volume of media up to 10 mL. This procedure was repeated the next day with the VLP supernatant harvested at 72 hours. The following day, cells were split 1:2 into new flasks and allowed to recover in the absence of drug selection for 24 hours. Media was then supplemented with 400 µg/mL of geneticin (to maintain CCR5 expression) and 300 µg/mL of hygromycin B (Invitrogen) to select for cells expressing CD4. The cultures were maintained in these conditions, changing media every 3 to 4 days until cultures that had not been exposed to VLPs were completely clear of cells (typically 10-14 days). Cultures were maintained thereafter with 150 µg/mL of hygromycin B.

The surviving tranduced cells were analyzed for levels of CD4 expression and were sorted on a FACSAria II cell sorter (BD Biosciences). Briefly, 1-3×10^6 cells were treated with 5 mM EDTA to remove them from the tissue culture flask, and washed in PBS containing 2% FBS. The cells were resuspended in 200 µL of PBS/2% FBS and incubated with 4 µL of APC conjugated mouse anti-human CD4 antibody (cat. no. 551980, BD Biosciences) at room temperature for 30 min. The cells were then washed again in PBS/2% FBS, resuspended to a concentration of 1×10^6 cells/mL in PBS/1% FBS/1 mM EDTA and filtered through a 35 µm nylon mesh cap (BD Falcon) before sorting. The cells with the highest levels of CD4 expression, representing the top 20-30% of CD4-expressing cells, were retained and used for infection assays.

Env clones and mutagenesis

Envelope clones Q23-17, Q23-17 A204E and Q23-17 G312V were described previously in Chapter 2.

Subtype A, B, C, and D Env clones that were isolated from HIV-infected individuals at acute or early (< 6 months) in infection have been described. The 12 Env clones in the subtype B acute/early panel (RHPA4259.7, REJO4541.67, SC422661.8, WITO4160.33, TRO.11, CAAN5342.A2, QH0692.42, AC10.0.29, THRO4156.18, TRJO4551.58, PVO.4, and 6535.3) and the 12 Env clones in the subtype C acute/early panel (ZM249M.PL1, Du156.12, ZM135M.PL10a, ZM214M.PL15, ZM53M.PB12, ZM233M.PB6, CAP45.2.00.G3, CAP210.2.00.E8, Du172.17, Du422.1, ZM109F.PB4, and ZM197M.PB7) have been described previously (104, 105) and were obtained from the NIH AIDS Reference and Reagent Program. An additional subtype C acute/early clone, QC406.70M.ENV.F3 (17), was also included in this study, and is referred to throughout as QC406F3. The 10 subtype A acute/early Env clones, which were chosen randomly from available clones for inclusion in the study, have been described previously in (17) [for QB726.70M.ENV.B3, QF495.23M.ENV.A3, QG984.21M.ENV.A3, QH343.21M.ENV.A10, and QH359.21M.ENV.C1] and (18, 110) [for Q259.d2.17, Q168b23, Q461e2, Q769h5, and Q842d16]. Similarly, the four subtype D acute/early Envs; QA013.70I.ENV.H1, QA465.59M.ENV.A1, QB857.110I.ENV.B3, and QD435.100M.ENV.B5 (17); were chosen randomly from available clones, and are referred to as QA013H1, QA465A1, QB857B3, and QD435B5 throughout the chapter.

Additional subtype A acute/early Envs used in this chapter were Q842d12, Q769d22, Q461d1 and Q769b9 (18), and Q461e2 TAIV, a clone of Q461e2 to which the T569A and I675V amino acid changes have been introduced to gp41 (19).

Env clones from BaL.01 (NIH AIDS Reagents program, submitted by Dr. John Mascola), YU-2 (106), SF162 (33), SF162P3, [(83); a gift from Dr. Cecilia Cheng-Mayer], the dual-tropic 89.6 (37) and SIV Mne CL8 (135, 139), were also used in this chapter.

Nucleotide changes encoding the A204E and G312V mutations were introduced to *env* QC406F3, QA013H1, QB857B3, and QD435B5 using primers designed in PrimerX (http://www.bioinformatics.org/primerx/, listed in Table 4.1) to amplify 100 ng of plasmid with Pfu Turbo (Invitrogen) and 125 ng of each primer under the following reaction conditions: 95°C for 5 min, followed by 18 cycles of 95°C for 30s, 55°C for 1 min, and 68°C for 16 min. The envelope mutants were sequenced through the entirety of the *env* open reading frame to verify that no undesired nucleotide changes had occurred. These *env* variants were created by Sandra Emery and Elizabeth Laws.

Preparation of GFP reporter pseudoviruses

GFP reporter pseudoviruses were generated in 293T cells. Twenty-four hours prior to transfection, 293T cells were plated at 5×10^5 cells/well in a 6-well dish. Cells were co-transfected with 667 ng of Q23Δ*env*-GFP (described in Chapter 2) and 333 ng of the Env clone of interest using Fugene 6 (Roche) at a ratio of 3 µL Fugene to 1 µg DNA as per the manufacturer's instructions. Pseudoviruses were harvested 72 hours after transfection, centrifuged at 1300 rpm for 5 min to clear cellular debris, aliquoted, and frozen at -80°C until use. To obtain an estimated titer for each virus, 2-10 µL of thawed viral supernatant was used to infect Cf2Th/syn CCR5 huCD4 cells, and cells were analyzed for GFP expression as described in more detail in the next section. GFP expression within the linear range of the assay (~0.5% to 25% GFP-positive cells) was used to determine the titer of the virus (where an MOI of 0.1 corresponds to 10% GFP positive Cf2Th/syn CCR5 huCD4 cells).

Infection and analysis of Cf2Th/syn CCR5-based cells and $CD4_{HIGH/LOW}$ cells

Cf2Th/syn CCR5 cells stably expressing CD4 were plated 24 hours prior to infection at 2.5×10^4 cells/well in 24-well tissue culture plates in 500 µL of drug-free media. Cells were infected in duplicate wells in the presence of 10 µg/mL DEAE dextran by spinoculation for 60-90 mins at 1200×*g* at an estimated MOI of 0.1 in 100 µL of media. Forty-eight hours later, the cells were washed in cold PBS, incubated with 100 µL of 5 mM EDTA until they lost adherence to the well, and fixed in 500 µL of 1% paraformaldehyde. The fixed cells were transferred to flow cytometry tubes

Table 4.1 Primers used for site-directed mutagenesis of subtype C and D *envs*

Envelope clone	Residue Change	Primer Sequence[a]
QC406F3	A204E	ATACCTCAGCCATTACACAAGAGTGTCCAAAGGTCTCTTTTGAC
	G312V	CAAGAGAAAGTATAGGGATAGTACCAGGACAAATGTTCTATGC
QA013H1	A204E	CTGCCATTACACAGGAATGTCCAAAGGTAAC
	G312V	GGTGAACATATGGTACCAGGGCGAGC
QB857B3	A204E	CAGCCATTACACAGGAGTGTCCAAAGGTAAG
	G312V	CAAAGTATCCGTATAGTACCAGGGCAAGCATTC
QD435B5	A204E	CAGCCATTACACAGGAGTGTCCAAAGGTAAG
	G312V	GGTACACACATAGTACCAGGGCGAGC

[a]The sequence of the primer in 5' to 3' orientation. The underlined nucleotide(s) represent the changes being introduced. Note that the reverse complement primer was also used to introduce the mutations of interest, as described in the text.

(BD Falcon) which were centrifuged at 800×*g* for 5 mins. The excess fix was carefully poured off, and the cells were analyzed for GFP expression on a BD FACSCalibur flow cytometer.

Data from approximately 1×10^4 live cells, as determined by side-scatter and forward-scatter profile, was analyzed using FlowJo version 9.4.6. Those cells whose GFP expression was greater than 200 fluorescence units were gated as GFP-positive. This fluorescence intensity was chosen because it limited false positives to only 0.01-0.02% (not shown).

Infections and analysis of the $CD4_{HIGH}$ and $CD4_{LOW}$ cells were performed as described for the Cf2Th/syn CCR5 cells with the exception that 4×10^4 cells were plated on the day prior to infection, 25 mM EDTA was used to remove the cells from the tissue culture well, and only approximately 5×10^3 live cells were analyzed for GFP expression. These infections and flow cytometry analyses were performed by Sandra Emery.

Amplification and sequencing of CD4 exons 3 and 4 from rhesus total genomic DNA

Total rhesus macaque genomic DNA was obtained from the Oregon Health and Science University Macaque DNA Bank with the help of Dr. Betsy Ferguson (59). Exons 3 and 4 were amplified by PCR in triplicate from 30 samples, 15 from the Indian subspecies and 15 from the Chinese subspecies using primers 5'-ATGGCGTGGGAGGAAAGGCAAAGGT -3' and 5'-TCTGTCAGCCCCAGCCCTCTGGGA-3' for exon 3 and 5'-CCACTGTCCCAGCCAGGTAAATGGA-3' and 5'-CTCGTCCCCACCTGCCCCACTC-3' for exon 4. Both of these primer sets bind to intronic regions flanking the exons and amplification with these primers resulted in amplicons of 370 bp and 371 bp, respectively. AmpliTaq DNA polymerase (Applied BioSystems) was used for PCR under the following thermocycler conditions: 95°C for 5 min, followed by 5 cycles of 95°C for 30s, 65°C for 30s, 72°C for 30s; 30 cycles of 95°C for 30s, 60°C for 30s, 72°C for 30s; and 72°C for 5 min.

The PCR amplicons were detected as single prominent bands by gel electrophoresis. All reactions were treated with ExoSap (Amersham Biosciences) and the amplicons were sequenced directly without gel purification. A portion of this analysis was performed by Elizabeth Laws.

Construction of Chimeric CD4 and CD4 mutants

There are NheI restriction sites at position 603 (encoding amino acid 176 after processing of the CD4 signal tag) of both huCD4 and ptCD4, and in the backbone of pBabe-puro, 3' of the CD4

stop codon (124). Digestion of pBabe-puro/ptCD4 with NheI results in an 1859 bp fragment comprising the entire ptCD4 ORF, with the exception of the D1 and D2 domains. This fragment was subcloned into a similarly digested pBabe-puro/huCD4 resulting in a chimeric ptCD4 that encompasses all of the amino acid differences in the D1 and D2 domains. The resulting clone was sequenced to ensure that the fragment had been cloned in the proper orientation.

Chimeric ptCD4 sequences containing the huCD4 D1, N-terminal D1 and C-terminal D1 domains were constructed using a two-step overlap extension PCR approach described previously (44) with some changes. In the first round, the huCD4 fragment of interest, as well as regions of ptCD4 overlapping with the huCD4 fragment and comprising the remainder of the ptCD4 open-reading frame were amplified by PCR from 25 ng of plasmid encoding huCD4 or ptCD4 (described in Chapter 2) for 25 cycles using the primers described in Table 4.2. The first-round products were electrophoresed on an agarose gel, purified using a Qiagen Gel Purification Kit, and, depending on yield, 1-4 uL of each product was pooled together for use as template in the second round. Similarly, the second round amplification was performed for 25 cycles with forward primer 5'-GATGGATCCATGAACCGGGGAATCCC-3' and reverse primer 5'-GGTGTCGACTCAAATGGGGCTACATG-3', except in the case of the chimeric ptCD4 expressing the C-terminus of the huCD4 D1 domain, in which 5'-GATGGATCCATGAACCGGGGAGTCCC-3' was used as a forward primer . The amplicons were purified using the QIAquick PCR Purification Kit, digested with BamHI and SalI (restriction sites underlined in the primers), gel purified, and ligated into the pBabe-Puro expression vector (124).

Site-directed mutagenesis was performed in huCD4 and ptCD4 using primers designed in PrimerX and the overlap extension protocol described above. All primers used to make and CD4 mutants are listed in Table 4.3.

Infection of cells transiently expressing CD4 and CCR5

Infection of 293T cells transiently expressing the CD4 constructs and CCR5 were performed as described in Chapter 2. Briefly, cells were transfected with CD4 and CCR5 expression plasmids in 6-well dishes using Fugene 6 transfection reagent. Forty hours later, cells were removed from the dish using 5 mM EDTA and plated at 80 000 cells/well in 500 µL in a 24-well dish, with $2\text{-}3\times10^5$ cells kept for analysis of CD4 and CCR5 expression by flow cytometry, as described in Chapter 2.

Four to five hours after plating cells, infections were performed in duplicate by spinoculation in the presence of 10 µg/mL DEAE-dextran with dilutions of GFP pseudovirus. Cells were fixed with cold PBS/1% formaldehyde/0.2% gluteraldehyde 72 hours after infection and GFP-positive cells were counted by eye to determine viral titer.

Data presentation and analysis

All data was plotted; and Mann-Whitey U Tests, Wilcoxon Signed Rank Tests, and Spearman correlations, were performed using Prism version 5.0a (GraphPad Software).

Table 4.2 Primers used to create chimeric CD4 variants

Chimeric CD4	Fragment (Start nt, End nt)[a]	Forward Primer Sequence	Reverse Primer Sequence
ptCD4 huD1	huD1 (1, 336) ptCD4 ΔD1 (307, 1377)	GATGGATCCATGAACCGGGGAATCCC GACTCAGATACTTACATCTGTGAAGTGGAG	CTCCACTTCACAGATGTAAGTATCTGAGTC GGTGTCGACTCAAATGGGGCTACATG
ptCD4 huD1 Nterm	huD1 Nterm (1, 228) ptCD4 ΔD1 Nterm (201, 1377)	GATGGATCCATGAACCGGGGAATCCC CTTCTTAACTAAAGGTCCATCCAAGCTG	CAGCTTGGATGGACCTTTAGTTAAGAAG GGTGTCGACTCAAATGGGGCTACATG
ptCD4 huD1 Cterm	ptCD4 D1 Nterm (1, 228) huD1 Cterm (201, 336) ptCD4 ΔD1 (307, 1377)	GATGGATCCATGAACCGGGGAGTCCC CTTCTTAACTAAAGGTCCATCCAAGCTG GACTCAGATACTTACATCTGTGAAGTGGAG	CAGCTTGGATGGACCTTTAGTTAAGAAG CTCCACTTCACAGATGTAAGTATCTGAGTC GGTGTCGACTCAAATGGGGCTACATG
huCD4 ptD1 Nterm	ptCD4 D1 Nterm (1, 228) huCD4 ΔD1 Nterm (201, 1377)	GATGGATCCATGAACCGGGGAGTCCC CTTCTTAACTAAAGGTCCATCCAAGCTG	CAGCTTGGATGGACCTTTAGTTAAGAAG GGTGTCGACTCAAATGGGGCTACATG

[a]The fragment of CD4 being amplified in the first round PCR. These fragments were later pooled for a second round PCR as described in the text. Numbering is relative to the CD4 open-reading frame.

Table 4.3 Mutagenesis primers used to make ptCD4 and huCD4 single and double mutants

CD4	Residue Change[a]	Mutation[b]	Primer Sequence[c]
ptCD4	N17T	C125A	GGAACTGACCTGTACTGCTTCGCAGAAG
	N23S	A143G	CTTCGCAGAAGAAGAGCACACAATTCCACTG
	T24I	C146T	GCTTCGCAGAAGAAGAACATACAATTCCACTGGAAAAAC
	NT23SI	A143G, C146T	AATGCTTCGCAGAAGAAGAGCATACAATTCCACTGGAAAAACT
	I39N	T191A	GATAAAGATTCTGGGAAATCAGGGCTCCTTCTTAAC
huCD4	T17N	126A, 127C	GGAACTGACCTGTAACGCTTCCCAGAAGAAG
	S23N	G143A	CTTCCCAGAAGAAGAACATACAATTCCACTGG
	I24T	T146C	CTTCCCAGAAGAAGAGCACACAATTCCACTGGAAAAAC
	SI23NT	G143A, T146C	GTACAGCTTCCCAGAAGAAGAACACACAATTCCACTGGAAAAACTC
	N39I	A191T	GATAAAGATTCTGGGAATTCAGGGCTCCTTCTTAAC

[a]The amino acid change being introduced (number is relative to the processed CD4 peptide in which the signal peptide comprised by the first 25 amino acids encoded by the CD4 open-reading frame has been removed)
[b]The nucleotide change(s) being introduced (numbering is relative to the CD4 open-reading frame)
[c]The sequence of the primer in 5' to 3' orientation. The underlined nucleotide(s) represent the changes being introduced. Note that the reverse complement primer was also used to introduce the mutations of interest, as described in the text.

Results

Creation of stable cell lines expressing human, pig-tailed and rhesus CD4

Cf2Th/syn CCR5 cells express high levels of codon optimized human CCR5 and support post-entry steps of HIV-1 replication (121). These cells were stably transduced to express human (hu), pig-tailed (pt), or rhesus (rh) CD4. Flow cytometric analysis using a monoclonal antibody that cross-reacts equally with macaque and human CD4 (BD BioSciences, personal communication) showed that CD4 surface expression was comparable across all three cell lines (Fig 4.1a). Importantly, infection of these cells with GFP reporter pseudoviruses carrying the Q23-17 wild-type, A204E and G312V Env variants recapitulated the results originally observed in HEK 293T cells transiently expressing hu and ptCD4 (Chapter 2). In order to normalize for any differences in overall virus titer that were not specific to macaque CD4 usage, the infectivity of viruses carrying each Env in the huCD4 cell line was set to 1 and infectivity in the macaque CD4 cell lines was expressed relative to this as 'fold infection'. Viruses expressing the wild-type Q23-17 Env were poorly infectious in Cf2Th/syn CCR5 cells expressing ptCD4 (< 0.01-fold infection), whereas viruses expressing the Q23-17 A204E and G312V Env variants infected cells expressing ptCD4 to comparable levels as cells expressing huCD4 (0.95-fold and 0.83-fold infection, respectively, Fig 4.1b). Viruses expressing the Q23-17 A204E and G312V Env variants also mediated increased infection of Cf2Th/syn CCR5 cells expressing rhCD4, compared to viruses expressing wild-type Q23-17, with the infectivity increasing from <0.01-fold, for the wild-type Q23-17 Env; to 0.76 and 0.65-fold, for the A204E and G312V variants, respectively.

Infection of huCD4, ptCD4 and rhCD4 cell lines with diverse SHIV, SIV, and HIV-1 Env variants

The ability of Env variants of SHIVs and SIVs that were previously shown to replicate in macaque cells and infect using Pt CD4 was examined. Among the strains tested were 89.6, BaL, SF162, and SF162p3, which have all been used to generate SHIVs that replicate in macaques *in vivo* (66, 112, 136, 149), and SIVMneCl8, which is also pathogenic in Pts *in vivo* (135). Although not tested *in vivo*, the YU-2 Env has previously been used in the context of a minimal SHIV that

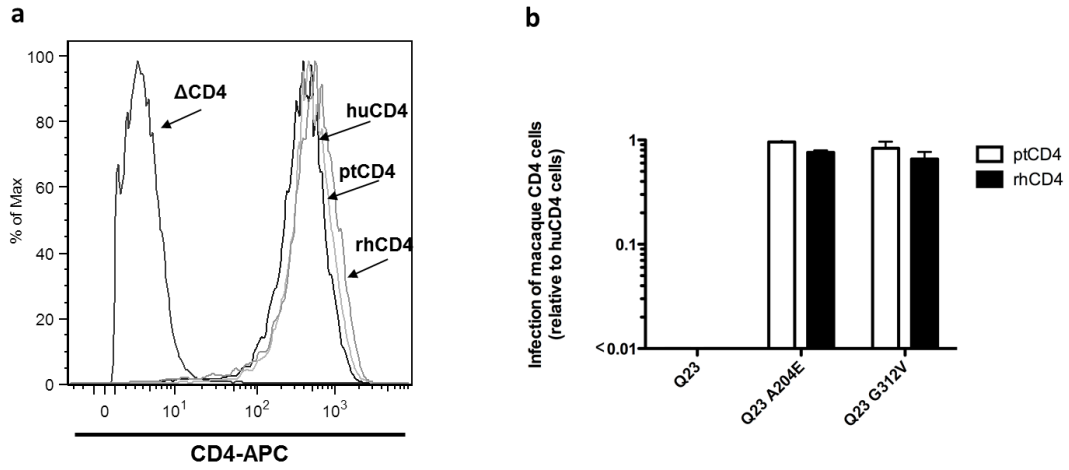

Fig 4.1 Stable expression of hu, pt, and rh CD4 in Cf2Th/syn CCR5 cells and infection with GFP reporter pseudoviruses expressing Q23-17 Env variants *(a)* Expression of hu, pt, and rh CD4 (indicated by arrows) on the surface of stably transduced CF2Th/syn CCR5. ΔCD4 refers to the parental Cf2Th/syn CCR5 cell line, which does not express CD4. *(b)* Infection of ptCD4 cells (white bars) and rhCD4 cells (black bars) relative to huCD4 cells with GFP pseudoviruses expressing the Q23-17, Q23-17 A204E and Q23-17 G312V Env variants (as denoted on the *x*-axis). The y-axis shows infection of cells expressing pt or rh CD4 relative to cells expressing huCD4, with infectivity of huCD4 cells set to 1. Error bars represent the standard deviation of the mean from two independent experiments.

replicated to high levels in macaque PBMCs *in vitro* (188). The ability of the SIV/SHIV Envs to mediate infection of macaque CD4 cells ranged from 0.30-fold (for 89.6) to 0.81-fold (for YU-2) in ptCD4 cells; and from 0.36-fold (for SIVMneCl8) to 0.89-fold (for SF162p3) in rhCD4 cells (Fig 4.2a). The median relative infection among SIV/SHIV strains was 0.61-fold for ptCD4 cells and 0.71-fold for rhCD4 cells.

Viruses expressing Envs from acute/early HIV-1 strains representing subtypes A through D were generally poorly infectious for cells expressing ptCD4. Of the 39 Envs tested, 34 (>87%) had a greater than 10-fold decrease in their ability to mediate infection of ptCD4 cells relative to their ability to infect huCD4 cells, and the median relative infection of ptCD4 cells for each of the four subtypes was significantly lower than SIV/SHIV Envs (Fig 4.2b, $p < 0.01$ for all subtypes, Mann-Whitney U Test). The median infection of ptCD4 cells relative to huCD4 amongst subtypes ranged from < 0.01-fold for subtypes C and D to 0.02-fold for subtype A. Differences between subtypes were not statistically significant ($p > 0.23$ for each comparison, Mann-Whitney U Test).

In general, viruses expressing acute/early Env variants had higher infectivity in cells expressing rhCD4 as compared to cells expressing ptCD4 (median 0.049-fold versus 0.018-fold relative to huCD4, $p = 0.0003$ by Wilcoxon Signed Rank test). Nonetheless, viruses expressing acute/early Envs from all subtypes showed decreased infectivity of rhCD4 cells as compared to the SIV/SHIV strains (Fig 4.2c, $p<0.01$ for all subtypes, Mann-Whitney U Test), with 29 of the 39 acute/early Envs tested (>74%) having a greater than 10-fold decrease in their ability to infect rhCD4 cells as compared to huCD4 cells. The median relative infection of rhCD4 cells for each subtype ranged from 0.01-fold for subtype C to 0.07-fold for subtype A strains. The difference in median infection between subtypes A and D was statistically significant ($p=0.0337$, Mann-Whitney U Test), however all other differences between subtypes were not statistically significant ($p > 0.08$ for other comparisons, Mann-Whitney U Test). Despite the statistically significant increase in differences, it is clear that acute/early Envs from all of the subtypes tested are impaired in their ability to utilize rhCD4, as was observed with ptCD4.

Association between macaque CD4 usage and sensitivity to b12, VRC01, and soluble CD4

In Chapter 2, initial studies of a small number of subtype A Envs suggested that Envs with the capacity to mediate the infection of cells expressing ptCD4 tended to be those that were sensitive soluble CD4 (sCD4), and potentially to neutralizing antibodies directed toward the CD4 binding site

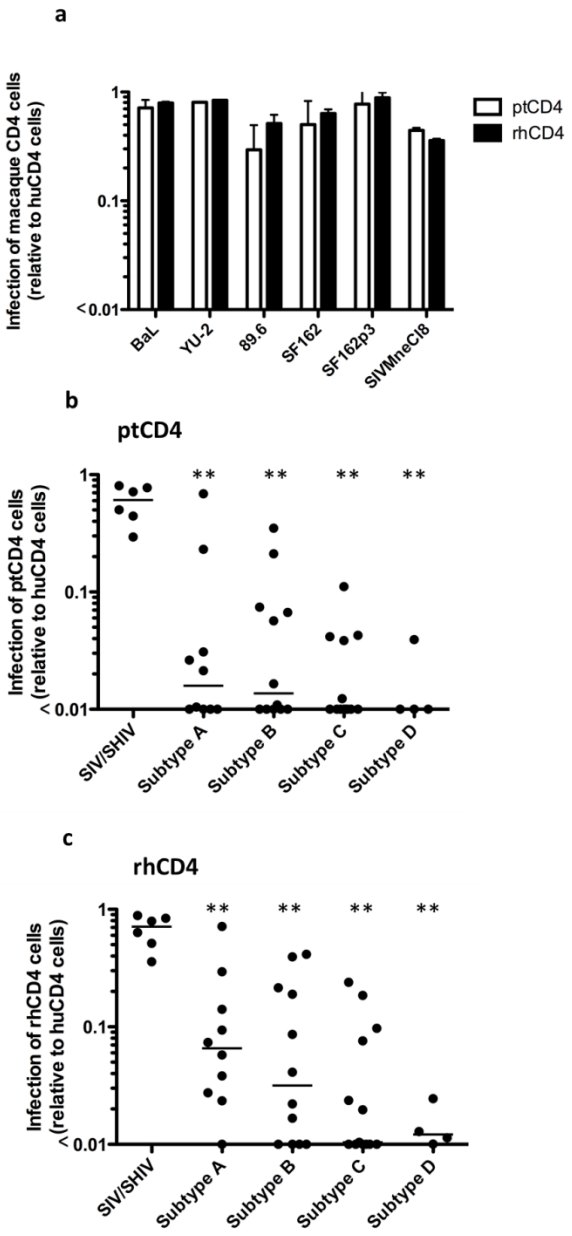

Fig 4.2 Infection of macaque CD4 cell lines with GFP reporter pseudoviruses expressing Envs from diverse HIV-1 and SIV strains *(a)* Infection of ptCD4 cells (white bars) and rhCD4 cells (black bars) relative to huCD4 cells with GFP pseudoviruses expressing Envs from SHIV/SIV strains denoted on the *x* axis. Error bars represent the standard deviation of the mean from two independent experiments. *(b)*, *(c)* Infection of ptCD4 cells *(b)* or rhCD4 cells *(c)* relative to huCD4 cells with GFP reporter pseudoviruses expressing Envs from acute/early HIV-1 Envs from subtypes A-D as indicated on the *x*-axis. Each point on the plot represents the mean infectivity of a given Env obtained from duplicate independent experiments, and the line represents the median measurement for each subtype. The SHIV/SIV Envs are the same as shown in part (a). **p<0.01 as compared to SIV/SHIV Envs, Mann-Whitney U Test.

(e.g. b12 and VRC01). This was examined using a larger data-set comprising neutralization data published by Wu et al (200). Including the SIV/SHIV Envs and the acute/early Envs, 30 of the 45 Env clones that were examined for their ability to use macaque CD4 in Figure 4.2 overlapped with this previously published data-set.

The association between relative infection of ptCD4 cells and neutralization by b12 or VRC01 was not significant as determined by Spearman correlation (Fig 4.3a and c), and the same was true for rhCD4 cells (Fig 4.3 b and d). However, there was a significant association between sensitivity to sCD4-Ig and the relative infection of both ptCD4 cells (Fig 4.3e; $p< 0.0001$) and rhCD4 cells (Fig 4.3f; $p< 0.0001$). Thus, increased sensitivity to sCD4-Ig was significantly associated with the ability to use macaque CD4.

Data in Figure 4.3 suggested that increased sensitivity to sCD4 may predict for an increased ability to use macaque CD4 by HIV-1 Env, potentially allowing for the identification of other acute/early Envs that may have an increased ability to utilize macaque CD4. To examine this idea in more detail, we made use of the fact that 3 subtype A Envs included among the 39 tested acute/early Envs had been characterized in a previous study for sensitivity to neutralization by sCD4 (18). Env clones Q842d16 and Q769h5, which were previously identified as being sensitive to sCD4, were outliers among subtype A Envs with their increased ability to use ptCD4 (0.68- and 0.23-fold infection; compared to a median of 0.02-fold for other subtype A Envs) and rhCD4 (0.71- and 0.39-fold; compared to a median of 0.07-fold for other subtype A Envs; Fig 4.4a and b). Conversely, Q461e2, which had been identified as being insensitive to sCD4, was severely limited in its ability to use macaque CD4 (0.01-fold for ptCD4 and 0.04-fold for rhCD4, Fig 4.4c). When other Env clones obtained from subjects Q842, Q769, and Q461 were tested for their ability to utilize macaque CD4, Envs Q769b9, Q769d22, and Q461d1, which had been identified as being sensitive to sCD4, had an increased ability to use ptCD4 (0.15-, 0.25-, and 0.59-fold infectivity) and rhCD4 (0.26-, 0.32-, and 0.57-fold infectivity, Fig 4.4a, b, and c), whereas Env Q842d12, which had previously been identified as being resistant to neutralization by sCD4, was limited in its ability to use macaque CD4 (<0.01 fold infectivity for ptCD4 and 0.02-fold infectivity for rhCD4, Fig 4.4a) .

The Q461e2 and Q461d1 Env clones have very similar sequences (0.16% difference on the nucleotide level, (17)) but have very different neutralization properties, with Q461d1 being much more sensitive to neutralization by sCD4. These differences in neutralization sensitivity have been mapped to two amino acid differences in gp41, T569A and I675V (19). An Env encoding these

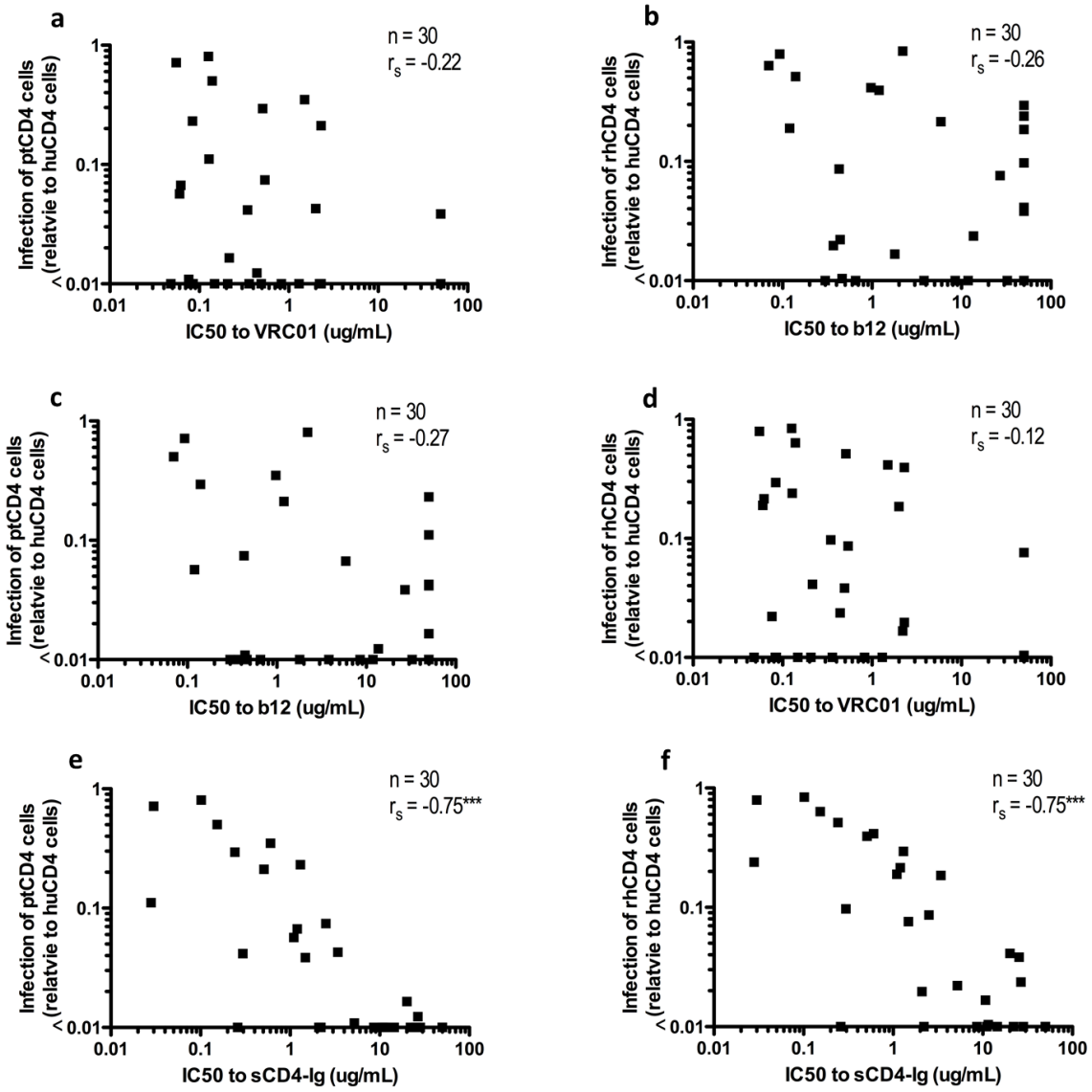

Fig. 4.3 Association between infection of cells expressing macaque CD4 and sensitivity to neutralization by CD4 binding site-directed monoclonal antibodies and sCD4-Ig. Association with infection of ptCD4 cells (panels *a*, *c*, and *e*) and infection of rhCD4 cells (panels *b*, *d*, and *f*) was determined using a subset of infectivity data from Figure 5.2 and neutralization data from a previous study describing the isolation and characterization of VRC01 (200). r_s represents the Spearman rank correlation coefficient. Each dot represents a variant tested for infection of cells expressing macaque CD4 in Fig. 4.2. *** $p < 0.0001$, Spearman correlation.

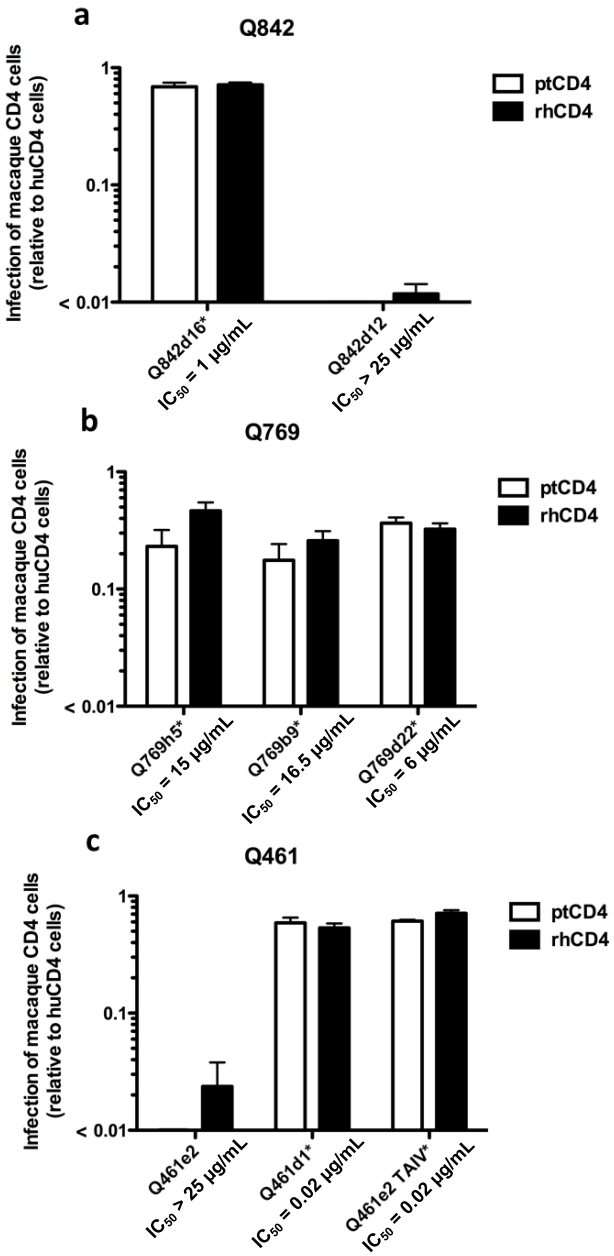

Fig. 4.4 Infection of macaque CD4 cell lines with pseudoviruses expressing subtype A Envs previously characterized for sensitivity to sCD4. Infection of ptCD4 cells (white bars) and rhCD4 cells (black bars) relative to huCD4 cells with GFP pseudoviruses expressing the Env clones from subjects Q842 *(a)*, Q769 *(b)*, and Q461 *(c)*. Error bars represent the standard deviation of the mean from two independent experiments. *represents those clones that were previously identified to have some sensitivity to sCD4 (18), the IC_{50} to sCD4 is shown underneath each Env clone.

changes, Q461e2 TAIV, was increased in its ability to use macaque CD4 by 33 to >60-fold (Fig 4.4c) relative to the parental Q461e2. Taken together, the data strongly suggests that the sensitivity of a given Env to sCD4 is predictive of its capacity to utilize macaque CD4 to mediate infection.

Association between macaque CD4 usage, the ability to use low levels of CD4, and naturally occurring polymorphisms flanking the CD4 binding site

Given that macaque CD4 usage was associated with sensitivity to sCD4, we also sought to determine whether it was associated with the ability to utilize low levels of cell surface CD4, another property that is attributed to increased CD4 binding site exposure (51-54, 65, 154, 178). To do this, the SIV/SHIV Envs and acute/early Envs were examined for their ability to mediate the infection of cells expressing low levels of CD4 relative to cells expressing high levels of CD4 ($CD4_{LOW}$ and $CD4_{HIGH}$ cells, as described in the Materials and Methods and performed by Sandra Emery). There was a strong association between both ptCD4 and rhCD4 usage and the ability to use low levels of huCD4 ($p< 0.0001$, Spearman Correlation, Fig 4.5a and b).

A number of naturally occurring polymorphisms in Env that flank the CD4 binding site have previously been shown to be associated with the ability to use low levels of CD4 to mediate infection. These polymorphisms include the presence of N362 (177), D279 (178) and N283 (53). Given the strong correlation between infection of $CD4_{LOW}$ cells and macaque CD4 usage, the acute/early Envs were examined for an association between the presence of these polymorphisms and increased macaque CD4 usage. Envs encoding these amino acid polymorphisms generally had only modest increases in median relative infection of macaque CD4 cells as compared to those Envs encoding other amino acids (Table 4.4). The presence of N283 had a slightly significant association with increased infection of rhCD4 cells ($p = 0.0439$, Mann-Whitney U Test), however this was not true in ptCD4 cells (Table 4.4).

Effect of amino acid changes outside of the CD4 binding site on macaque CD4 usage

To determine if the A204E and G312V amino acid changes had the same effect of increasing macaque CD4 usage in other global subtypes, we introduced the changes to 3 subtype D Envs (QB857b3, QA013h1, and QD435b5) and a subtype C Env (Q406f3). Unlike with subtype A Envs, introduction of the A204E and G312V changes to the subtype C and D Envs was much more deleterious to Env function, often resulting in viruses with very low titers in huCD4 cells. For this

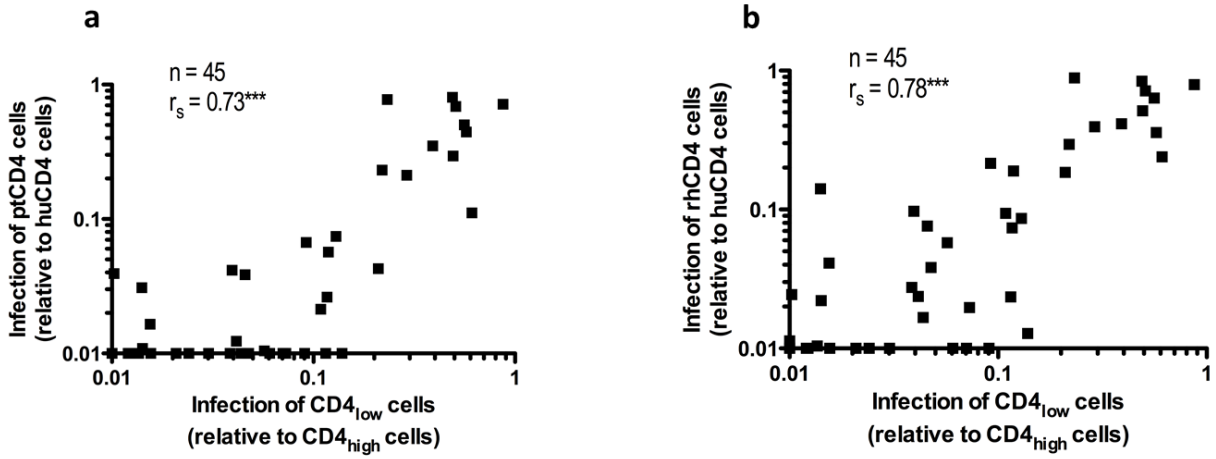

Fig. 4.5 Association between infection of cells expressing macaque CD4 and infection of cells expressing low levels of CD4. Association between infection of cells expressing *(a)* ptCD4, and *(b)* rhCD4 was and cells expressing low levels of CD4 was determined using infectivity data from Figure 4.2 and $CD4_{low/high}$ infectivity data obtained from Sandra Emery. r_s represents the Spearman rank correlation coefficient. *** $p < 0.0001$, Spearman correlation.

Table 4.4 Association of D279, N362 and N283 polymorphisms in gp120 with macaque CD4 usage

Presence of polymorphism[a]	n[b]	ptCD4 usage[c]		rhCD4 usage[c]	
		median (range)	p-value[d]	median (range)	p-value[d]
+ D279	12	0.03 (0.01, 0.11)	0.7188	0.02 (0.01, 0.24)	0.3749
- D279	31	0.01 (0.01, 0.69)		0.01 (0.01, 0.71)	
+ N362	17	0.01 (0.01, 0.35)	0.3162	0.05 (0.01, 0.41)	0.7902
- N362	26	0.01 (0.01, 0.69)		0.02 (0.01, 0.71	
+ N283	10	0.03 (0.01, 0.59)	0.2391	0.12 (0.01, 0.57)	0.0439
- N283	33	0.01 (0.01, 0.69)		0.02 (0.01, 0.71)	

[a]Indicates the presence (+) or absence (-) of the indicated polymorphism
[b]Number of Envs with or without the indicated polymorphism
[c]Infection of cells expressing macaque CD4 relative to cells expressing huCD4
[d]As calculated by a Mann-Whitney U Test comparing the medians

reason the infectivity of the QC406f3 G312V, QB857b3 A204E, and QD435b5 G312V variants could not be determined. For the remaining Env variants, the G312V and A204E amino acid changes increased the Env's ability to utilize macaque CD4 ranging from a >17-fold increase for the QD435b5 A204E variant in ptCD4 cells to a > 80-fold increase for the QA013h1 A204E variant in rhCD4 cells (Fig 4.6a). The G312V change had variable effects on the two Envs in which it was tested. In the case of QB857b3, introduction of the G312V change increased infection in ptCD4 and rhCD4 cells by approximately 20-fold and 30-fold, respectively. Surprisingly, the change had very little impact on the ability of the QA013h1 Env to use macaque CD4, with increases in infection of <2-fold in macaque CD4 cells.

Amino acid determinants of huCD4 allowing for increased usage by circulating HIV-1 strains

An amino acid alignment of the D1 and D2 domains of CD4 shows that there are 21 amino acid differences between humans and macaque CD4 in this region, including non-conservative changes at critical residues S23 (N23 in macaques) and N52 (S52 in macaques, Fig. 4.7a). While the rhCD4 sequence used in this study was completely identical to ptCD4 in the D1 and D2 domains, the most commonly used reference sequence for rhCD4 (GenBank accession no. NM_001042662) differs from the ptCD4 sequence at 3 residues in this region, including a serine to leucine change at position 42, a residue critical to gp120 binding. To examine whether these differences might indicate the existence of polymorphisms, the 3rd and 4th exons, which encode the entirety of the D1 and D2 domains, were amplified from genomic DNA from 15 unrelated Indian Rhs and 15 unrelated Chinese Rhs, and sequenced. Despite this extensive analysis, no polymorphisms were identified in the sequences, with all of the 30 animals surveyed encoded identical residues in these regions (data not shown) that were consistent with sequence shown in Fig. 4.7a, and with previously published sequences (60).

To investigate which of the amino acid differences between huCD4 and ptCD4 were most critical for mediating infection by circulating HIV-1 strains, chimeric CD4s were constructed to look for a gain of function induced by introducing human residues to ptCD4 (Fig 4.7b). Chimeric CD4s containing the human D1-D2 region (huD1D2) and D1 region (huD1) performed as well as huCD4 at mediating infection by a pseudovirus expressing the Q23-17 Env (Fig 4.7b). Thus, the critical residues for mediating infection by Q23-17 reside within the D1 domain. Amino acid differences were further dissected by making ptCD4 clones including human residues in the N-terminal of D1 (encompassing changes N17T, N23S, T24I, and I39N) or the C-terminal of D1 (encompassing changes S52N, K59R, C66N, S68P, and M69L). Chimeric ptCD4 encoding the N-terminus of huD1

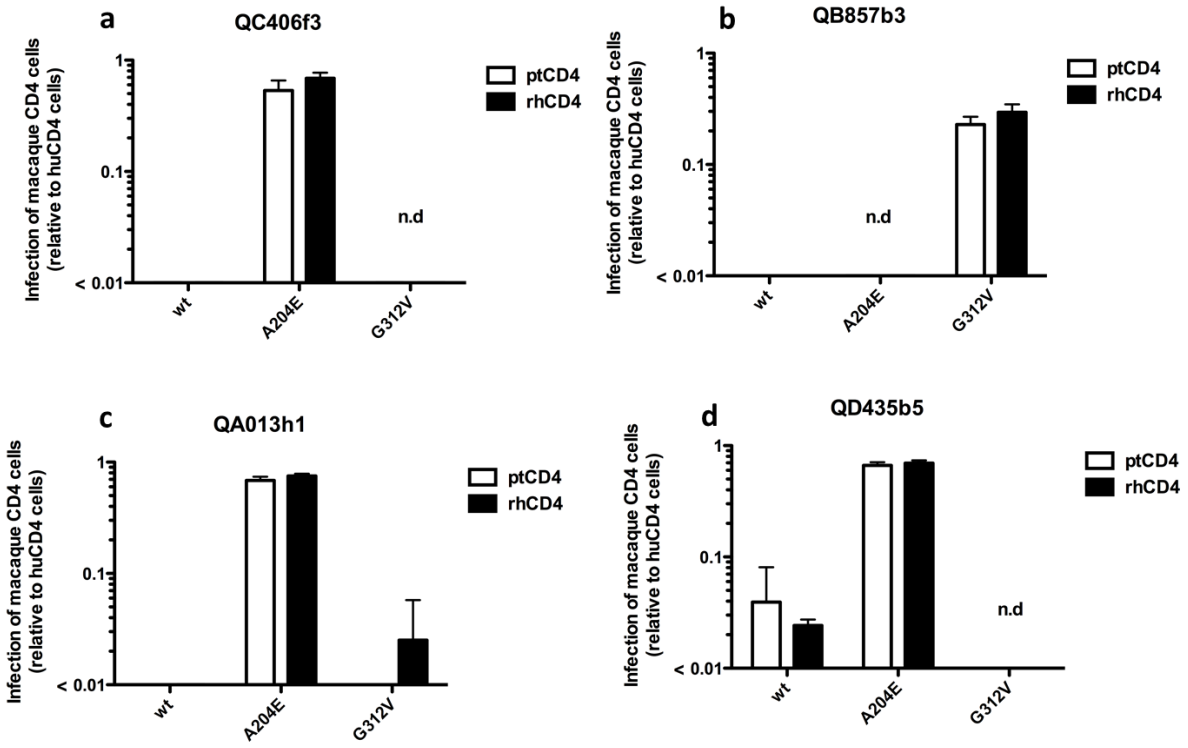

Fig. 4.6 Infection of macaque CD4 cell lines with pseudoviruses expressing subtype C and D A204E and G312V Env variants. Infection of ptCD4 cells (white bars) and rhCD4 cells (black bars) relative to huCD4 cells with GFP pseudoviruses expressing wild-type (wt), A204E, and G312V Env variants derived from clones QC406f3 *(a)*, Q857b3 *(b)*, QA013h1 *(c)*, and QD435b5 *(d)*. Error bars represent the standard deviation of the mean from two independent experiments. n.d., not determined due to insufficient titer.

mediated infection nearly as well as huCD4, increasing infection by approximately 50-fold relative to ptCD4. Conversely, ptCD4 encoding the C-terminus of huD1 was more limited in its ability to allow entry by the Q23-17 Env with entry decreased by approximately 6-fold compared to huCD4, but still increased relative to ptCD4 by approximately 10-fold (Fig 4.7b). Thus the residues most critical for increased infection by Q23-17 reside in the N-terminal of the D1 domain of CD4 (including T17, S23, I24, and N39), however there also appears to be some contribution from residues in the C terminus of the D1 domain.

Examination of a crystal structure of the D1 and D2 domain of CD4 did not reveal a role that any of the T17N, S23N, I24T, or N39I amino acid differences could be playing individually (Fig 4.8a). None of the residues are found in close proximity to the critical F43 or R59 residues in human CD4, that are thought to form hydrogen bonds with gp120 to stabilize binding (98). Interestingly, the S23 and I24 residues cluster spatially with the N39 residue, raising the possibility that these residues may interact together to modulate binding by gp120 (Fig. 4.8a).

To further investigate which of these amino acid changes was sufficient to allow infection by Q23-17, ptCD4 mutants encoding the individual T17N, S23N, I24T, and N39I changes, in addition to a double mutant encoding both the S23N and I24T changes, were created. The N17T, S23N, I24T and S23N/I24T changes to ptCD4 did not increase infectivity by a pseudovirus expressing the Q23-17 Env relative to ptCD4, however the I39N change increased infection to levels that were comparable to huCD4 (Fig. 5.8b). Thus, the I39N change to ptCD4 was sufficient to allow infection by viruses expressing the Q23-17 Env.

Reciprocal mutations encoding all four amino acid changes, as well as the single and double mutants described above were introduced into huCD4 to determine if these residues were necessary to allow infection by Q23-17. The N17T, N23S, T24I, and N23S/T24I changes all mediated infection by the Q23-17 Env to levels that were comparable to wild-type huCD4, thus supporting the data from the ptCD4 mutants that none of these amino acids plays a significant role as determinants of infection by Q23-17 (Fig. 5.8b). Unfortunately, both huCD4 expressing the N-terminus of ptD1 and huCD4 N39I were expressed poorly on the surface of cells (Fig. 5.8c), thus precluding them from analysis. Thus, while residue N39 is sufficient when introduced into ptCD4 to allow for infection mediated by the Q23-17 Env, its absolute necessity for mediating infection and the role of amino acids other than T17, S23, and I24 could not be determined.

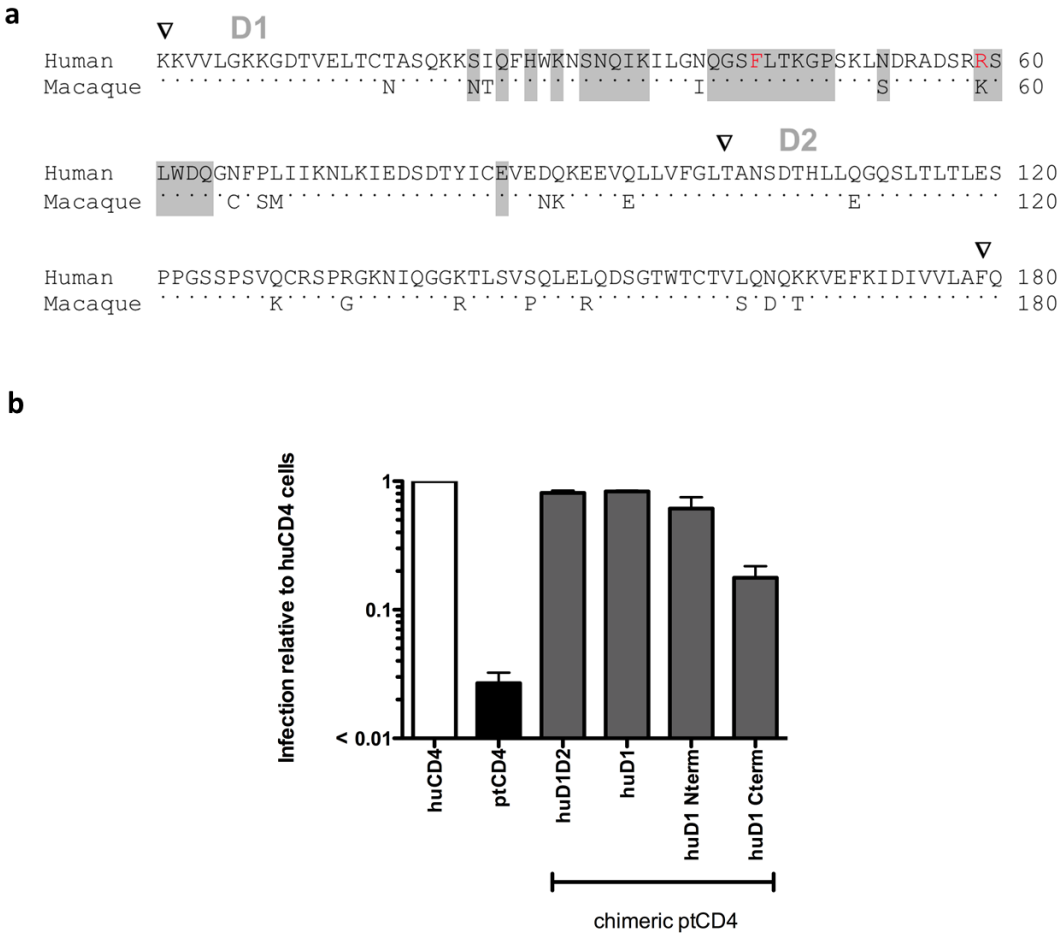

Fig. 4.7 Identification of subdomains in huCD4 sufficient for infection mediated by Q23-17 Env. *(a)* Amino acid alignment of the D1 and D2 domains of human and macaque CD4. Arrowheads above the alignment denote the beginning and end of the D1 and D2 domains. Dots indicate conserved residues, and positions where macaque and human sequence differ are shown as letters. The residues that are highlighted in grey have previously been implicated in gp120 binding (5), and residues highlighted in red are thought to form hydrogen bonds with gp120 (98). The black arrow indicates the breakpoint differentiating the N and C-terminal of huD1 in making chimeric ptCD4 expressing huD1 Nterm and huD1 Cterm. *(b)* Single-cycle infection of 293T cells transiently expressing huCCR5 and huCD4, ptCD4, or chimeric ptCD4 variants with GFP reporter pseudoviruses bearing the Q23-17 Env. The y-axis represents viral infection relative to huCD4, which is set to 1 for reference. Error bars represent the standard deviation of the mean from duplicate independent experiments.

To determine if introducing the I39N change to ptCD4 increased infection by other circulating HIV-1 strains, Cf2Th/syn CCR5 cells were engineered to stably express ptCD4 I39N. Infection by viruses carrying subtype A, B, C and D Envs identified in Figure 4.2b as being limited in their ability to utilize ptCD4 (<0.01-fold relative infection) was increased by >90 to 120-fold in ptCD4 I39N cells (Fig. 5.8d). Thus, the introduction of the I39N amino acid change to ptCD4 is sufficient to allow for increased infection by a wide range of circulating HIV-1 strains.

Discussion

In this chapter, inefficient entry of cells via macaque CD4 was identified as a significant barrier to HIV-1 replication including infection by viruses expressing diverse acute/early HIV-1 Envs. This defines a mechanism that may explain a major impediment to designing relevant SHIVs for use in macaque studies. Both sensitivity to sCD4 and infection of cells expressing low levels of surface CD4 were determined to be predictors of the ability of a given Env to use macaque CD4. Importantly, we show that the property of sensitivity to sCD4 provides a means of identifying HIV-1 Envs with increased capacity to use macaque CD4, which may allow for the further identification of Envs for use in the construction of SHIVs. Surprisingly, the inefficient usage of macaque CD4 could be relieved by introducing a single amino acid change to macaque CD4, which increased infection by in all strains tested.

The association of sensitivity to sCD4 and the ability to infect cells expressing macaque CD4 provides further support for a hypothesis that we proposed in Chapter 2 based on a limited number of subtype A Envs, suggesting that sensitivity to sCD4 may act as a predictor for the ability to use macaque CD4. The property of increased sensitivity to sCD4 enabled us to identify other acute/early subtype A Envs that have an increased ability to use macaque CD4. Additionally, we found that infection using macaque CD4 correlates with the ability to utilize low levels of human CD4, suggesting that the ability to utilize low levels of human CD4 can also be used as a predictor for the ability to mediate infection using macaque CD4. Previous studies showing the high correlation between sensitivity to sCD4 and the ability to use low levels of surface CD4 also support this hypothesis (51, 177, 178).

Naturally occurring polymorphisms flanking the CD4 binding site have previously been found to be associated with the ability to use low levels of CD4 (53, 177, 178), a property we found to be highly correlated with macaque CD4 usage. These polymorphisms were only nominally

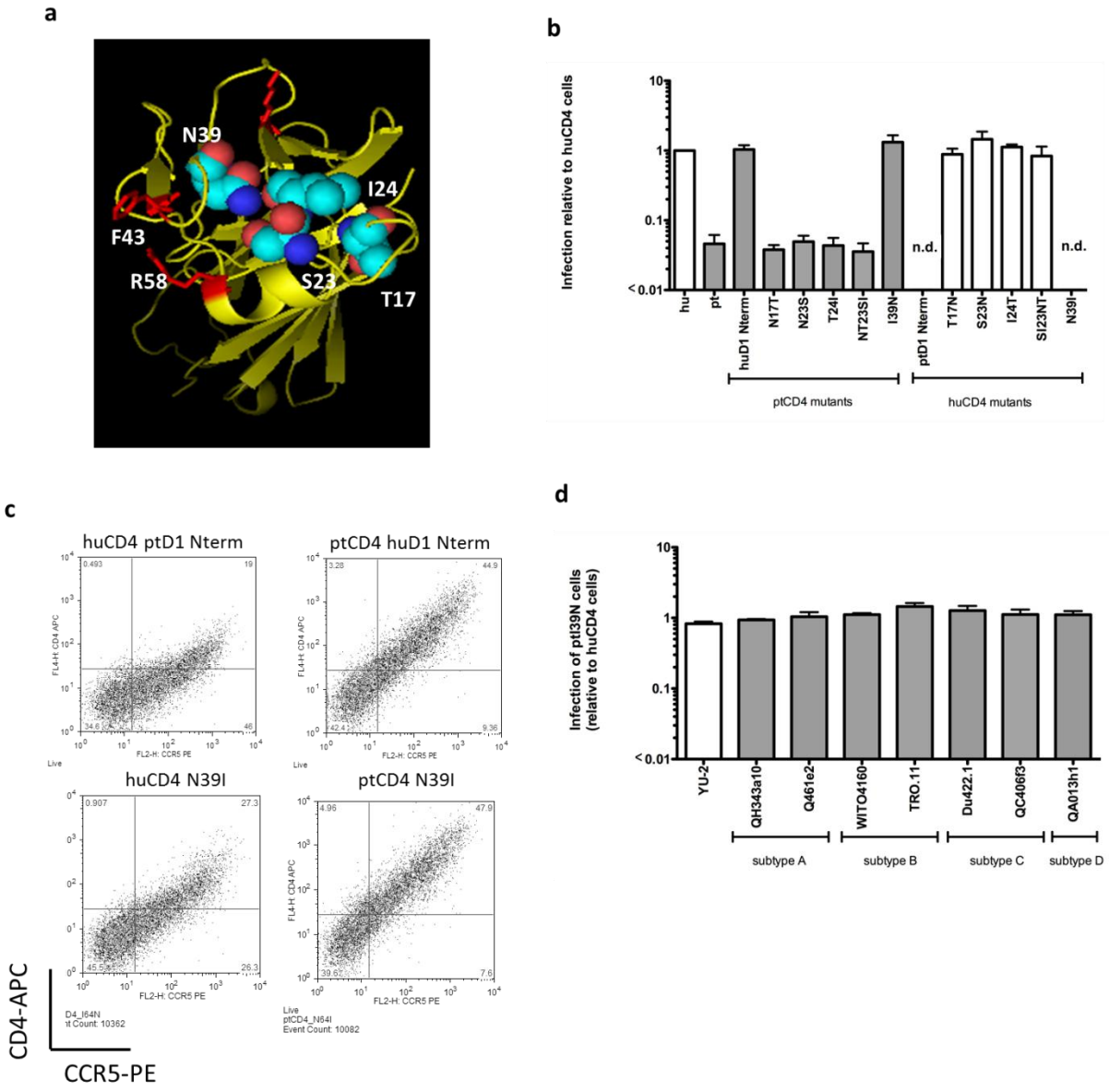
a
N39
I24
F43
R58
S23
T17
b
Infection relative to huCD4 cells
10
1
0.1
< 0.01
n.d.
n.d.
hu-
pt-
huD1 Nterm-
N17T-
N23S-
T24I-
NT23SI-
I39N-
ptD1 Nterm-
T17N-
S23N-
I24T-
SI23NT-
N39I-
ptCD4 mutants
huCD4 mutants
c
huCD4 ptD1 Nterm
ptCD4 huD1 Nterm
huCD4 N39I
ptCD4 N39I
FL4-H: CD4 APC
FL2-H: CCR5 PE
Live
0.493
19
34.6
46
3.28
44.9
42.4
9.36
0.907
27.3
45.5
26.3
4.96
47.9
39.6
7.6
D4_I64N
Event Count: 10362
Live
ptCD4_N64I
Event Count: 10082
CD4-APC
CCR5-PE
d
Infection of ptI39N cells
(relative to huCD4 cells)
10
1
0.1
< 0.01
YU-2
QH343a10
Q461e2
WITO4160
TRO.11
Du422.1
QC406f3
QA013h1
subtype A
subtype B
subtype C
subtype D

Fig 4.8 Effect of single residue changes in CD4 on infection mediated by Q23-17 Env and other circulating HIV-1 Envs. *(a)* Location of N39, I24, S23, and T17 in the crystal structure of the D1 and D2 domains of huCD4. The critical residues identified in Fig. 4.7 are shown in space-filling mode, while the rest of CD4 is shown as a yellow ribbon diagram. Two previously identified critical residues, F43 and R58 (98), are shown in red. The crystal structure co-ordinates were obtained from a published crystal structure of the CCR5 N-terminus, YU-2 gp120, and CD4 [(84), PDB ID:2QAD], and were visualized in PyMol. *(b)* Single-cycle infection of 293T cells transiently expressing huCCR5 and huCD4, ptCD4, or huCD4 and ptCD4 point mutants with GFP reporter pseudoviruses bearing the Q23-17 Env. The y-axis represents viral infection relative to huCD4, which is set to 1 for reference. Error bars represent the standard deviation of the mean from duplicate independent experiments. *n.d.*, not determined due to insufficient CD4 expression. *(c)* Transient expression of huCCR5 and huCD4 or ptCD4 variants in 293T cells. The y-axis represent CD4 expression and the x-axis represents CCR5 expression as determined by flow cytometry. The CD4 variant is labeled at the top of each dot-plot. These plots are representative of at least three independent experiments. *(d)* Infection of Cf2Th/syn CCR5 cells stably expressing ptCD4 I39N relative to huCD4-expressing cells with GFP pseudoviruses expressing the YU-2 Env, and acute/early Envs from subtypes A to D (as denoted on the *x*-axis). Error bars represent the standard deviation of the mean from two independent experiments.

associated with macaque CD4 usage, and, in contrast to previous studies (53, 177, 178), we did not observe a statistically significant association between the presence of these polymorphisms and infection of $CD4_{LOW}$ cells (not shown). One reason for this difference may be that these polymorphisms were originally identified in late-stage subtype B variants (53, 177, 178) and it is possible that there are other Env determinants that are required to further modulate the influence of these polymorphisms that are not present in acute/early Envs from other subtypes (51).

Attempts to directly induce macaque CD4-tropism in subtype C and D Env variants by introduction of the previously identified A204E and G312V changes met with mixed results compared to the subtype A A204E and G312V variants described in Chapter 2. Introduction of the G312V or A204E amino acid changes had a negative impact on the functionality of subtype C and D Envs, with viruses expressing 2/4 of the G312V variants, and 1/4 of the A204E variants having low titers that precluded them from further examination. Moreover, the QA013H1 G312V Env variant showed only a minimal (< 2-fold) increase in macaque CD4 usage. These results may indicate that other determinants are needed to complement the A204E and G312V amino acid changes in subtype C and D Envs either to allow for basic functionality, or to increase usage of macaque CD4. Nonetheless, the QC406f3 A204E, QB857b3 G312V, QA013h1 A204E and QD435b5 A204E variants all had a >40-fold increased ability to mediate infection using macaque CD4 as compared to wild-type. These Envs warrant further characterization for their replicative and neutralization phenotypes as they may be good candidates for use in the development of SHIV-Cs and SHIV-Ds.

Additionally, we identified a single amino acid change, I39N, to macaque CD4 that increased infection >30-fold by viruses carrying acute/early HIV-1 Envs from all subtypes tested. The mechanism by which I39N increases ptCD4-mediated infection is unclear, however, given its proximity to a loop adjacent to F43, the CD4 residue thought to have the largest area of interaction with gp120 (98), it may involve global conformational changes that influence the positioning of F43. Surprisingly, huCD4 encoding the reciprocal N39I change did not express well on the cell surface, indicating the presence of other epistatic interactions that may be required to accommodate the N39I change. Residue 39 of CD4 has been found to be under positive selection (205), thus it is possible that other residues that have also been found to be under positive selection which were not directly addressed in this study, such as S52 or C65, may have co-evolved with I39 to allow proper folding and expression of macaque CD4. This idea is further supported by the fact that chimeric ptCD4 encoding the C-terminal portion of huD1, which includes these residues, conferred a partial increase in entry by viruses expressing the Q23-17 Env. The observation that viruses expressing acute/early

Envs displayed slightly increased infection in rhCD4 cells versus ptCD4 cells is puzzling as it is inconsistent with the mapping data given the fact that rhCD4 and ptCD4 are identical in critical regions for gp120 binding. There have been previous studies identifying amino acid changes outside of the D1 and D2 domains of CD4 that can modulate fusion after binding (26, 122), thus it is possible that the sole amino acid difference between rhCD4 (T324) and ptCD4 (A324), located in the D4 domain, may modulate fusion by HIV-1 Env.

The utility of macaques as models to study HIV-1 transmission and develop strategies to prevent infection depends in part on the relevance of SHIVs used in the infection of these animals to HIV-1 in humans. The construction of relevant SHIVs will require the incorporation of HIV-1 Envs, such as those obtained from early in infection, that are able to recapitulate aspects of HIV-1 transmission, neutralization phenotypes, and early infection in humans. In this chapter we have found that inefficient usage of macaque CD4 imposes a severe limitation on which acute/early HIV-1 Envs may be used as the basis for SHIVs. However, we have identified properties of acute/early Envs, including sensitivity to neutralization by sCD4, as predictors of macaque CD4 usage. Importantly, we have used this property to identify acute/early subtype A Envs whose increased ability to use macaque CD4 make them good candidates for use in relevant SHIV models. Furthermore, we have identified a single amino acid change to macaque CD4 that relieves the inefficient usage of macaque CD4 by acute/early HIV-1 Envs. This finding provides insight into novel CD4 determinants necessary to support infection by acute/early HIV-1 strains, and suggests that lab adapted and late-stage HIV-1 Envs may have significantly different mechanisms of interaction with CD4.

Chapter 5

Conclusions and Future Directions

The creation of SHIVs expressing HIV-1 envelopes (Envs) that are representative of the HIV-1 strains circulating among humans is a pressing need in order to establish macaque models of HIV-1/AIDS that are more relevant to human infection (56, 194). Such viruses are required to provide heterologous challenge viruses for use in macaque trials, such as in evaluating the breadth and potency of the immune response elicited by vaccine strategies. Unfortunately the incorporation of circulating HIV-1 Envs into existing SHIVs has been difficult because these SHIVs are often unable to establish infection in macaque cells (78), despite being able to infect human cells. The reasons for these species-specific limitations to the Envs that can be used in SHIVs, and the properties that differentiate Envs that successfully mediate the infection of macaque cells versus those that do not has not been well-explored. Thus, the goals of this thesis were to identify causes of the species-specific limitations to infection of macaque cells, and to identify characteristics of HIV-1 Envs that predict for better infection of macaque cells, thus allowing for the development of additional SHIVs that include HIV-1 Envs of interest. In this chapter, the key findings of the thesis are identified, and an expanded discussion of the future directions and implications stemming from these findings is provided.

In Vivo Adaptation of SHIV-As

In Chapter 2 we identified amino acid changes G312V and A204E to subtype A HIV-1 Envs that allowed for increased replication in Pt cells. Following this, we characterized SHIVs expressing the G312V and A204E subtype A Env variants (SHIV-As) for their ability to replicate in human and macaque cells. We identified two viruses, SHIV QF495 A204E and SHIV MG505 G312V, that we believe warrant further development for use *in vivo*. All pathogenic SHIVs that have been developed to date have required further adaptation *in vivo*, regardless of their ability to mediate infection in macaque cells *in vitro* [for example, (130, 173-175)].

The first step in developing pathogenic SHIV-As will be to infect macaques with the SHIV-As we characterized *in vitro* to determine if they have any ability to replicate *in vivo*. Viral loads will be monitored in the blood by real-time PCR and memory CD4+ T cells, the preferential target cells of CCR5-tropic viruses, can be monitored by flow cytometry. Based on the performance of other CCR5-tropic SHIVs, it is quite probable that our SHIV-As will be poorly infectious *in vivo* and that

we will not see any depletion of target cells. If this is the case, then the infected animals can be depleted of their CD8+ T cells, at which point we would expect to see more sustained levels of viral replication and decreases in the memory CD4+ T cell population (130). This will at least indicate that the SHIV-As are able to establish infection in an immunocompromised animal.

From there we will need to begin the process of adapting the virus for growth *in vivo*. Typically two different approaches are used: rapid passaging, or the more time-consuming serial passage (130, 173, 174). Rapid passaging takes virus harvested from one animal during the peak levels of infection in the acute phase (~1-2 weeks post-infection) and uses that virus to inoculate the next animal. This process is much less time consuming than serial passaging, but it is disadvantageous because the virus that adapts for replication in these animals does so in the absence of the humoral immune response. As a result, the adapted viruses may also evolve to become more sensitive to neutralization by plasma antibodies (130, 173, 174). Thus, to avoid this fate, the SHIV-As will be serially passaged through multiple animals. This process involves the inoculation of an animal which will be followed for several weeks for the emergence of viral replication and disease progression. If viral replication becomes attenuated, the infected macaque can be depleted of CD8+ T cells to enable increased viral replication and to maximize the chances of viral outgrowth, before serial passage of large amounts of infected cells and cell-free virus to subsequent macaques. This procedure has been used in the past to generate pathogenic SHIV populations in approximately 1 year (130).

The adaptation of SHIV-As to become pathogenic *in vivo* will be informative for future studies. In particular, CCR5-tropic SHIVs that have been adapted to date by serial passage *in vivo* have expressed HIV-1 envelopes that were sensitive to neutralization. Upon adaptation, these viruses displayed a large number of amino acid changes in Env that ultimately resulted in variants that were less sensitive to neutralization than the parental Env (83, 175). It is possible then, that adaptation of SHIV-As expressing Envs that are already relatively insensitive to neutralization, such as the MG505.H3 G312V Env, for pathogenic replication *in vivo* may require only minimal changes, and so the adaptation process might occur more quickly with less changes to the Env phenotype. This would bode well for the adaptation of additonal SHIVs expressing Envs with moderate or low sensitivity to neutralization that were predicted based on their sensitivity to soluble CD4 to allow for increased infection of macaque cells, because it would suggest that the neutralization phenotypes of the *in vivo* adapted Envs may not change greatly, thus increasing the rapidity with which pathogenic SHIVs

expressing diverse Envs with neutralization phenotypes more reflective of circulating HIV-1 strains can be established.

Future experiments to perform with an expanded repertoire of relevant SHIVs

One of the major applications of SHIVs is to provide an indication of the correlates of protection that will required of a successful HIV-1 vaccine. Results from a number of studies have indicated that relatively little passively transferred neutralizing antibodies are required to protect macaques against SHIV challenges (24, 74-76). However, these results do not correspond with data from HIV-1 infection in humans, which suggests that the presence of relatively high levels of neutralizing antibodies is not protective against HIV-1 infection (16, 113). A major caveat of many of the macaque studies performed to date is that the SHIV being tested is often matched to a neutralizing antibody to which it is especially susceptible, such as in the case of $SHIV_{SF162P3}$ and b12 (74), and so may grossly underestimate the amount of neutralizing antibody that will be required to elicit for a protective HIV-1 vaccine. That being said, the ability to perform these infusion studies more robustly is currently limited by the lack of SHIV models that express HIV-1 Envs whose sensitivity to neutralization if more reflective of circulating HIV-1 strains. In Chapter 4 we were able to identify acute/early Envs based on their sensitivity to sCD4 that had an increased ability to use macaque CD4, but still have decreased sensitivity to b12 that is more reflective of circulating strains. Moreover, the MG505 G312V Env, which we assessed in Chapter 3 for its ability to mediate infection in macaque cells when expressed in the setting of a traditional SHIV, also displays decreased sensitivity to b12. Thus, the creation of SHIVs expressing some acute/early Envs, including the subtype A Env Q769h5 and the subtype C Env ZM109F.PB4, in addition to the SHIV MG505 G312V may result in challenge viruses that provide more realistic benchmarks for use in passive infusion studies in macaques.

The identification of additional HIV-1 Envs with an increased ability to use macaque CD4 will eventually lead to an expanded repertoire of diverse SHIVs representing several different global subtypes. One application for which this expanded repertoire of SHIVs could be especially useful is in the discovery of broadly neutralizing antibodies. Neutralizing antibodies have previously been characterized and isolated from macaques infected with $SHIV_{162P3}$ (96, 196). Interestingly, the breadth of the neutralizing antibody response has been associated with increased Env diversity (138). In recognition of this idea, it has been suggested that individuals infected with more than one strain of HIV-1 (superinfected) may have a greater propensity to produce broadly neutralizing antibodies. With an increased repertoire of diverse SHIVs, it will be possible to infect macaques with multiple

diverse SHIVs (induced superinfection). This strategy of induced superinfection will allow for superinfection of a large number of animals by a greater number of viruses expressing more diverse Envs than what is normally observed in human infection. After induced superinfection, macaques could be followed for the emergence of broad and potent neutralizing antibodies, and any antibodies of interest could then be isolated for further study and potential therapeutic applications.

Future experiments to address limitations in 'minimal' SHIV development

The development of a 'minimal' SHIV that establishes pathogenic infection in macaques remains an elusive goal as these SHIVs have so far failed to establish productive, high levels of infection *in vivo* (70, 187, 188). One potential explanation for the lack of success of minimal SHIVs *in vivo* may be due to the absence of a viral antagonist to macaque tetherin (86, 129, 162) or other interferon-stimulated gene products whose effects are generally not examined in the evaluation of SHIVs for infection of macaque cells. Interestingly, it has been demonstrated in *in vitro* assays that some HIV-1 Vpu proteins display an increased ability to antagonize macaque tetherin, and that excluding Vpu from traditional SHIVs can result in decreased pathogenesis (77, 82, 156, 171, 176). Thus, it is possible that the Vpu encoded by the Pt cell-adapted $HIVA_{Q23}/SIV_{vif}$ may have some ability to counteract macaque tetherin. This idea could be directly assayed *in vitro* by examining the ability of the Vpu protein encoded by Q23-17 to mediate viral release in cells expressing Pt tetherin. If the Q23-17 Vpu does show some propensity to counteract Pt tetherin, then the Pt cell-adapted $HIVA_{Q23}/SIV_{vif}$ would warrant further *in vivo* evaluation. Alternatively, if the Q23-17 Vpu protein does not display any ability to downregulate Pt tetherin, then $HIVA_{Q23}/SIV_{vif}$ could be further engineered to express the Vpu from an HIV-1 strain, such as DH12, which is known to have an increased effect against macaque tetherin (171), and this new virus could then be further assessed *in vivo*.

Future experiments to address limitations in 'traditional' SHIV Development

We were surprised to find that a number of the traditional SHIVs expressing the A204E and G312V Env variants replicated poorly in primary human and Rh cells, despite the fact that these same Env variants were able to establish robust spreading infection in the context of a minimal SHIV. Importantly this finding was not species-specific as all traditional SHIVs replicated in immortalized Pt cells, suggesting that the reasons for the failed replication go beyond the ability of the Envs to utilize macaque CD4. The increased sensitivity of the G312V variants to TAK779 hints at the fact that their affinity for CCR5 and fusion properties may have been altered in the process of adapting them to immortalized Pt cells. While these altered properties may not be detrimental in the setting of

a minimal SHIV replicating in cells with high CCR5 expression, they may pose a problem to growth in primary cells, in particular if Env expression in the context of a traditional SHIV is not optimal.

It is possible that intraviral protein interactions that take place in the traditional SHIV, such as the interaction between matrix (MA) and gp41 thought to be required for proper Env incorporation into the nascent virion [as reviewed in (28)], are not optimal in the setting of traditional SHIVs in which the SIV matrix must interact with the HIV-1 gp41. Interestingly, the idea that inefficient MA-gp41 interactions may be prohibiting SHIV replication has been hinted at in previous reports of traditional SHIVs that failed establish replication even in human T cells lines (31, 93, 147). However the authors of these studies suggested the Rev and Tat proteins as being the major determinants in SHIVs that allow for increased replication. This explanation seems somewhat unlikely given the high conservation of the Tat and Rev proteins between HIV-1 strains. Moreover, in creating SHIVs expressing heterologous Rev proteins to investigate the role of Rev in increasing SHIV infection, these studies cannot rule out the role that heterologous gp41 might play in increasing infection given that the portion of *env* open reading frame encoding gp41 overlaps with the second exon of *rev* (as pictured in Figure 1.1a) (31, 93, 147). Thus the role that efficient MA-gp41 interactions play in the successful development of traditional SHIVs warrants another look. The specific determinants of HIV-1 gp41 that allow for efficient interaction with the SIV MA could be mapped using gp41 sequences from HIV-1 strains that work well in the setting of traditional SHIVs, such as SF162, compared to HIV-1 strains that do not work well in the setting of traditional SHIVs, such as Q23-17.

Further characterization of the A204E and G312V Env variants and potential implications for immunogen design and antibody discovery

The G312V change occurred at the very tip of the V3 loop and the A204E change occurred adjacent to the bridging sheet, and these particular residues are very highly conserved across all HIV-1 subtypes. The introduction of the A204E and G312V amino acid changes to subtype A Envs greatly increased their sensitivity to inhibition by soluble CD4 (sCD4) and in some cases, neutralization by the b12 monoclonal antibody, suggesting increased exposure of the CD4 binding site. This is significant because the CD4 binding site is an especially attractive target for broadly neutralizing antibodies (25, 200), so further understanding of how to create immunogens that focus the immune response to the CD4 binding site, as well as how the virus is able to mask the CD4 binding site, are important questions. Many amino acid changes that greatly increase the sensitivity of HIV-1 Env to sCD4 and CD4-binding site directed antibodies have been identified [as in (51, 53, 177), for example]. However these changes are typically context-specific, meaning they only

increase the neutralization sensitivity when introduced to a limited number of closely-related Envs. Thus, the A204E and G312V amino acid changes are of special interest given the conservation of their effect on the ability to use macaque CD4 in a wide range of subtype A Envs and even Envs from other subtypes.

The mechanism of action of the A204E and G312V amino acid changes is not especially apparent given that they are not believed to be in close proximity to the CD4-binding site, as ascertained by crystal structures of CD4-bound monomeric gp120 (98). This suggests that their influence may be on the overall quaternary structure of the HIV-1 Env. This hypothesis can be tested formally by evaluating sCD4 or b12 binding imparted by the A204E or G312V changes in the context of monomeric gp120 as compared to cell surface expressed Envs. If these amino acid changes are acting on the level of quaternary structure then one would expect to see differences in binding only with surface expressed Env, and not with gp120 monomers. The determinants and specific intraprotomer interactions that occur in conjunction with the A204E or G312V change to induce the exposure of the CD4 binding site could then be examined in more detail by characterizing heterogeneous wild-type and mutant Env trimers, as has been done in other studies characterizing the influence of the V1/V2 loops on masking epitopes on V3 (157).

Surprisingly, the phenotype of the G312V variants may have implications for naturally circulating HIV-1 variants. In Brazil, a high frequency of circulating subtype B Env variants carrying a ‘GWG’ motif at the tip of the V3 loop have been described (27, 40, 101). Much like the G312V change, the substitution of a tryptophan in the context of the highly conserved GPG motif might be expected to disrupt this motif, and could similarly lead to a more exposed CD4 binding site as appears to be the case with the G312V variants. Interestingly, individuals naturally infected by the GWG variants tend to display slower disease progression and better viral control compared to individuals harboring viruses expressing the more typical GPG motif (27, 40, 101). Given the increased sensitivity to neutralization of many of the G312V variants, one hypothesis for why the circulating GWG variants are better controlled is that they are more sensitive to neutralization by CD4-binding site directed antibodies, and this idea can readily be tested by examining the sensitivity of GWG variants to neutralization to sCD4, b12, and other monoclonal antibodies. If it is true that the GWG variants are more sensitive to neutralization due to a more exposed binding site, then another interesting implication of the circulating GWG variants is that in individuals naturally harboring the GWG variants, the viruses may be acting a natural immunogens that elicit neutralizing antibodies directed towards the CD4-binding site. If this is the case, then individuals harboring the GWG

variants may have an enriched pool of CD4-binding site directed antibodies, and isolation of antibodies from these individuals may result in the discovery of broadly neutralizing antibodies with potential therapeutic applications.

Inefficient usage of non-human primate CD4 as a bottle-neck to the cross-species transmission of other lentiviruses

In Chapter 4, we found that the significant block imposed by inefficient usage of ptCD4 on HIV-1 replication could be relieved by introducing a single amino acid change, I39N, to ptCD4. Interestingly, this residue and others in CD4 have been identified as being under positive selection in the primate lineage, although the functional consequences of this have not been directly explored (205) . Much like in human CD4, sooty mangabey CD4 retains an asparagine at position 39 (61, 205). Although the SIV_{MAC}s that were originally introduced to macaques arose from SIV_{SM} (2, 3), it has been demonstrated that there is a severe bottle-neck that occurs in adapting SIV_{SM} for replication in macaques (45). Notably, the variants that emerged in the rhesus macaques all had lost a glycosylation site in the V1 loop, and loss of this glycosylation site had previously been associated with CD4 independence (45). This suggests, much like what was seen when we adapted our minimal SHIV-A for replication in macaque cells, that differences in CD4 between macaques and sooty mangabey may have driven the adaptation of SIV_{SM} to rhesus macaques, and so it is possible that similar CD4-induced bottlenecks as the one imposed by macaque CD4 on infection by HIV-1 may be present in the transmission of lentiviruses between other non-human primate species. This hypothesis can be examined in more detail by assaying for the ability of viruses expressing SIV_{SM} Envs to mediate infection of cells expressing macaque CD4 as compared to cells expressing sooty mangabey CD4.

Interestingly, the bottle-neck imposed by macaque CD4 on HIV-1 Env may also be true of CD4 from other non-human primates. In particular, African green monkeys (AGMs) encode a lysine at position 39 of CD4 (61, 205). Thus, one would predict that inefficient usage of AGM CD4 may also impose a limitation on infection mediated by HIV-1 Envs. This can be tested by making cells stably expressing AGM CD4 and assaying for the ability of HIV-1 Envs to mediate infection of these cells. Further sequencing of CD4 from additional non-human primates and assaying of Envs from SIVs that are endemic in other non-human primates may uncover additional clues into the role that amino acid differences in CD4 play in limiting the cross-species transmission of lentiviruses.

Conclusion

In conclusion, we have identified inefficient usage of macaque CD4 as a barrier to infection mediated by circulating HIV-1 envelopes. Furthermore, we have identified sensitivity to soluble CD4 as a property of HIV-1 envelopes that predicts for increased ability to use macaque CD4, and have uncovered specific amino acid changes to the HIV-1 envelope that allow for increased usage of macaque CD4. These findings have immediate implications on the construction of SHIVs that include clinically relevant HIV-1 envelopes. Such SHIVs will provide improved models of critical aspects of HIV-1 biology, and so, will be important for use in future vaccine trials and pathogenesis studies in macaques.

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Curriculum Vitæ

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Personal Information

Date of Birth: 25/07/1982
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Education

2006-2012 PhD - Molecular and Cellular Biology Program
University of Washington, Seattle, Washington, USA
Dissertation: Adaptation of HIV-1 envelope to macaque cells and implications for the design of improved models of HIV/AIDS.
Supervisor: Dr. Julie Overbaugh, Human Biology Division, Fred Hutchinson Cancer Research Center

2001-2006 Bachelor of Science - Biology and Bioinformatics
University of Waterloo, Waterloo, Ontario, Canada

Research Experience

June 2007 – Feb. 2012 *Research Assistant - Overbaugh Lab, Human Biology Division, FHCRC*

Jan. – Apr. 2007 *Rotating Graduate Student – Tewari Lab, Human Biology Division, FHCRC*

Oct. – Dec. 2006 *Rotating Graduate Student – Geballe Lab, Human Biology Division, FHCRC*

May – Aug. 2006 *Summer Internship - Mousseau Lab, Neuropsychiatry Research Unit, University of Saskatchewan, Saskatoon, SK*

May – Aug. 2005 *Summer Internship – Haston Lab, Meakins-Christie Laboratories, McGill University, Montreal, PQ*

Jan. – Apr. 2005, Sept. – Dec. 2005 *Undergraduate Research Assistant – Zacharewski Lab, Dept. of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI*

Sept. –Dec. 2003, May – Aug. 2004 *Undergraduate Research Assistant – McKerlie Lab, Physiology and Experimental Medicine Program, Hospital For Sick Children, Toronto, ON*

Publications

Humes, D., and J. Overbaugh. 2011. "Adaptation of subtype a human immunodeficiency virus type 1 envelope to pig-tailed macaque cells". J. Virol. 85(9):4409-20.

Piantadosi, A., **D. Humes**, B. Chohan, R. S. McClelland, and J. Overbaugh. 2009. "Analysis of the percentage of human immunodeficiency virus type 1 sequences that are hypermutated and markers of disease progression in a longitudinal cohort, including one individual with a partially defective Vif". J. Virol. 83:7805-7814.

Publications (continued)

Nemati, B., W. Atmodjo, S. Gagnon, **D. Humes**, C. McKerlie, F. Kaplan, and N. B. Sweezey. 2008. "Glucocorticoid receptor disruption delays structural maturation in the lungs of newborn mice". Pediatr. Pulmonol. 43:125-133.

Haston, C. K., **D. G. Humes**, and M. Lafleur. 2007. "X chromosome transmission ratio distortion in Cftr +/- intercross-derived mice". BMC Genet. 8:23.

Canale-Zambrano, J. C., M. C. Poffenberger, S. M. Cory, **D. G. Humes**, and C. K. Haston. 2007. "Intestinal phenotype of variable-weight cystic fibrosis knockout mice". Am. J. Physiol. Gastrointest. Liver Physiol. 293:G222-9.

Boverhof, D. R., J. C. Kwekel, **D. G. Humes**, L. D. Burgoon, and T. R. Zacharewski. 2006. "Dioxin induces an estrogen-like, estrogen receptor-dependent gene expression response in the murine uterus". Mol. Pharmacol. 69:1599-1606.

Gagnon, S., W. Atmodjo, **D. Humes**, C. McKerlie, F. Kaplan, and N. B. Sweezey. 2006. "Transgenic glucocorticoid receptor expression driven by the SP-C promoter reduces neonatal lung cellularity and midkine expression in GRhypo mice". Biol. Neonate. 90:46-57.

Yi, M., R. P. Jankov, R. Belcastro, **D. Humes**, I. Copland, S. Shek, N. B. Sweezey, M. Post, K. H. Albertine, R. L. Auten, and A. K. Tanswell. 2004. "Opposing effects of 60% oxygen and neutrophil influx on alveologenesis in the neonatal rat". Am. J. Respir. Crit. Care Med. 170:1188-1196.

Conference Presentations

Humes, D and Julie Overbaugh. "Inefficient use of macaque CD4 limits infection by circulating HIV-1 strains: implications for SHIV development". Talk presented at the 29th Annual Symosium on Nonhuman Primate Models for AIDS (October 2011).

Humes, D and Julie Overbaugh. "Progress Towards Developing a Subtype A SHIV". Poster presented at the Conference on Retroviruses and Opportunistic Infections (February 2011).

Humes, D and Julie Overbaugh. "Identification of amino acid changes in the envelope surface unit, gp120, of HIV-1 subtype A variants that allow for infection of pig-tailed macaque lymphocytes". Poster presented at the Keystone HIV Biology and Pathogenesis Symposium (January 2010).

Humes, D and Julie Overbaugh. "Engineering a CCR5-tropic HIV-1/HIV-2 chimera for infection of non-human primate cells". Poster presented at the West Coast Retrovirus Meeting (October 2007).

Honours and Awards

2011 Early Investigator Award
Awarded by: NHP Models for AIDS Scientific Committee

Young Investigator Award
Awarded by: CROI Scientific Program Committee

2010 Keystone Symposia Travel Scholarship
Awarded by: National Institute of Allergy and Infectious Diseases

2006 Dean's Honours List
Awarded by: University of Waterloo