Developing improved challenge viruses for macaque models of HIV-1 infection

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Abstract

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Infection of macaques with SIV/HIV chimeric viruses (SHIVs) is often used as a model of HIV-1 infection for pre-clinical vaccine studies. Although SHIV infection of macaques serves as the gatekeeper of promising vaccine candidates, it is limited by the fact there are a very small number of SHIVs available, and they do not represent the large genetic diversity of globally circulating HIV-1 variants. In addition, SHIVs that are able to establish persistent infection in macaques are a highly selected subset of viruses that require multiple rounds of adaption in lab culture or by serial animal-animal passage. This thesis describes the changes that occur in SHIVs as they are adapted for replication in macaques as well as the selective pressures in macaques that drive these changes.

First, we investigated the antigenic changes that occur in the envelope protein (Env) as HIV-1 is adapted for replication in macaques. Mutations that permit HIV-1 Env to use the macaque CD4 receptor for entry disrupted quaternary epitopes in the Env trimer that are key targets of current vaccine strategies. SHIVs that were adapted by serial passage in macaques and are currently used in pre-clinical vaccine trials exhibited antigenic properties similar to HIV-1 Envs adapted to macaque CD4. These results indicate that there are antigenic consequences of adapting HIV-1 for replication in macaques and that adaptation to the

macaque CD4 receptor compromises the ability to test vaccines that target key quaternary epitopes on HIV-1 Env.

Next, we investigated the viral determinants that contribute to the ability of SHIVs to replicate in macaques and establish persistent infection. We compared the replication capacity in macaque cells of nine SHIVs encoding HIV-1 sequences that represent circulating HIV-1 variants, lab-cultured variants and macaque-passaged variants. By generating chimeras between a macaque-passaged SHIV with high replication capacity and a circulating SHIV with low replication capacity, we determined that HIV-1 Env is a critical determinant of the ability to replicate to high levels in macaque cells. In addition, the amount of Env present in virions and expressed in infected macaque cells predicts the ability to replicate to high levels.

Finally, we studied the ability of SHIVs to resist the type-I interferon (IFN-I) response, an important component of innate immunity to viruses. Pathogenic SHIVs that have been adapted by macaque-passage were highly resistant to treatment with IFN-I compared to SHIVs encoding circulating HIV-1 Envs. Both replication capacity in macaque cells and virion-associated Env content predicted the ability of SHIVS to overcome the macaque IFN-I response. These results suggested that the process of adaptation for replication in macaques selects for variants that are resistant to the IFN-I response.

Overall, these studies describe the changes that occur in SHIVs as they are adapted for replication in macaques and the host pressures that contribute to these changes. Improved understanding of this process of adaption may allow for rational design of pathogenic SHIVs that are more representative of HIV-1 variants circulating in people.

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List of Abbreviations

AIDS: acquired immunodeficiency syndrome

bNAb: broadly neutralizing antibody

CD4bs: CD4 binding site

Env: envelope protein

HIV: human immunodeficiency virus

IFN-I: type-I interferon

mAb: monoclonal antibody

MPER: membrane proximal external region

SHIV: SIV/HIV chimeric virus

SIV: simian immunodeficiency virus

T/F: transmitted/founder variants

V1V2: variable regions 1 and 2 of HIV-1 envelope

V3: variable region 3 of HIV-1 envelope

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Chapter I: Introduction

The global HIV-1 epidemic remains a major public health challenge. There are an estimated 35 million people currently living with HIV-1 worldwide (UNAIDS Global Report 2013). The HIV-1 epidemic disproportionately affects low- and middle-income countries, especially those in sub-Saharan Africa where 70% of new infections occur. One of the major achievements in the field of HIV/AIDS research has been the development and administration of highly effective treatment regimens for HIV-1 infection. As the number of people living with HIV-1 has increased due to new infections, the number of deaths due to HIV/AIDS has decreased since the early 2000's because more people are receiving life-saving drug therapy (UNAIDS Global Report 2013). Since 2001, there has been a steady decrease in the number of new infections each year. Interventions including education programs, counseling of HIV-1 positive individuals, and improved access to drug treatment, among others, have likely all contributed to this decrease in new infections. Despite these successes, there are still ~2 million new infections each year worldwide. In addition to the successful interventions already being implemented, new interventions, such as the administration of a safe and effective vaccine, will be needed in order to end the HIV-1 epidemic.

HIV-1 Origins

There are two different human immunodeficiency viruses (HIV) that infect people, HIV-1 and HIV-2. Both viruses entered the human population from cross-species transmission of

different simian immunodeficiency viruses (SIVs) from non-human primates to people. HIV-1 is most closely related to an SIV that infects great apes [1] and is further divided into groups M, N, O and P, each of which resulted from separate transmission events of SIV from great apes to people [1,2]. HIV-1 group M is responsible for the vast majority of HIV cases worldwide and resulted from the transmission of an SIV that naturally infected chimpanzees (SIVcpz) to people. Infections with HIV-1 groups N, O and P are rare [3]. Phylogenetic data supports the hypothesis that HIV-1 group M entered the human population in the late 19th or early 20th century in what is now the Democratic Republic of the Congo and has since diversified greatly [4,5]. Based on phylogeny, HIV-1 group M is further divided into nine distinct subtypes, A, B, C, D, F, G, H, J, K and several circulating recombinant forms (Fig. 1.1). On the amino acid level, there is 8 – 17% genetic variation within a subtype and 17 – 35% variation between subtypes depending on the genome region analyzed [6]. The prevalence of each subtype depends on geographic region, and the highest amount of diversity is observed in central sub-Saharan Africa, where the current epidemic originated [6]. In sub-Saharan Africa, the most common subtypes are A and C while subtype B is most common in the United States and Europe [1,6]. The large genetic diversity of HIV-1 presents a major challenge to the development of intervention strategies, such as a protective vaccine.

In comparison to HIV-1, HIV-2 is less pathogenic in people and accounts for a much smaller number of new infections. HIV-2 is most closely related to a SIV that naturally infects sooty mangabeys (SIVsm) (Fig. 1.1) [1,2,7-9]. Thus, HIV-1 and HIV-2 are distinct viruses that entered the human population from different sources.

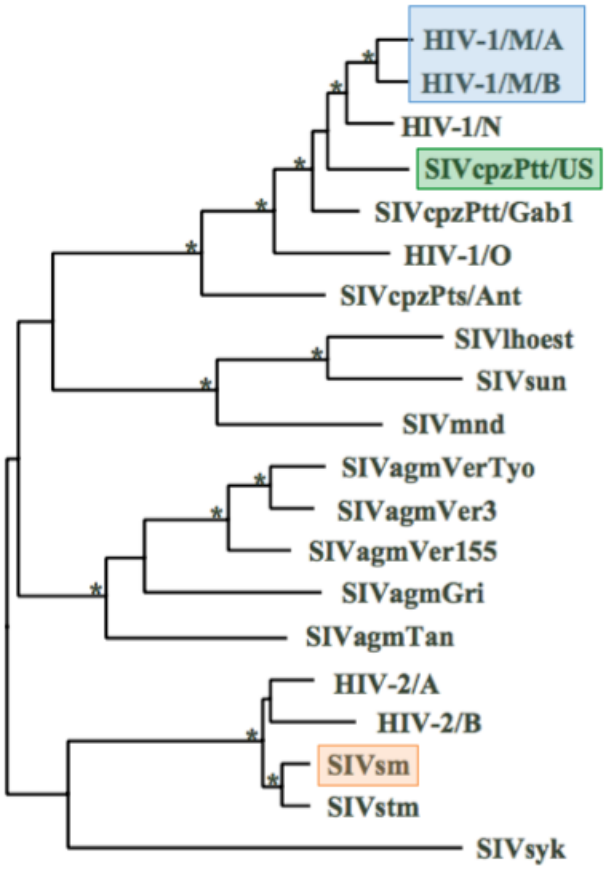

Figure. 1.1. Phylogenetic relationship between HIV-1 and SIVs. Image is adapted from Beer *et al* Human Retroviruses and AIDS 1999. The phylogenetic tree represents a maximum likelihood analysis of HIV/SIV pol sequences. HIV-1 Group M clades, responsible for the current pandemic, are highlighted in blue. SIVcpz, from which HIV-1 Group M is derived, is highlighted in green. SIVsm, which naturally infected sooty mangabeys, and is used to model HIV-1 infection in non-human primates is highlighted in green. Asterisks indicate clades supported with at least 80% of bootstrap replicates, and branch lengths are drawn to scale.

Genomic Organization and Proteins

HIV-1 belongs to the genus *lentivirus* of the *Retorviridae* family. As reviewed in Fields Viriology Edition 6, an HIV-1 virion contains two single-stranded, plus-sense RNA genomes. The HIV-1 genome encodes nine genes and long-terminal repeats (LTRs) that flank the RNA sequence (Fig. 1.2). Three of these genes (*gag*, *pol* and *env*) are common to all retroviruses. Group-specific antigen (Gag) is a polypeptide that is proteolytically cleaved into three structural proteins – matrix (MA), capsid (CA), nucleocapsid (NC) and p6 – which form the core of the virion that houses the RNA genome. Polymerase (Pol) contains three enzymatic proteins – protease (PR), reverse transcriptase (RT) and integrase (IN) - which cleave polypeptides into functional proteins, synthesize complementary DNA (cDNA) from the RNA genome and facilitate the integration of the viral cDNA into the genome of the host cell. Envelope (Env) is a surface glycoprotein composed of two non-covalently linked subunits (gp120 and gp41) that facilitate binding of a virion to the host cell receptors and fusion of the viral and host membranes to enter a target cell. The HIV-1 genome also encodes a number of accessory proteins that have been shown to be dispensable for viral replication in some cell culture systems, but are important for infection and pathogenesis *in vivo* [10]. These accessory proteins include viral infectivity factor (Vif), viral protein unique (Vpu), trans-activator of transcription (Tat), regulator of virion (Rev) and negative factor (Nef). The requirement for Vif for HIV-1 replication is cell-type dependent, and the primary role of Vif in infection is thought to be the antagonism of an anti-viral host cell factor. Vif, Vpu and Nef are all important for antagonizing host cell factors with antiviral activity. In addition, Vpu is involved in degradation of the host cell receptor CD4 and promotes virion release while Nef downregulates cell-surface molecules, including CD4 and

major histocompatibility complex-I (MHC-I), and modulates cellular activation pathways. Tat and Rev are regulatory proteins involved in viral RNA transcription and export of viral mRNA from the nucleus, respectively. Tat increases the steady-state levels of viral mRNA by interacting with the RNA polymerase transcription complex and increasing its processivity resulting in efficient viral RNA transcription. Rev facilitates the nuclear export of viral mRNAs containing a Rev-response element (RRE), a sequence of viral mRNA with a secondary loop structure, present in un-spliced and partially spliced transcripts.

There are some notable differences between HIV-1 and HIV-2 with respect to genomic organization. In contrast to HIV-1, HIV-2 does not encode a *vpu* gene. HIV-2, however, does encode the accessory gene *vpx*. These differences in genomic organization are due to the fact that HIV-1 and HIV-2 evolved from distinct SIV variants (HIV-1 from SIVcpz and HIV-2 from SIVsmm) that also differ in the genes that they encode (Fig. 1.2).

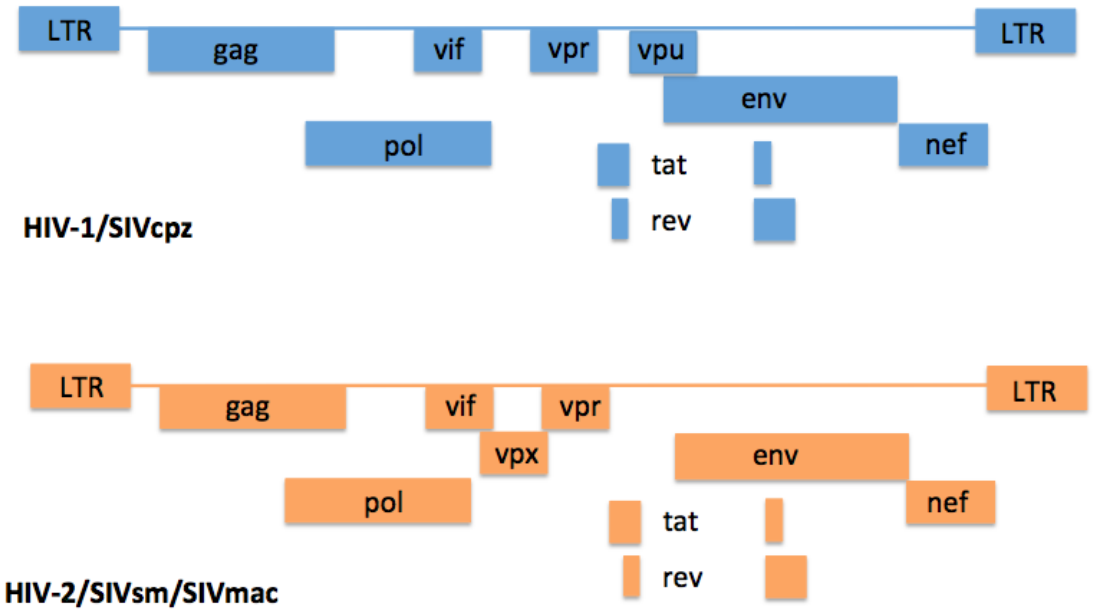

Figure. 1.2. Genomic organization of HIV-1/SIVcpz and HIV-2/SIVsm/SIVmac. Each block indicates a distinct genomic region with the identity of the region indicated for each.

HIV-1 Env structure

In addition to playing a critical role in host cell receptor binding and membrane fusion, the HIV-1 Env protein is also the major target of the host antibody response. HIV-1 Env is the only protein displayed on the surface of the virion, and experiences significant pressure from the host immune response. Thus, HIV-1 is a highly variable protein. HIV-1 Env is translated in the endoplasmic reticulum as polypeptide precursor, known as gp160. In the Golgi, the gp160 precursor is then cleaved by the cellular protease furin into its functional subunits, gp120 and gp41, which remain non-covalently linked [11]. In its functional form, HIV-1 Env exists on the surface of a virion as a trimer spike made up of three Env monomers with each monomer consisting of a gp120 surface subunit and a gp41 transmembrane subunit. The gp120 subunit is organized into five conserved regions (C1 – C5) and five variable regions (V1 – V5) while the gp41 subunit is organized into six different domains, including the fusion peptide (FP), heptad repeat regions 1 and 2 (HR1 and HR2), the membrane proximal external region (MPER), the transmembrane region (TM) and the cytoplasmic tail (CT) (Fig. 1.3). Each gp120 subunit contains nine cysteine bridge disulfide bonds that demarcate the heavily glycosylated variable loops. These variable loops also occlude the more conserved regions of gp120 including the highly conserved CD4 binding site [12].

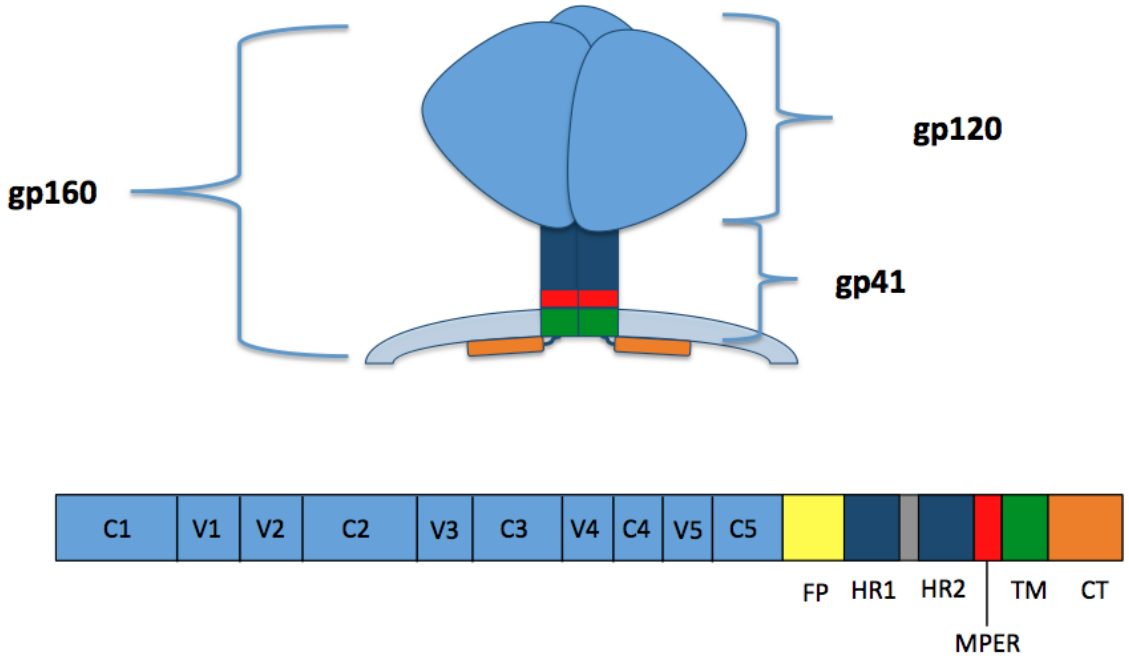

Figure. 1.3. Genetic organization and structure of the HIV-1 envelope protein (Env). The HIV-1 Env is depicted in its trimeric form found on the surface of virions. The HJIV-1 Env trimer consists of three gp120 surface subunits (light blue) non-covalently linked to three gp41 transmembrane subunits. The gp120 subunit is divided into five constant (C1 – C5) and five variable (V1 – V5) regions. The gp41 subunit is divided into six domains: the fusion peptide (FP, yellow), two heptad repeats (HR1 and HR2, dark blue), the membrane-proximal external region (MPER, red), the transmembrane domain (TM, green) and the cytoplasmic tail (CT, orange).

HIV-1 Life Cycle

All of the viral proteins described above interact with host cell factors that together complete the HIV-1 life cycle. HIV-1 primarily infects CD4+ T cell lymphocytes. Although some HIV-1 variants are able to infect monocyte-derived macrophages (MDMs) and may be able to infect dendritic cells (DCs), both cell types are typically more resistant to infection [13,14]. The first step of the HIV-1 life cycle is the process of entry into target cells. The gp120 subunit of the HIV-1 Env protein binds to the main CD4 receptor on target cells. This interaction induces conformational changes in Env that facilitate binding to one of two physiologically important co-receptors, c-c chemokine receptor type 5 (CCR5) or c-x-c chemokine receptor type 4 (CXCR4) [15-17]. Engagement of the co-receptor leads to fusion of the viral and host cell membranes. Upon entry into a cell, the virion core is released into the cytoplasm. HIV-1, then, undergoes reverse transcription of its single-stranded RNA genome into double-stranded DNA and un-coating of the virion core [18].

Reverse transcription is carried out by the enzyme reverse transcriptase (RT), an RNA dependent DNA polymerase. RT synthesizes DNA from RNA and also contains an RNase H domain that degrades the transfer RNA (tRNA) template, thus initiating reverse transcription. The process of reverse transcription is highly error-prone, due to the fact that it lacks a proofreading mechanism, and contributes to the high mutation rate and large genetic diversity of HIV-1 [19]. The newly synthesized viral DNA is part of the pre-integration complex (PIC). The PIC is composed of both viral and host proteins that direct import into the nucleus and facilitate integration of viral DNA, referred to as the provirus, into the host genome [20]. The viral protein

integrase (IN) is critical in this process [21]. Interestingly, it has also been demonstrated that HIV-1 tends to integrate in regions of the host genome that are actively transcribed [22]

Once integrated into the host genome, HIV-1 utilizes host cell machinery to transcribe viral genes and synthesize viral proteins. An ~600bp region of the provirus, termed the long terminal repeat (LTR), is the site of initiation of transcription and contains both promoter and enhancer elements, including a binding site of the transcription factor NFκB, for viral gene expression [23]. At the LTR, most transcription complexes initially fail to elongate and produce full-length mRNA transcripts. The Tat protein increases the processivity of these complexes by interacting with an RNA secondary structure at the 5' end of the viral transcripts, known at the transactivation response region (TAR) [24]. Thus, the presence of Tat increases the steady-state levels of viral mRNA. In order to generate all of the viral proteins from initiation at a single promoter, HIV-1 differentially splices the viral transcript into different mRNAs. Splicing is an inefficient process, and fully spliced, partially spliced and un-spliced transcripts exist in the nucleus [25]. The Rev protein is critical for the efficient nuclear export of partially spliced and un-spliced mRNAs that contain introns and encode the RRE [10]. Once exported from the nucleus, viral mRNAs are translated into polypeptides by cellular machinery in the endoplasmic reticulum.

Using cellular machinery, HIV-1 then assembles its viral proteins and genome. The process of assembly, including timing, sequence, and host/viral factors involved, is an active area of research. Briefly, Gag subunits multimerize and interact with the viral genome. At the cellular membrane, Env is incorporated into virions, and the viral particles buds from the host cell. Once they bud from the host cell, HIV-1 virions undergo a process of maturation, after which they are capable of infecting naïve target cells [26].

Innate immune defense: anti-retroviral restriction and resistance factors

During the last decade, it has come to be appreciated that the ability of HIV-1 to propagate in a host depends not only on the ability to utilize cellular machinery but also to evade or antagonize a number of anti-viral proteins that act at multiple stages of the viral life cycle. These host anti-viral proteins have been termed 'restriction factors' because they restrict the ability of the virus to replicate in a particular cell type expressing the protein. Restriction factors act in a dominant manner, are constitutively expressed and inducible by innate immune responses, and, in contrast to components of the adaptive immune response, do not undergo gene rearrangements or somatic mutation [27]. Because restriction factors are effective at inhibiting viral replication, viruses have evolved mechanisms to evade or antagonize their activity. As different lentiviruses have evolved to replicate in their natural hosts, they have developed viral antagonists that act in a species-specific manner. Thus, restriction factors have been rendered inactive against the wild-type viruses due to these viral antagonists. Because of the species-specificity that has been demonstrated for restriction factors, they have also been implicated in preventing or limiting cross-species transmissions of lentiviruses. For example, HIV-1 is unable to replicate in other non-human primate species in part due to the activity of restriction factors that are not effectively antagonized by HIV-1 proteins. The species-specificity of this viral antagonism also poses a major challenge to the development of animal models of HIV-1 infection and has limited the number of models available.

To date four anti-lentiviral restriction factors that inhibit cross-species infection have been well characterized: tripartite motif-containing protein 5α (TRIM5α), apolipopotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G), sterile α motif domain and

histidine aspartic acid (HD) domain 1 (SAMHD1), and bone marrow stromal antigen 2 (BST2, more commonly known as *tetherin*). TRIM5α is a post-entry restriction factor that recognizes CA on the virion core and leads to premature un-coating and inhibition of reverse transcription [28]. APOBEC3G, a cytidine deaminase, is packaged into virions and associates with the reverse transcription complex. In a newly infected cell, APOBEC3G interferes with the processivity of reverse transcription and hypermutates viral cDNA, often rendering the provirus replication incompetent [29,30]. SAMHD1 is a 2'-deoxynycleoside 5'-triphosphate (dNTP) triphosphohydrolase the function of which is to deplete available dNTP pools in non-dividing cells. In the presence of SAMHD1, lentiviruses are unable to undergo efficient reverse transcription of the RNA genome into cDNA because of the lack of sufficient levels of dNTPs [31,32]. Tetherin is a transmembrane protein that forms bridges between viral and cellular membranes and prevents budding and release of virions [33].

For each of these restriction factors, mechanisms of viral evasion or antagonism have been well defined. These mechanisms of viral evasion/antagonism have developed as lentiviruses have adapted for replication in their particular hosts and contribute to the establishment of persistent infection despite the presence of restriction factors. As TRIM5α recognizes CA on the virion core, mutations in CA render TRIM5α ineffective against a lentivirus that naturally infect a particular host, for example HIV-1 infection in humans; APOBEC3G is targeted for degradation by the lentiviral protein Vif [29]; SAMHD1 is antagonized by either of two related SIV accessory proteins Vpx or Vpr, although an HIV-1 antagonist has not been identified [31,34,35]; and, tetherin is antagonized by HIV-1 Vpu in humans or by SIV Nef in non-human primates [33,36]. As an example of the importance of restriction factor antagonism for cross-

species transmission, the ability of Vpu from SIVcpz to antagonize human tetherin appears to have played a critical role in HIV-1 group M becoming pandemic in the human population [37].

Another critical feature of anti-lentiviral restriction factors is that their expression is induced by the type-I interferon (IFN-I) signaling pathway. IFN-I is a group of cytokines produced by cells that have sensed the presence of viral infection through a variety of mechanisms. IFN-I signaling leads to the localization of transcription factors that results in the transcription of hundreds of host genes with anti-viral potential. In this way, the IFN-I response acts as one of the first lines of defense against viral infection. It is likely that the restriction described above represent only a few of the IFN-I-induced proteins that have the ability to inhibit replication of lentiviruses. In just the last several years, three additional IFN-I-induced host factors with anti-lentiviral activity have been described. These factors include schlafen 11 (SLFN11) [38], which acts at the step of translation to inhibit viral protein synthesis, myxovirus resistance 2 (MX2) [39-41], which inhibits viral replication sometime after reverse transcription, and the IFN-induced transmembrane proteins (IFITMs) [42,43], which inhibit at the step of viral entry. Because viral antagonists of these host factors have not yet been identified, it has been suggested that they be referred to as ‘resistance factors’ in order to distinguish them from well defined ‘restriction factors’ [27]. Recent studies have provided evidence that the IFN-I response and up-regulation of restriction factors are important for limiting viral replication in the context of infection in both people and non-human primates [44,45]. Thus, even when a particular lentivirus is equipped with the necessary viral antagonists to establish persistent infection in a particular host species, up-regulation of restriction/resistance factors upon IFN-I induction can still inhibit viral replication and affect the course of infection within an individual.

Given that restriction factors act in a species-specific manner and are inducible by the IFN-I response, they pose an important barrier to cross-species transmissions and the development of animal models of HIV-1 infection. A successful challenge virus for a non-human primate model of HIV-1 infection will require the necessary viral antagonists of restriction factors of the particular host species and will need to be able to resist the IFN-I response to establish initial infection.

Course of HIV-1 Infection and Adaptive Immune Responses

Even in the face of the potential barriers described above, lentiviruses are able to establish a persistent, chronic infection in their natural hosts. The ability to establish a persistent chronic infection is likely due to the fact that the virus rapidly diversifies upon initial infection and quickly establishes a latent reservoir that persists in the host [19,46]. Acute HIV-1 infection in humans is characterized by high levels of virus, reaching a peak viral load around 21 days after initial infection [47]. Increase in virus levels is quickly followed by the induction of the IFN-I response reaching peak levels within the first 10 days of infection [48,49]. Robust viral replication during acute infection results in a depletion of CD4+ T cells. These virus levels rapidly decline to a steady-state viral load set point during chronic infection [50]. As with all infections, the host responds to the presence of virus limit the spread of infection. Cytotoxic T cell lymphocytes (CTLs) are detectable several days after initial infection and their expansion coincides with the dramatic decrease in viremia. Thus, CTLs are thought to play a role in killing infected cells and decreasing the plasma levels of virus [51-54].

Infection also elicits an antibody response, and HIV-1-specific antibodies are able to recognize a number of viral proteins. Non-neutralizing antibodies that are capable of binding

viral proteins are induced early in infection. Despite their inability to neutralize cell-free virus, these binding antibodies are capable of killing of infected cells by antibody-dependent cellular cytotoxicity (ADCC) and have been associated with protection from infection and delay in disease progression [55]. Only antibodies that recognize the Env protein, however, are able to neutralize virus and prevent infection of target cells. Despite a host antibody response to infection and the presence of antibodies that are capable of neutralizing the infecting variant, these antibodies do little, if anything, to help control infection in an individual. HIV-1 is able to evade the host neutralizing antibody (NAb) response by a number of mechanisms [56-59]. As previously mentioned, HIV-1 exhibits a very high mutation rate and rapidly diversifies upon infecting an individual [19]. As an individual develops NAbs that recognize the infecting variant, HIV-1 quickly mutates to evade detection by these antibodies. In addition to genetic variation, there are a number of characteristics of the Env protein that help the virus evade the NAb response. HIV-1 Env is a highly dynamic protein that shifts in conformation exposing and occluding epitopes targeted by NAbs. Importantly, HIV-1 Env conformationally masks conserved epitopes critical for receptor binding that are targeted by a class of broad and potent NAbs [60]. Conformational masking of distal epitopes in Env has also been implicated in neutralization antibody escape during transmission [61]. HIV-1 Env is also heavily glycosylated with ~50% of the molecular weight of the protein composed of glycans [62]. Glycans are derived from the host cell and are typically not immunogenic, and the high levels of glycosylation "shield" immunogenic epitopes on Env from the host immune system. In addition, glycosylation can be heterogeneous within a given Env variant affecting NAb recognition [63,64]. Thus, HIV-1 Env is a constantly changing target for the host immune response.

Broadly neutralizing antibodies

Despite the mechanisms by which HIV-1 Env evades detection by neutralizing antibodies, a small percentage (~10-30%) of infected individuals do develop antibodies that are able to potently neutralize a broad range of diverse HIV-1 variants [65-69]. Although these broadly neutralizing antibodies (bNAbs) do little, if anything, to reduce the amount of virus circulating within an infected individual, it is thought that bNAbs would protect against initial infection if present at the time of exposure. Indeed, a number of studies have demonstrated that passive immunization of macaques with broadly neutralizing antibodies can provide sterilizing immunity against viral challenge, even at low doses of antibody [70-72]. These antibodies may also have a therapeutic benefit as administration of bNAbs to macaques dramatically, although transiently, reduces the amount of virus circulating in the blood [73,74]. In addition to the potential benefit of translating these findings to the clinic, the isolation of monoclonal bNAbs has provided an important set of tools that can be used to study the epitopes that they target. To date, greater than 20 bNAbs have been isolated that target a wide range of different epitopes on HIV-1 Env [75]. As will be discussed in Chapter II of this thesis, monoclonal bNAbs can be used to detect structural changes in HIV-1 Env resulting from the introduction of point mutations.

HIV-1 Transmission

Critical to developing a safe and effective vaccine against HIV-1 is the understanding of transmission of the virus to a naïve individual. The most common route of HIV-1 transmission globally is by sexual contact [76], but transmission also occurs by contact with infected blood, often by intravenous drug use, and by vertical transmission from an infected mother to an infant *in utero*, during childbirth or post-partum via breast milk [77]. During transmission to a newly

infected individual, HIV-1 experiences a severe genetic bottleneck. While a transmitting individual harbors a large pool of genetically diverse HIV-1 variants, a very limited number of variants are transmitted to a newly infected person [78-82]. There are a number of factors, for example the route of transmission, recipient gender and inflammation at the site of exposure, that influence the number of variants transmitted [83-85]. In the case of heterosexual transmission, typically just one or two HIV-1 variants transmitted [83,86,87]. These transmitted variants that establish infection are commonly referred to as transmitted/founder (T/F) variants. A number of recent studies have identified unique characteristics of the T/F viruses. These characteristics include: CCR5 co-receptor tropism [87-90], lower levels of glycosylation [86,91,92], poor replication in macrophage cultures [93], increased resistance to CD4-binding site NAbs [87], and resistance to IFN-I [94,95]. This genetic bottleneck represents an opportunity to prevent transmission because, in theory, a vaccine would only need to protect against the relatively smaller number of T/F variants by targeting these unique characteristics. In order to develop such a vaccine, the models of HIV-1 infection used to test different strategies would need to capture these unique characteristics of the T/F viruses circulating in people.

Animal Models of HIV-1 Infection

Despite three decades of research, there still is not a safe and effective vaccine for HIV-1. One of the major limitations in the HIV-1 vaccine field has been the lack of an animal model that captures all of the most important aspects of HIV-1 transmission and infection. While HIV-1 infects people, it does not infect other animals, including non-human primates and mice, both of which are often used in experimental studies. As described above, the difficulty in developing an animal model of HIV-1 infection is in part due to the presence of restriction factor that act in a

species-specific manner and limit cross-species infections. There are more than 40 species of African primate species that are naturally infected with distinct strains of SIV [3]. Infection of a natural primate host with SIV is typically not pathogenic, in contrast to HIV-1 infection in people [96]. Interestingly, natural SIV hosts typically do not develop disease despite chronic infection with high levels of circulating virus [96]. Infection of non-natural hosts, such as Asian macaques, with certain SIVs can be pathogenic and resemble HIV-1 infection in people [97,98]. SIV infections of three macaque species (*Macaca mulatta*, *Macaca nemestrina* and *Macaca fascicularis*) are the most commonly used models of infection and have been critical for studies of transmission and pathogenesis of lentiviruses. The pig-tailed macaque (*Macaca nemestrina*) has recently gained increasing interest as a model of HIV-1 infection because it has been demonstrated that these animals are more susceptible to infection [98], in part due to the fact that pig-tailed macaques lack a functional TRIM5α gene [99,100].

There are, however, some important limitations to the SIV/macaque model of HIV-1 infection. The SIV strain that is most commonly used to model HIV-1 infection in macaques is a molecular clone of *SIVmac*, a virus that was derived from experimental infection of rhesus macaques with a *SIVsmm*, a SIV strain that naturally infects sooty mangabeys [97]. While the HIV-1 epidemic is the result of cross-species transmissions of SIVs from non-human primates to people, HIV-1 is most closely related to *SIVcpz*, a strain that naturally infects chimpanzees [1]. Representative variants of HIV-1 and SIVmac are only about 50% identical on the nucleotide level as distinct selective pressures have shaped the evolution of these viruses in their respective hosts [101]. In terms of HIV-1 vaccine development, differences between HIV-1 and SIVmac Env proteins are of particular importance. HIV-1 and SIV Envs differ in their functional properties that likely reflect important differences in their structures. For example, compared to

HIV-1, SIVs are more promiscuous with respect to co-receptor usage and undergo co-receptor switching less frequently over the course of infection [102,103]. In addition, antibodies that recognize HIV-1 Env rarely cross-react with SIV Envs, in particular SIVmac, reflecting differences in amino acid sequence and in protein conformation.

SIV/HIV chimeric viruses

During the last several decades, a number of groups have constructed SIV/HIV chimeric viruses (SHIVs) with the goal of developing challenge viruses that display some of the antigenic properties of HIV-1 while maintaining the ability to replicate in macaques. Importantly, these viruses encode SIV antagonists of the well-characterized macaque restriction factors. There are a number of variations of SHIVs that have been constructed, and this thesis will focus on SHIVs that encode the HIV-1 *env* gene (including the second exons of *tat* and *rev*) and a partial or full *vpu* gene (Fig. 1.4). Over the years, SHIV development has proven to be a challenging task. To generate a SHIV, typically the HIV-1 *env* gene is cloned into an SIV provirus, and the resulting SHIV is passaged in cell culture. These viruses initially replicated poorly in macaques and required multiple serial passages in the animals to become pathogenic and mimic HIV-1 infection in people [98]. These multiple rounds of passage result in mutations, including changes in HIV-1 Env, that increase replication capacity and pathogenesis in macaques [104-106]. The process of adaptation also affects the antigenic properties of the SHIV so that the adapted virus has a different antigenic profile than the parental virus [107-109].

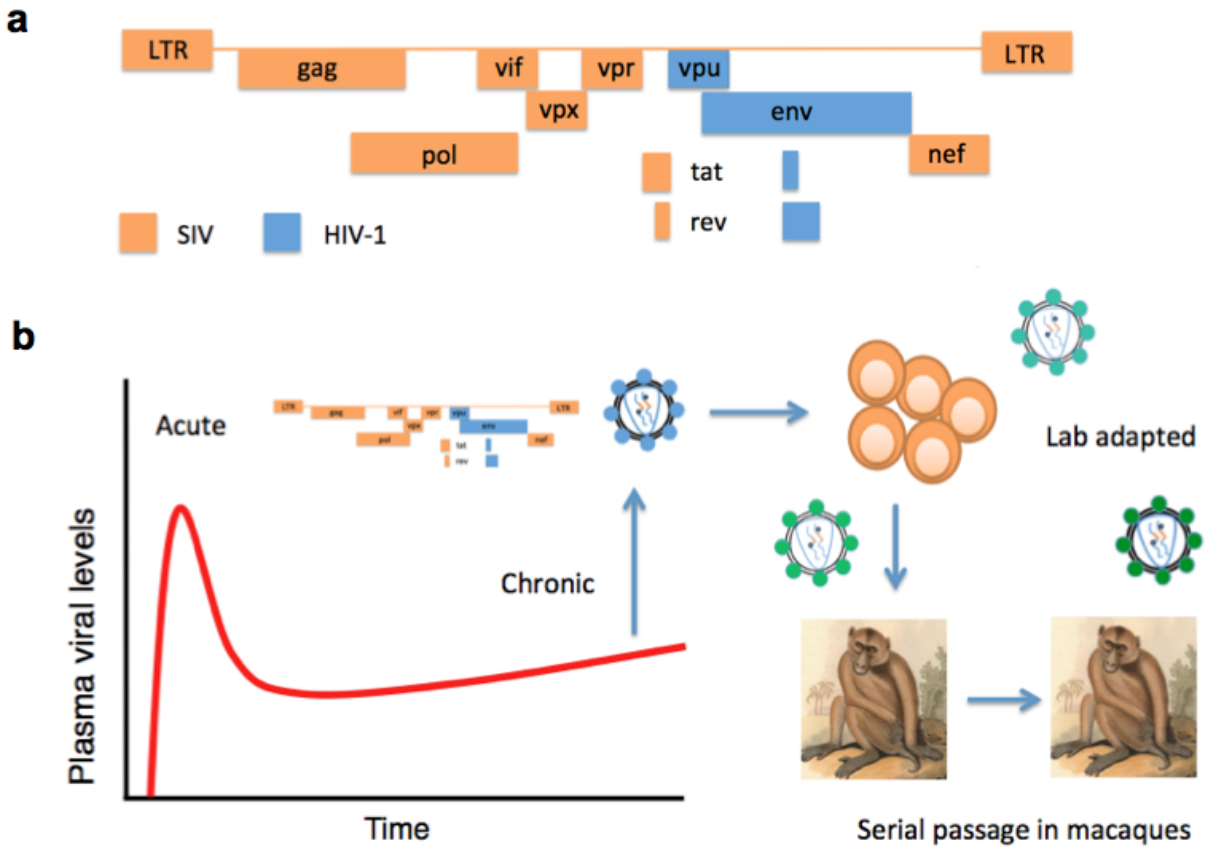

Figure. 1.4. Construction and development of pathogenic SIV/HIV chimeric viruses (SHIVs). (a) Genomic organization of SHIVs. Genes derived from SIV are indicated in orange while genes derived from HIV-1 are indicated in blue. (b) Schematic of the adaptation process for pathogenic SHIVs. The chart represents the coarse of HIV-1 infection in an individual with acute and chronic stages indicated. HIV-1 variants are typically isolated during late-stage chronic infection and cultured *in vitro*. HIV-1 sequences are then cloned into an SIV provirus and further adapted for replication in cell culture. SHIVs are then serially passaged animal-to-animal in order to select for pathogenic variants. The darker color-coding of the viruses indicates phenotypic changes in the SHIV as it is adapted to macaques.

Because SHIVs encode the HIV-1 *env* gene and have antigenic properties more similar to those of HIV-1, SHIV infection of macaques has been the preferred model for pre-clinical vaccine studies in animals [98]. The SHIV/macaque model, however, has some important limitations. First, because it has been so difficult to generate pathogenic SHIVs that mimic HIV-1 infection in humans, there are a very limited number of pathogenic SHIVs available representing only two, B and C, of the major subtypes of HIV-1 [110-112]. Thus, this limited number of SHIVs currently used in pre-clinical vaccine studies does not represent the large genetic diversity of HIV-1 variants circulating in people. Moreover, because such pathogenic SHIVs are so difficult to generate, typically one Env variant is used to predict the efficacy of a vaccine candidate against circulating HIV-1 variants that vary by ~30% in sequence [6]. Second, pathogenic SHIVs are a highly selected subset of viruses that likely have unique characteristics that allowed them to progress through the various stages of adaptation. These viruses are not representative of variants that are transmitted between people against which an effective vaccine would need to protect.

The first generation of SHIVs were developed using *env* sequences from HIV-1 variants that were isolated from late-stage chronic infection and used the CXCR4 co-receptor. A number of studies have now demonstrated that HIV-1 variants that use the CXCR4 co-receptor are rarely transmitted [87,89,90] and co-receptor usage can influence the progression of HIV-1 infection in people [113]. Such SHIVs are less ideal for pre-clinical vaccine studies. As an example, an early pathogenic virus, SHIV 89.6P, encodes HIV-1 sequences originally isolated from late-stage chronic infection and uses the CXCR4 co-receptor [114]. SHIV 89.6 was propagated in lab culture and then passaged in macaques to generate a SHIV with increased pathogenicity, SHIV 89.6P [104,115,116]. SHIV 89.6P was used as challenge virus for vaccine candidates in a

number of pre-clinical vaccine trials that showed some promise of protection of macaques against progression to disease [117,118]. Despite these promising results in the SHIV/macaque model, protection in people was not observed using the same vaccine candidate in a larger clinical trial. Following the efficacy trial in people, there was general consensus that improved SHIV models of infection were needed [119].

There has been progress in generating CCR5-tropic SHIVs that are pathogenic in macaques. Most of these SHIVs were derived from HIV-1 variants that were isolated from the later stages of chronic infection. For example, the commonly used SHIV SF162P3 encodes the HIV-1 Env variant SF162, which was isolated from the central nervous system during late-stage chronic infection [120]. HIV-1 SF162 is particularly neutralization sensitive, in contrast to transmitted variants, which tend to be relatively more resistant to neutralization [121]. Serial passage of the parental SHIV SF162 in macaques resulted in SHIV SF162P3, which accumulated a number of mutations in the HIV-1 *env* gene that contributed to its pathogenicity and also resulted in increased resistance to neutralization [122].

In the last several years, exciting progress has been made with respect to the development of challenge viruses that are more representative of HIV-1. The identification of restriction factors and the corresponding species-specific viral antagonist has opened the possibility of rationally designing SHIVs that encode the necessary viral antagonists to replicate in macaques. Certain lab-adapted HIV-1 strains that encode SIV Vif, the antagonist of macaque APOBEC3G, are able to replicate to modest levels in pig-tailed macaques (PTMs), although these viruses do not establish persistent infection in the host [123,124]. Interestingly, serial passage of an HIV-1 variant encoding SIV Vif in PTMs resulted in adaptive mutations in HIV-1 Vpu that improved its ability to antagonize macaque tetherin [125]. Taken together, these data indicate that

restriction factors play a critical role in inhibiting the replication of HIV-1 in macaques *in vivo* and will be important to consider when generating novel pathogenic SHIVs. Progress has also been made towards developing SHIVs that are more representative of T/F HIV-1 variants. By pooling large numbers of SHIV clones based on T/F HIV-1 sequences, at least two groups have identified several T/F SHIVs that are able to replicate in macaques and causes disease without animal-to-animal serial passage [126,127]. In each study, only one or two "winner" SHIVs were able to establish persistent infection in the animals suggesting that these viruses had certain properties for which they were selected during propagation *in vitro* or infection *in vivo*. It remains unclear what properties allow SHIVs to establish persistent infection in macaques.

Goals of thesis

There is an urgent need to develop challenge viruses for animal models infection that are more representative of HIV-1 variants circulating in people in order to test vaccine and therapeutic strategies. The overall goal of my thesis was to identify the viral and host factors that contribute to the ability of SIV/HIV chimeric viruses to establish persistent infection in macaques. First, in Chapter II, I describe the effect that adaptation to the macaque CD4 receptor has on the antigenic properties of HIV-1 envelope. In Chapter III, I investigate the viral determinants of high replication capacity of SHIVs in macaques and describe a possible mechanism by which pathogenic SHIVs are able to replicate to such high levels. Next, I characterize the ability of different SHIVs to evade or antagonize the innate immune response, in particular the effect of treatment with type-I interferon, in Chapter IV. Finally, in Chapter V, I will discuss the implications of this work for the development of improved SHIVs for macaque models of HIV-1 infection and how such models will be useful for evaluating HIV-1 vaccine candidates.

Chapter II: Antigenic Consequences of Adapting HIV-1 Env to Macaque CD4

Introduction

Macaque models of HIV-1 infection have been critical to pre-clinical vaccine and passive immunization studies and to the understanding of HIV-1 pathogenesis. HIV-1 does not persistently infect macaques due to several species-specific host factors that prevent infection or inhibit viral replication [98]. SIV/HIV chimeric viruses (SHIVs) encode SIV antagonists of these macaque restriction factors, and such SHIVs serve as surrogates of HIV-1 infection in macaques. Despite the fact that SHIVs incorporate the critical SIV antagonists of known macaque restriction factors, they require additional passage *in vivo* in order to replicate to high levels and cause persistent infection in macaques [98]. Even with the improved understanding of host-virus interactions, there has been variable success in generating SHIVs capable of establishing infection in macaques, and this process remains expensive and labor-intensive.

SHIVs that incorporate the gene for the envelope glycoprotein (Env) of HIV-1 are particularly important for HIV-1 vaccine and passive immunization studies in macaques because Env is the major target of the host antibody response. Thus, Envs from viruses representing those that were transmitted and/or successfully spreading in the population would be ideal; however, all but two SHIVs in current use encode Env sequences derived from chronic infection [98,109,111]. Moreover, currently available pathogenic SHIVs represent only two of the major circulating HIV-1 subtypes, B and C [98,109,111,116,122,128-130]. Identifying pathogenic SHIVs based on other subtypes has been hindered by the fact that not all SHIV chimeras replicate in macaque lymphocytes [131]. Thus, the current limited collection of SHIVs does not represent the genetic diversity of circulating HIV-1 viruses.

All but two of the SHIVs in current use – both encoding a subtype C *env* [109,111] –were generated using virus that was first amplified by replication in culture. Among the SHIVs that have been tested for infection in macaques, all required serial passage to further adapt to cause persistent infection and disease [109,111,116,122,128-130]. Several studies have shown that this process of serial passage resulted in mutations in both the constant and variable regions of Env [106-108,130,132-135]. A number of these studies focused on CXCR4 and dual-tropic variants of HIV-1 and showed that the passaged viruses have neutralization profiles that differ from the un-passaged viruses from which they were derived, suggesting that adaptation of HIV-1 Env to macaques may alter its antigenicity. In general, the CXCR4- and dual-tropic HIV-1 Envs that were passaged in macaques were more resistant to monoclonal antibodies. However, there has not been a systematic evaluation of how the process of macaque adaptation impacts the antigenic properties of SHIVs representing transmitted HIV-1 Envs, which use the CCR5 co-receptor. Likewise, the role of adaptation of HIV-1 Env to the mCD4 receptor in this process has not been examined.

The requirement for adaptation of SHIVs is not surprising given that species-specific differences between the human and macaque CD4 (mCD4) receptor restrict the ability of HIV-1 Env variants to infect macaque cells [136,137]. Specifically, a single polymorphism at position 39 in the macaque (isoleucine) versus human (asparagine) CD4 receptor leads to a 1 – 2 log reduction in entry of circulating HIV-1 variants [137]. The SHIVs in common use all have the ability to use the mCD4 receptor more efficiently than circulating HIV-1 variants [137], suggesting that they are able to tolerate this amino acid difference in the mCD4 receptor. Recently, two independent point mutations in Env were identified that increased entry mediated by mCD4 by ~100-fold for most of the viruses tested [136]. Interestingly, these two mutations

occur outside of the CD4 binding site in the C2 region (A204E) and the V3 loop (G312V) of the gp120 subunit of Env. A SHIV encoding the G312V change has also been selected in macaques [125].

It is critical that SHIVs that are used in preclinical models for vaccine and passive immunization studies retain the antigenic features of HIV-1 Env circulating in humans. The recent identification of broadly neutralizing antibodies (bNAbs) against HIV-1 has provided a set of highly sensitive probes to assess changes to the conformation of the Env trimer at distinct epitopes that were not available in earlier studies of SHIV evolution [107,133-135,138]. These bNAbs include the following: CD4 binding site (CD4bs) antibodies, exemplified by VRC01 and VRC03 [139]; a class of antibodies that recognize glycan-dependent epitopes in the V1V2 region, such as PG9, PG16 and PGT145 [138,140]; antibodies that recognize glycan-dependent epitopes in the V3 loop, PGT121 and PGT128 [138]; and those that target the membrane-proximal external region (MPER), a recent example of which is 10E8 [141]. A number of these bNAbs target quaternary epitopes formed by the interaction of multiple Env protomers and preferentially bind the trimeric form of Env [140,142,143]. Such bNAbs are useful in detecting conformational changes in the Env trimer.

It is unclear what, if any, effect the changes that result from adaptation of HIV-1 Envs circulating in humans for entry using the macaque CD4 receptor have on the biological and immunological properties of the Env protein. If the process of increasing replication fitness by adapting to mCD4 leads to major biological and/or antigenic changes in Env, then SHIVs based on these Envs may not faithfully predict key features of HIV-1 Envs circulating in human populations. Thus, understanding how the adaptation impacts Env is a critical consideration in the use of these model systems for screening HIV-1 vaccines and prevention methods. The goals

of the present study were to assess the antigenic properties of HIV-1 Envs adapted to replication in macaques and to determine if there are conformational changes in the Env trimer that facilitate this adaptation. Our results indicate that Envs capable of entry using mCD4 exhibit a pattern of increased resistance to antibodies that recognize quaternary epitopes when compared to the parental Env while becoming more sensitive to epitopes that are further exposed upon CD4 binding. We propose that adaptation to mCD4 results in conformational changes that allow the Env trimer to more readily adopt the CD4-bound state, disrupting quaternary epitopes in the native Env trimer and increasing sensitivity to antibodies that recognize epitopes exposed upon binding to CD4.

Materials and Methods

Envelope clones and mutagenesis

The following envelope clones and their corresponding A204E and G312V mutants were described previously [136,137]: 5 subtype A clones (Q23ENV.17, MG505.W0M.ENV.H3, BG505.W6M.ENV.B1, QF495.23M.ENV.A3), 1 subtype C clone (QC406.70M.ENV.F3), and 1 subtype D clone (QA013.70I.ENV.H1). For the present study, nucleotide changes were introduced by site-directed mutagenesis to generate A204E and G312V Env mutants for the envelope clone QA255.662M.C [144] by methods similar to those previously described [136]. QA255.662M.C CD4-independnet (CD4i) Env clones (QA255-CD4iA and QA255-CD4iB) were a gift from Beth Haggarty, Andrea Jordan, Michael Hogan and James Hoxie) [145].

The following wild type envelope clones were also used: 7 subtype A clones (QA255.21P.ENV.A15, Q461.d1, QH209.A2, QH359.21M.ENV.C1, QF495.23M.ENV.B2, Q842.d16, QH343.21M.ENV.A10) [88,146,147], 7 subtype B clones (RHPA.4259.7,

WITO4160.B33, AD8, TRJO4551.58, CAAN5342.A2, QH0692.42, PVO.4) [148], 6 subtype C clones (ZM233M.PB6, ZM249.PB1, CAP210.2.00.E8, CAP45.2.00.G3, DU156.12, DU422.1) [148,149], and 3 A/D recombinant subtype clones (QG393.60M.ENV.B7, QA790.204I.ENV.A4, QA790.204I.ENV.C1) [146].

The following SHIV envelope clones were used: SHIV AD8-EO [112] and SF162P3 [122]. SHIV AD8-EO Env was cloned from a full-length proviral clone into the pCI-neo vector using *EcoRI* and *SalI* restriction enzymes.

Production of virus

Pseudoviruses were generated by co-transfecting 293T cells with 0.5 μg of each envelope clone of interest with 1.0 μg of an env-deficient subtype A proviral plasmid (Q23Δenv) [88]. For these studies, $4x10^5$ 293T cells were plated in a 6-well dish ~24 hours prior to transfection in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine (DMEM complete media). For each transfection, plasmid DNA was mixed with 6 ul of Fugene 6 transfection reagent (Roche). Pseudoviruses were harvested 48 hours post-transfection. Supernatants were spun at 1200 x g for 5 minutes at room temperature to remove cell debris, and aliquots of the pseudovirus stocks were stored at -80°C. GFP reporter viruses were generated by co-transfecting $5x10^5$ 293T cells, plated the day prior, with 333 ng of each envelope clone and 667 ng of Q23Δenv-GFP – a derivative of the subtype A Q23Δenv provirus that encodes GFP [88]. Transfections were performed as described for pseudoviruses, and supernatants were harvested 72 hours post-transfection. The viral titer of each transfection supernatant was determined by infecting TZM-bl cells and counting the number of blue cells at 48 hours post-infection after staining for β-galactosidase activity [150].

Viral stocks of full-length, replication-competent SHIV 1157ipEL-p [111] were generated by expanding the virus in immortalized pig-tailed macaque lymphocytes as described [151].

Neutralization assay

Approximately 500 infectious pseudoviral particles in 25 ul of DMEM complete media were incubated with 5-fold serial dilutions of each monoclonal antibody or sCD4 in duplicate for 60 minutes at 37°C. $1x10^4$ TZM-bl cells in 100 ul of DMEM complete media were added to each dilution in the presence of DEAE-dextran at a final concentration of 10 ug/ml. For assays using SHIV 1157ipEL-p, full-length, replication-competent virus was used. Each monoclonal antibody (MAb) – VRC01, VRC03 [139], PG9, PG16, PGT145 [140], PGT121, PGT128 [138], 10E8 [141], 447-52D [152], and 697-30D [153] – was tested at a starting concentration of 10 ug/ml. The following V3 MAbs were tested at a starting concentration of 50 μg/ml: 447-52D [152], 2219 [154], 2557 [155] , 3074 [155], 3869 [156], 2424 [157], 10-188 [158] and 1-79 [159]. At 48 hours post-infection, β-galactosidase activity was measured using the Galacto-Lite system (Applied Biosystems). For assays using MAb 17b [160], pseudovirus was incubated with antibody dilutions for 6 hours before infection of TZM-bl cells. Pooled plasma from 30 HIV-1+ individuals from Kenya [161] was tested at 2-fold serial dilutions starting at 1:100. The IC50 value was determined as the concentration of antibody or reciprocal plasma dilutions at which 50% of the pseudovirus input was neutralized as previously described (Wu et al JVI 2006). IC50 values represent the average of at least 2 independent experiments performed in duplicate.

Generation of pseudoviruses incorporating heterotrimeric Env proteins

Pseudoviruses incorporating heterotrimeric Env proteins were generated by co-transfecting varying ratios of Q23.17 A204E mutant Env and Q23.17 parental Env plasmids. Two independent DNA preparations were generated and tested for each mutant and parental plasmid. The concentration of DNA in each preparation was determined using the Nanodrop 2000 spectrophotometer (Thermo Scientific). The probability of any given trimeric protein incorporating a mutant monomer defined as: i number of mutant protomers with f fraction of mutant transfected, assuming random assortment of mutant and parental protomers, was determined using the following formula: $\binom{3}{i} f^i (1-f)^{3-i}$ [162].

Infection of mCD4 and huCD4 cells

Cf2Th/syn CCR5 cells that stably express human CCR5 and either pig-tailed macaque (m) CD4 or human (hu) CD4 were plated in a 24-well plate ~24 hours prior to infection at $4x10^4$ cells/well in 500 ul of DMEM complete media [137]. Cells were infected with HIV-1 Env pseudotyped Q23ΔenvGFP reporter viruses at an estimated MOI of 0.1 in 100 ul of DMEM by spinoculation for 90 minutes at 1,200 x g in the presence of DEAE-dextran at a final concentration of 10 ug/ml. After incubation for 48 hours, the cells were washed with 1x PBS and then incubated with 100 ul of 5 mM EDTA to remove the cells from the plate. The cells were then fixed with 500 ul of 1% paraformaldehyde. Cells were analyzed for GFP expression by flow cytometry on the Canto II (BD Biosciences).

Data presentation and analysis. Data were plotted, and Spearman correlations were performed using Prism version 6.0c (GraphPad Software). A204 and G312 residues were highlighted on the BG505 SOSIP structure (PDB: 4NCO) [163] using MacPyMOL.

Results

A204E and G312V mutations that permit entry with macaque CD4 affect antibody recognition of the V1V2 region

We assessed whether mutations at A204E and G312V, which lead to increased entry using mCD4 [137], affect the antigenic properties of HIV-1 Env. Seven Envs that represent the most common subtypes circulating in sub-Saharan Africa (A, C and D) were tested: three that were isolated recently after sexual transmission (23 – 70 days), two isolated in the second year of infection, one from an infant at the first HIV positive time after birth (42 days), and one from the corresponding mother during chronic infection (Fig. 2.1a). For five of the seven Env variants tested, independent introduction of the A204E and G312V mutations yielded functional Envs capable of mediating infection using both the human and macaque CD4 receptor [136,137]. For two of the Envs (QA013.H1 and QC406.F3), only the A204E mutants encoded an Env capable of mediating entry; the G312V mutation yielded an Env that could not mediate entry into cells using either human or macaque CD4, and these two non-functional variants were not analyzed in this study. Simultaneous introduction of the A204E and G312V mutations did not produce a functional Env for any of the variants tested. The functional Envs harboring either the A204E or

G312V mutation, which were selected by passage *in vitro*, are referred to here as mCD4-adapted Envs.

The lab previously reported that the introduction of either the A204E or G312V mutation resulted in increased sensitivity to soluble CD4 (sCD4) [136]. Despite this change in sensitivity to sCD4, introduction of these mutations to parental envelopes had little (<6-fold) to no effect on neutralization by the CD4-binding site antibody VRC01 (Fig.1 a,b). Similarly, for six of the seven Envs tested, the mCD4-adapted mutants exhibited a relatively modest change (<10-fold) in IC50 value for the PGT antibodies recognizing the V3 loop and the MPER antibody 10E8 (Fig. 2.1a, c, d). The exception to this was BG505 for which the mCD4-adapted Envs were resistant to PGT121 and/or PGT128. Introduction of A204E or G312V resulted in an increase in resistance to the quaternary V1V2 antibodies PG9, PG16 and PGT 145. This was true for all four Envs that were originally neutralization sensitive. For three of these four mCD4-adapted Envs, the increase in IC50 value for PG9 and PG16 was greater than 300-fold compared to parental (Fig. 2.1a, e). For the other three Envs, that were initially resistant to these MAbs, the mCD4-adapted Envs showed no detectable change in neutralization sensitivity. In one such case, an Env (QF495.A3) that has the N160 residue, but does not have the full glycosylation sequon (N-X-S/T) [140,164] that is required for glycan-dependent recognition by PG9 and PG16, was resistant to neutralization, whether or not the A204E or G312V mutation was present. Two of the envelopes (QA013.H1 and QC406.F3) that maintained the N160 glycosylation site and were still resistant to PG9 and PG16 remained resistant when the mutations were introduced.

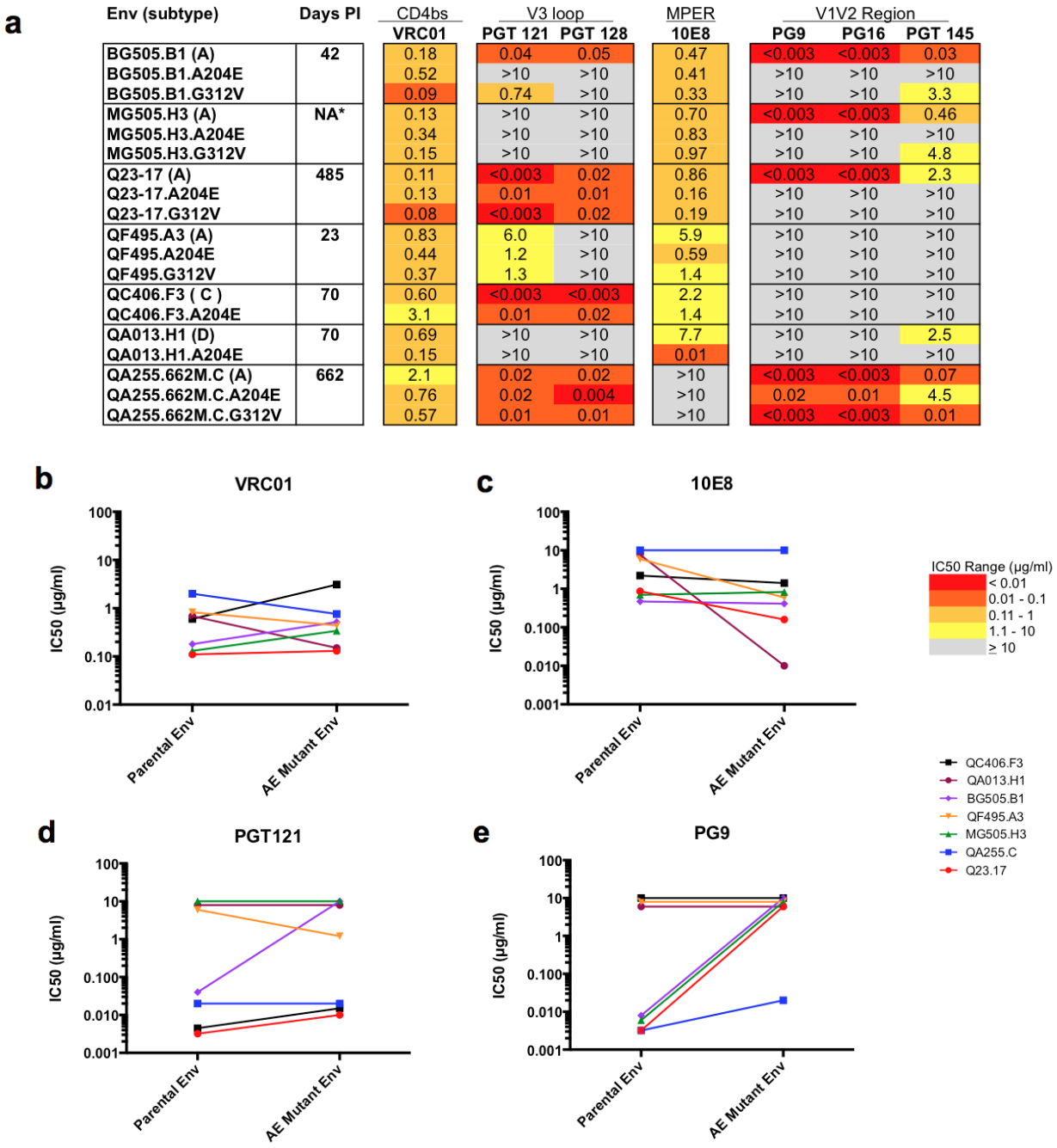

Figure 2.1. (a) Summary of neutralization profiles of seven parental HIV-1 Envs and corresponding A204E/G312V mutants. Patient ID, virus subtype in parentheses, and estimated days post-infection are indicated in the first two columns. NA indicates that days post-infection is not available. The A204E and G312V mutants are indicated below the corresponding parental Env. Darker shading indicates increasing sensitivity to the MAb tested, according to the key at the bottom. Gray shading indicates that 50% neutralization was not reach at the highest concentration tested (10 µg/ml). The IC50 values indicated in the chart represent averages from at least two independent experiments performed in duplicate. Comparison of IC50 values for parental and A204E mutant Envs for the MAbs VRC01 (b), 10E8 (c), PGT121 (d) and PG9 (e). IC50 values (µg/ml) are plotted for the parental (left) and corresponding A204E mutant (right) Envs. The key at the right indicates the color coding for each parental and mutant Env pair tested.

Thus, while the A204E and G312V mutations had little effect on recognition by the CD4bs antibody VRC01 and V3 and MPER antibodies – MAbs that are directed to epitopes that are largely found within the monomeric protein [138,141]– these mutations disrupted recognition by MAbs targeting quaternary V1V2 epitopes (PG9, PG16 and PGT 145) in Envs that were sensitive to these MAbs.

A204E mutants are sensitive to CD4-induced antibody 17b

Our initial results showing dramatic increases in resistance to PG9, PG16 and PGT145 in the mCD4-adapted Envs compared to the parental Env, and the fact that the mutations are found outside the characterized contact residues of these antibodies prompted us to explore whether these mutations cause structural perturbations using additional antibodies as probes. The epitopes of PG9 and PG16 depend on interactions involving neighboring gp120 subunits that come into contact in the envelope trimer [140,143,164]. Of note, the interactions between adjacent gp120 subunits in the Env trimer are also disrupted in the context of CD4 binding [165]. We hypothesized that introduction of the A204E and G312V mutations resulted in an open trimer conformation, similar to that observed upon CD4 engagement. To explore this hypothesis, we tested the mCD4-adapted Envs for neutralization sensitivity to the monoclonal antibody 17b that recognizes a CD4-induced epitope [160]. The neutralization assay was performed in the absence of sCD4 in order to assess the sensitivity of the native trimer to neutralization by 17b. None of the parental envelopes were sensitive to 17b at the highest concentration of antibody tested, 10 μg/ml. For five of the seven envelopes tested, the A204E mutant was more sensitive to neutralization by 17b than the corresponding parental Env (Fig. 2.2a). None of the G312V

mutants exhibited increased sensitivity to 17b compared to the parental envelope (Fig. 2.2a, b). The IC50 value for 17b correlated with the reciprocal dilution IC50 value for pooled HIV-1+ plasma (Spearman $r = -0.81$, $p<0.001$) suggesting that CD4-induced antibodies similar to 17b may mediate the increased sensitivity to pooled plasma for these A204E Envs.

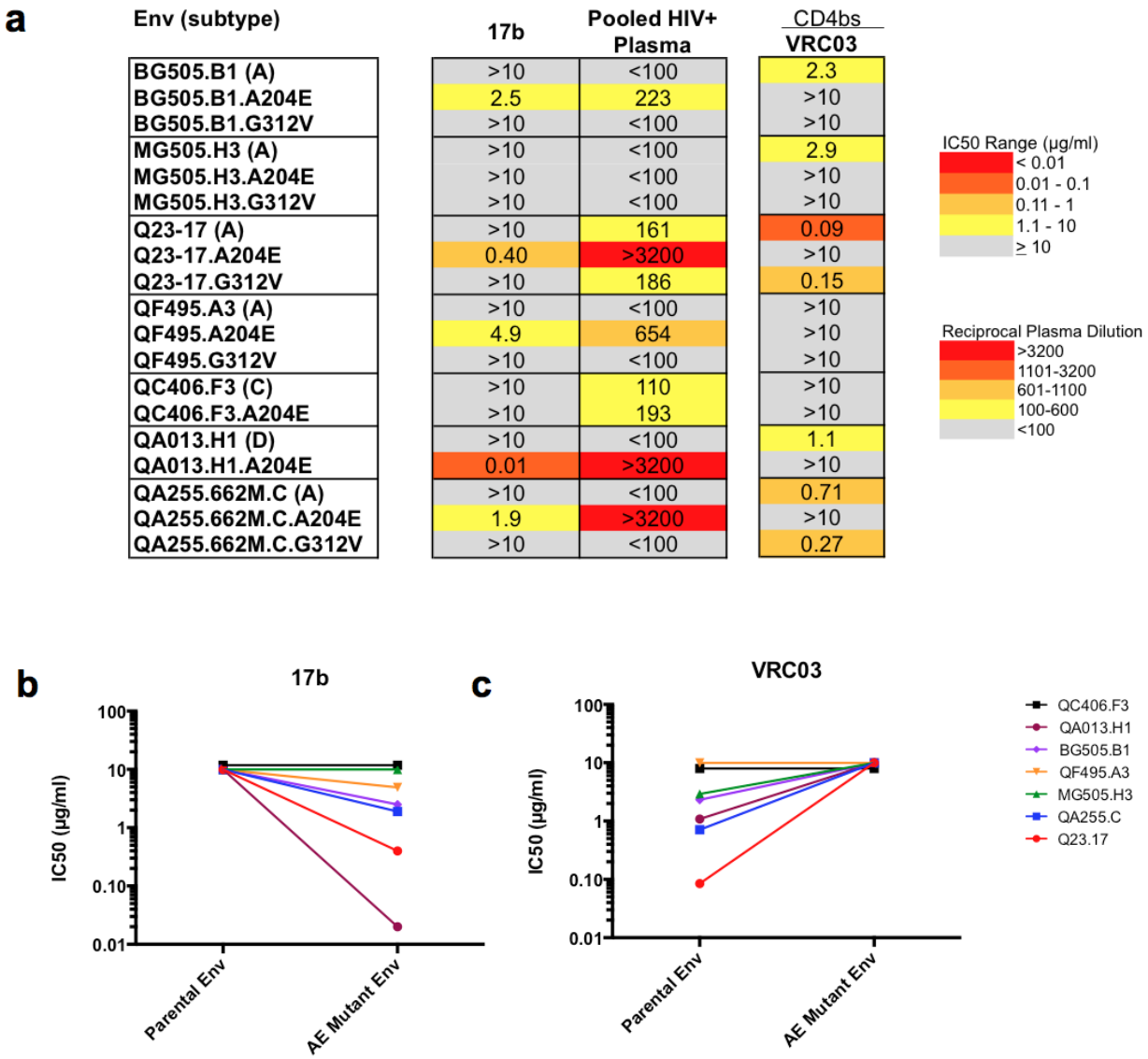

Figure 2.2. (a) Summary of neutralization sensitivities of parental and corresponding A204E/G312V mutant Envs to the MAbs 17b and VRC03 and to HIV+ pooled plasma. Patient ID is indicated in the first column. The A204E and G312V mutants are indicated below the corresponding parental Env. Darker shading indicates increasing sensitivity to the MAb tested or increasing sensitivity to HIV+ pooled plasma, according to the key at the bottom. Gray shading indicates that 50% neutralization was not reach at the highest concentration tested (10 µg/ml) for MAbs or the highest dilution of plasma tested (1:100). Comparison of IC50 values for parental and A204E mutant Envs for the Mabs 17b (b) and VRC03 (c). IC50 values (µg/ml) are plotted for the parental (left) and corresponding A204E mutant (right) Envs. The key at the right indicates the color-coding for each parental and mutant Env pair tested.

The A204E mutation disrupts quaternary epitopes on the Env trimer

In order to investigate further the structural changes induced by these adaptive mutations, we first tested a MAb that is predicted to make contacts with the adjacent protomer near the CD4bs (VRC03) [142]. VRC03 is a broad and potent neutralizing antibody, and recently, Lyumkis *et al*. described the quaternary nature of the epitope recognized by VRC03 [139,142]. Five of the Envs that were initially sensitive to VRC03, and in all five cases, introduction of the A204E mutation rendered these Envs resistant to neutralization by VRC03 at the highest antibody concentration tested (10 μg/ml) (Fig. 2.2a, c). The subtype A variant Q23.17 exhibited the greatest effect with a >100-fold increase in IC50 value. For four of these Env pairs, the A204E mutation also increased resistance to quaternary V1V2 antibodies (PG9/PG16 and PGT145) (Fig 2.1a). The G312V mutation had a more selective effect on VRC03 neutralization, with only two of four initially sensitive variants becoming resistant, both of which also acquired resistance to PG9/PG16 MAbs (Fig 2.1a). The Envs that were initially resistant to VRC03 remained resistant when mutated to either A204E or G312V.

The A204E mutation exposes epitopes in V2 and V3 hidden in the parental Env

The above results and the observation that portions of the V2 and V3 domains are occluded in the Env structure [142,163] led us to hypothesize that the V2 and V3 domains may be more exposed in the mCD4-adapted Envs. To address this hypothesis, we tested antibodies that target non-quaternary, conformational epitopes in V2 (697-30D) [153] and V3 (447-52D) [152] , 2219 [154], 2557 [155] , 3074 [155], 3869 [156], 2424 [157], 10-188 [158] and 1-79

[159]. All of the parental Env clones tested were resistant to neutralization by 697-30D and the panel of V3 MAbs with IC50 values greater than 10 μg/ml (Fig. 2.3). Six of the seven Envs tested (Q23.17, BG505.B1, MG505.H3, QF495.A3, QA255.662M.C and QA013.H1) became sensitive to at least two of the V3 MAbs tested upon introduction of the A204E mutation (Fig. 2.3). All five of the subtype A Envs became sensitive to at least five of the V3 MAbs tested. We did not observe increased sensitivity of QC406.F3, a subtype C Env, to any of the V2 or V3 MAbs. Of note, QA013.H1 and QC406.F3 have rare amino acid residues in the V3 loop that may affect binding of MAbs that target this region (Zolla-Pazner et al 2015 *submitted*).

We tested the G312V mutant Envs against only the V2 MAb 697-30D and the V3 MAb 447-52D. The G312V mutation did not affect sensitivity to either of these antibodies. In the case of 447-52D, this is not unexpected given that the G312 residue is critical for the binding of 447-52D to the V3 loop of Env [152]. Overall, these results indicate that introduction of the A204E mutation disrupts quaternary interactions at the apex of the trimer and at the CD4bs, as evidenced by resistance to PG9/16 and VRC03, and expose regions within the variable loops, as evidenced by sensitivity to MAbs targeting the V2 and V3 loops.

Env (subtype)	447-52D	697-30D	2219	2557	3074	3869	2424	10-188	1-79	CD4-IgG2	1418
BG505.B1 (A)	>50	>10	>50	>50	>50	>50	>50	>50	>50	3.6	>50
BG505.B1 A204E	16.0	>10	0.64	1.4	0.21	1.1	>50	0.82	1.3	0.002	>50
MG505.H3 (A)	>50	>10	>50	>50	>50	>50	>50	>50	>50	8.9	>50
MG505.H3 A204E	>50	>10	4.7	9.0	4.7	5.0	>50	4.1	21	0.03	>50
Q23.17 (A)	>50	>10	>50	>50	>50	>50	>50	>50	>50	0.13	>50
Q23.17 A204E	0.08	6.1	0.003	0.006	0.002	0.002	>50	0.004	0.008	0.001	>50
QF495.A3 (A)	>50	>10	>50	>50	>50	>50	>50	>50	>50	2.2	>50
QF495.A3 A204E	4.9	8.9	1.1	1.0	0.17	0.52	>50	0.46	11	0.006	>50
QC406.F3 (C)	>50	>10	>50	>50	>50	>50	>50	>50	>50	37	>50
QC406.F3 A204E	>50	>10	>50	>50	>50	>50	>50	>50	>50	0.007	>50
QA013.H1 (D)	>50	>10	>50	>50	>50	>50	>50	>50	>50	18	>50
QA013.H1 A204E	0.05	1.4	>50	>50	>50	>50	<0.0005	>50	>50	<0.0005	>50
QA255.662M.C (A)	>50	>10	>50	>50	>50	>50	>50	>50	>50	38	>50
QA255.662M.C A204E	0.82	>10	23	>50	0.01	11	>50	0.05	>50	0.006	>50

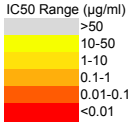

Figure 2.3. Summary of neutralization sensitivities of parental and corresponding A204E/G312V mutant Envs to the V3 MAbs and positive (CD4-IgG2) and negative (1418) controls. Patient ID is indicated in the first column. The A204E mutants are indicated below the corresponding parental Env. Darker shading indicates increasing sensitivity to the MAb tested according to the key at the bottom. Gray shading indicates that 50% neutralization was not reach at the highest concentration tested (50 or 10 µg/ml).

Disruption of quaternary epitopes in heterotrimeric Envs

The trimeric conformation of Env is maintained by metastable interactions between protomers. In order to determine whether disruption of the quaternary epitopes in the trimer requires that all protomers encode the determinants for mCD4 entry, we generated pseudoviruses expressing heterotrimeric Envs by co-transfecting plasmids encoding mutant Q23.17 A204E and parental Q23.17 Envs at fractions of 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 (mutant Env/total Env). For each population of heterotrimeric pseudoviruses resulting from the co-transfections, we determined the probability of any given heterotrimeric protein containing >0, >1 and exactly 3 mutant protomers, assuming random assortment of parental and mutant Env proteins [140,162,166]. For example, at a fraction of 0.2 mutant Env/total Env transfected, the probabilities that a given trimer has >0, >1 and exactly 3 mutant protomers are 0.49, 0.10 and 0.008, respectively.

IC50 values for VRC03 were determined for each set of heterotrimeric Envs. We plotted the VRC03 IC50 values of each population of heterotrimeric pseudoviruses independently against the probabilities of producing a protein with >0, >1 and exactly 3 mutant protomers (Fig. 2.4a, b, c). As expected, increasing the amount of A204E mutant transfected resulted in an increase in the VRC03 IC50 value indicating resistance to neutralization. For the plot of the probability of any given heterotrimeric protein having >1 (2 or 3) mutant protomer, the curve revealed a nearly log-linear relationship between the probability of having >1 mutant protomer and the VRC03 IC50 value (Fig. 2.4b). These results suggest that the heterotrimeric proteins containing two or three mutant protomers are largely responsible for the observed increases in

IC50 value. Thus, the disruption of quaternary interactions between adjacent protomers likely requires the presence of at least two mutant A204E protomers.

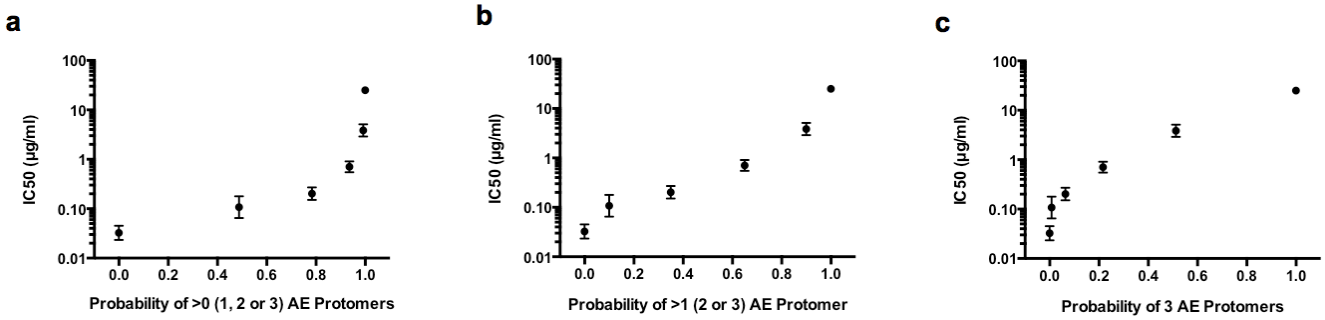

Figure 2.4. Sensitivity of heterotrimeric pseudoviruses to neutralization by VRC03. IC50 values are plotted on the y-axis versus the probability of any given trimer containing > 0 (a), >1 (b) or 3 (c) mutant protomers. Probabilities were calculated according to the fraction of mutant plasmid transfected (0, 0.2, 0.4, 0.6, 0.8 or 1). Two independent sets of DNA preps were generated and tested.

Neutralization profiles of Envs from pathogenic SHIV variants

Because the Env variants encoding changes that conferred increased replication in macaque lymphocytes showed substantial differences in their antigenic profiles compared to parental viruses, we assessed the neutralization profiles of three Env variants from pathogenic SHIVs (SHIV SF162P3, SHIV 1157ipEL-p, and SHIV AD8-EO) that were generated by serial passage in rhesus macaques [111,112,122] (Fig. 2.5a,b). All Envs from pathogenic SHIVs that we have tested were able to use mCD4 efficiently, similar to the Envs encoding the A204E and G312V mutations [137]. Similar to the mCD4-adapted Envs, two of the Envs from pathogenic SHIVs, both derived from subtype B HIV-1 variants (SHIV AD8-EO and SHIV SF162P3) were resistant to quaternary V1V2 Mabs (PG9/PG16) (Fig. 2.5a). The AD8 pair showed a similar profile with PG9/PG16 as the parental and A204E/G312V mCD4-adapted pairs – the parental Env (AD8) was sensitive to these quaternary MAbs, whereas the macaque-adapted variant (AD8-EO) was not (Fig. 2.5a). There was also a 17-fold decrease in sensitivity to PGT145 for this pair. In the case of SF162, the parental Env was also resistant to PG9/PG16, reflecting the fact that this variant encodes a K rather an N at position 160 [106,140]. SHIV SF162P3 does, however, encode an N at position 160; thus, the lack of a potential N-linked glycosylation site does not explain its resistance to PG MAbs [106]. The SF162 and AD8 Envs were all sensitive to VRC03 and resistant to 697-30D and 447-52D (Fig. 2.5b).

Env from SHIV 1157ipEL-p exhibited a different antigenic profile than the subtype B variants [111]. Similar to the mCD4-adpated Envs, this Env was sensitive to 17b and resistant to VRC03. In contrast to SHIV AD8-EO and SHIV SF162P3, Env from SHIV 1157ipEL-p was sensitive to PG9/PG16 (Fig. 2.5a). Thus, each of the Envs from animal-passaged, pathogenic

SHIVs was resistant to some MAbs directed to quaternary epitopes, although they were not uniformly or consistently resistant to particular MAbs of this type.

a

Env (subtype)	CD4bs	MPER	V3 loop		V1V2 Region		
	VRC01	10E8	PGT 121	PGT 128	PG9	PG16	PGT 145
AD8 (B)	0.32	1.4	<0.003	<0.003	<0.003	<0.003	0.05
AD8-EO	0.40	0.49	0.009	<0.003	>10	>10	0.87
SF162 (B)	0.36	0.16	0.004	<0.003	>10	>10	>10
SF162P3	0.20	0.46	<0.003	0.007	>10	>10	>10
1157ipEL-p (C)	0.53	>10	0.007	0.05	<0.003	<0.003	0.01

b

Env	17b	Pooled HIV+ Plasma	V2-specific 697-30D	V3 loop 447-52D	CD4bs VRC03
AD8 (B)	>10	<100	>10	>10	0.05
AD8-EO	>10	150	>10	>10	0.08
SF162 (B)	>10	>3200	>10	0.59	0.04
SF162P3	>10	119	>10	>10	0.07
1157ipEL-p (C)	6.7	351	>10	>10	>10

IC50 Range (µg/ml)
< 0.01
0.01 - 0.1
0.11 - 1
1.1 - 10
≥ 10

Figure 2.5. Summary of neutralization profiles of Envs from pathogenic SHIVs. The pathogenic SHIV from which the Envs were derived are indicated in the first column with subtype in parentheses (a, b). Darker shading indicates increasing sensitivity to the MAb tested or increasing sensitivity to HIV+ pooled plasma, according to the key at the right. Gray shading indicates that 50% neutralization was not reach at the highest concentration tested (10 µg/ml) for MAbs or the highest dilution of plasma tested (1:100).

Association between sensitivity to antibodies recognizing quaternary epitopes and macaque CD4 infectivity

The ability to use the mCD4 receptor for entry is a prerequisite for establishing persistent infection in macaques. Our neutralization data for the mCD4-adapted Envs suggest that adaptation of HIV-1 Envs to macaque CD4 *in vitro* and *in vivo* results in disruption of quaternary epitopes that are recognized by some bNAbs within the context of the trimer. These results raise the possibility that HIV-1 Env variants that use mCD4 as a receptor may not be targeted by these neutralizing antibodies, limiting the potential utility of the SHIV model in cases where the immune response targets more complex epitopes. To address this possibility, we investigated whether the ability of HIV-1 Envs to use the mCD4 receptor for entry predicts their sensitivity to a MAb that targets a quaternary epitope. We previously tested the ability of 30 HIV-1 Envs isolated from patients recently after transmission to enter cells using the mCD4 receptor [137]. In the present study, we tested an additional 18 HIV-1 Env variants isolated soon after infection for a total of 48 variants. Forty-three of the HIV-1 Envs tested were isolated recently after infection. We focused on recently transmitted variants because SHIVs encoding such Envs may be more representative of variants against which an effective vaccine would need to protect compared to lab-adapted Envs or those isolated later in infection. Entry was measured comparing infection of cells expressing mCD4 relative to those expressing human CD4 (Fig. 2.6). There was no correlation between sensitivity or resistance to PG9 (Spearman $r = 0.12$, $p = 0.42$) or PG16 (Spearman $r = 0.16$, $p = 0.27$) and entry using mCD4 suggesting that the ability to use mCD4 for entry is not a determinant of sensitivity to PG9/PG16 for HIV-1 Envs. For 83% (40/48) of the HIV-1 Envs tested, relative infection of mCD4 cells compared to human CD4 cells was $\leq$ 10%.

The eight HIV-1 Envs that used the mCD4 receptor more efficiently (relative infection of >10%) exhibited a wide range of neutralization sensitivity to PG9/16 (0.003 – 50 µg/ml). Among these variants, two Envs were neutralized at concentrations of 0.5 µg/ml, which is comparable to the median IC50 value of 0.2 µg/ml for variants tested against PG9 [138]. Overall, these results indicate that there is a subset of HIV-1 Envs that are able to use the mCD4 receptor for entry and maintain the quaternary epitope targeted by PG9/PG16.

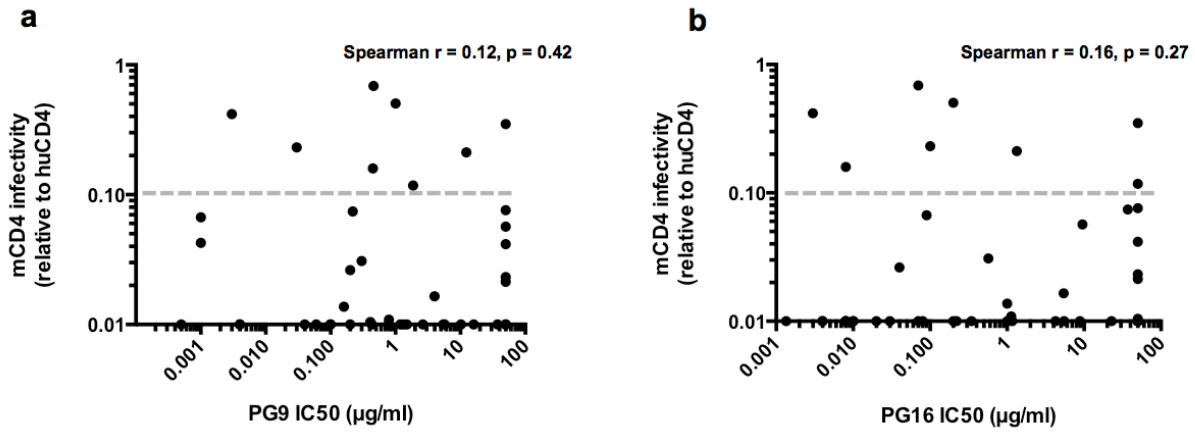

Figure 2.6. Relationship between infection of cells expressing macaque CD4 and sensitivity to MAbs targeting quaternary epitopes in the V1V2 region, PG9 (a) and PG16 (b). The association between infection of cells expressing macaque CD4 and sensitivity to neutralization by PG9 or PG16 was determined using infectivity data as described previously (Humes et al JVI 2012). Each dot represents a different Env variant tested. The identities of the Env variants are indicated in Supplemental Table 1. The gray dotted line represents the threshold (10% relative infection) used to identify HIV-1 Envs that might be suitable for SHIV development.

Discussion

Most HIV-1 Envs do not mediate entry using macaque CD4, or do so poorly, in particular those representing circulating HIV-1 variants spreading in humans [137]. In the context of a SHIV, Env can be readily adapted to increase replication fitness of the virus in macaque cells, with as little as a single point mutation required to increase infectivity [137]. Importantly, SHIVs currently used for vaccine and transmission studies have all undergone adaptation for increased replication fitness in macaques [98]. Here we show that the cost of the adaptation of HIV-1 for replication in macaques is a shift in antibody recognition. In particular, our data suggest that the quaternary structure of Env is altered, making adapted Envs generally resistant to antibodies, such as PG9/PG16, and VRC03 that recognize quaternary epitopes or make contacts with the adjacent protomer. However, there are some examples of circulating HIV-1 variants that can use the mCD4 receptor for entry and retain recognition by quaternary antibodies. We propose that the ability to use mCD4 as a functional receptor with limited disruption of quaternary epitopes should be used as criteria for the rational design of SIV/HIV chimeric viruses that best recapitulate features of Envs of biologically relevant HIV-1 variants.

Previous studies from the lab reported that two independent mutations, A204E and G312V, that were selected as a subtype A-derived SHIV adapted to replicate in macaque cells significantly increase mCD4-mediated entry [137]. Although the A204E and G312V mutations were identified by adapting a SHIV encoding a single HIV-1 Env variant (Q23.17), the mutations increased mCD4-mediated entry and replication for HIV-1 Envs representing globally circulating HIV-1 variants of diverse subtypes [136,137]. The G312V mutation was also observed in minimally modified HIV-1 passaged in macaques [125]. In this study, we assessed the conformational changes induced during adaptation to mCD4 across seven HIV-1 Env

variants, including six cloned directly from the infected individual and several representing transmitted variants. For parental Envs that were initially sensitive to the antibodies tested, the mCD4-adapted Envs were generally more resistant to MAbs that recognize quaternary epitopes on the Env trimer. These antibodies included PG9, PG16 and PGT145, which recognize complex epitopes in the V1V2 region [138,140], and VRC03, which targets the CD4bs [139] and is predicted to have more contact with adjacent protomers than other CD4bs MAbs such as VRC01 [142].

Interestingly, we detected differences in the antibody recognition profiles of the A204E compared to the G312V mutant. The A204E mutation caused a more global change in antibody recognition that included resistance to MAbs targeting quaternary epitopes in V1V2 (PG/PG16 and PGT145) and the CD4bs (VRC03), increase in sensitivity to sCD4 and 17b, and for a subset of cases, sensitivity to MAbs that target epitopes in V2 and V3 that may be hidden in the parental Env [167,168]. The G312V mutants also showed resistance to MAbs targeting quaternary V1V2 epitopes (PG9/PG16 and PGT145), but only some variants showed resistance to VRC03. In addition, G312V did not affect the sensitivity to 17b or the V2 and V3 MAbs for any of the Envs tested suggesting that there are more limited structural changes due to the G312V than the A204E mutation.

Recently, the structure of a soluble, stabilized form of the Env trimer (SOSIP) of a subtype A variant used in this study (BG505) was determined [142,163]. Fig. 2.7 highlights the A204E and G312V mutations mapped onto the SOSIP trimer model (PDB 4NCO). Previous studies demonstrated that disruption of interactions between V1V2 and V3 within a gp120 relaxed the monomer to a state similar to that observed upon CD4 binding, with formation of the bridging sheet and a more ordered CD4 binding site [169,170]. Thus, there are likely at least two

constraints on Env in the context of the trimer: 1) quaternary interactions at the apex of the trimer formed by V1V2 and V3 from adjacent protomers, and 2) tertiary interactions within gp120 subunits including V1V2 and V3 within a given protomer. The observation that the A204E mutation causes both resistance to antibodies that target quaternary epitopes and sensitivity to antibodies that target CD4-induced epitopes suggest that this change is not simply causing local perturbations of a single epitope (Fig. 2.8). Rather, the data are consistent with a model in which this mutation relaxes both constraints on Env in the context of the trimer. Analysis of heterotrimeric Envs suggests that at least two mutant protomers are required to disrupt quaternary interactions within a heterotrimeric protein. The G312V mutation caused resistance to quaternary V1V2 antibodies, but did not cause increased sensitivity to antibodies targeting CD4-induced epitopes. Thus, the G312V mutation may loosen quaternary contacts between adjacent protomers without disrupting the tertiary interactions within gp120 subunits. The differences in the neutralization profiles of the A204E and G312V mutants suggest that there is a spectrum of conformational changes that can occur as HIV-1 Envs adapt to use mCD4 for entry. The A204E mutants may represent mCD4-adapted Envs that exhibit more extensive conformational changes similar to those observed for CD4-independent Envs while the G312V mutants represent mCD4-adapted Envs with changes limited to the quaternary contacts between adjacent protomers. These findings suggest that it may be possible to identify Env variants that can bind mCD4 and yet retain many of the structural and antigenic features of circulating HIV-1 variants.

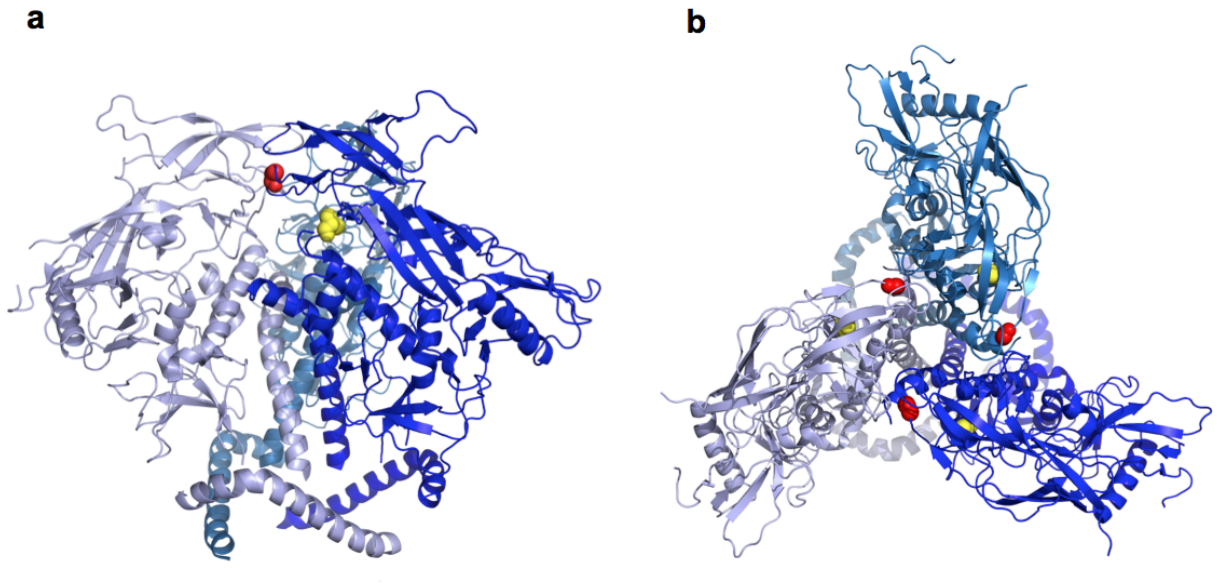

Figure 2.7. Location of A204E and G312V mutations in the context of the structure of the Env trimer. (a) Side view of two gp120 subunits of the BG505 SOSIP structure (Julien et al Science 2013) with the A204 residue highlighted in yellow and the G312 residue in red using MacPyMOL. (b) Top view of all three protomers of the BG505 SOSIP structure with the A204 and G312 residues highlighted.

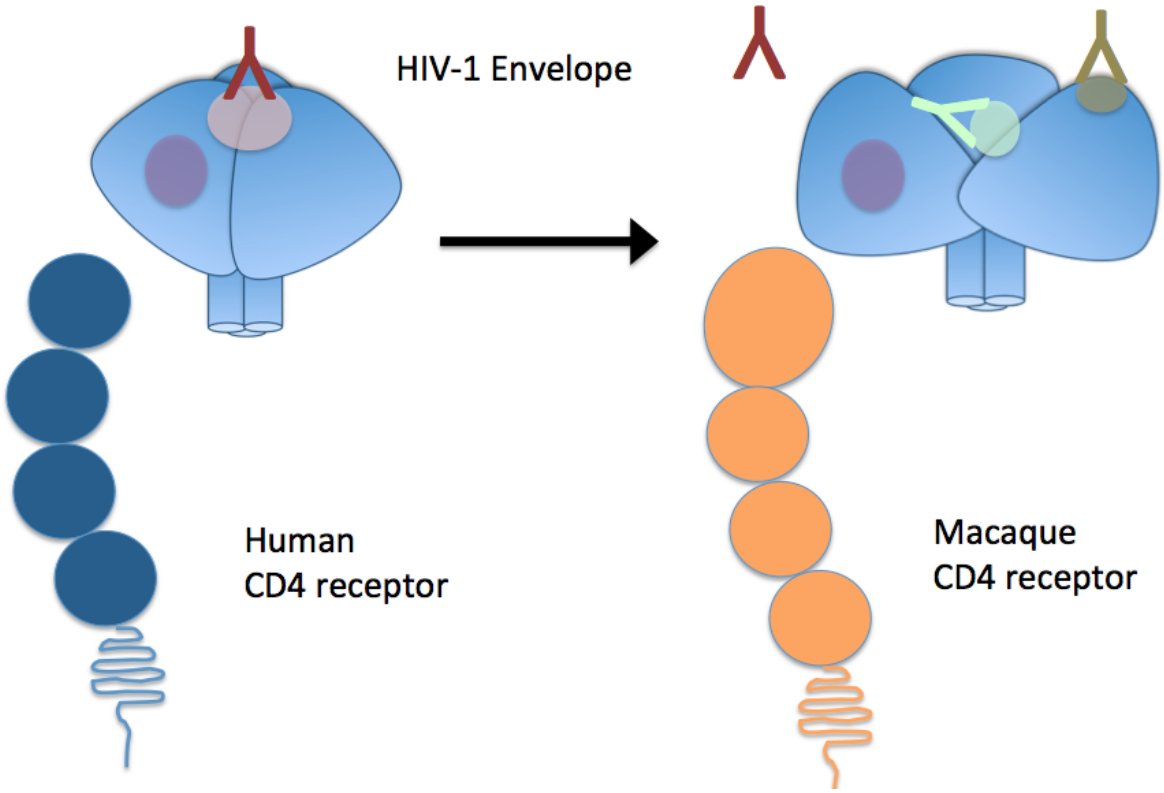

Figure 2.8. Proposed model of adaptation of HIV-1 Env to the macaque CD4 receptor and associated antigenic changes. Circulating HIV-1 Envs that use the human CD4 receptor maintain a more "closed" conformation and quaternary epitopes such as those in the V1V2 region indicated in red. Upon adaptation to macaque CD4, HIV-1 Envs adopt a more "open" conformation disrupting quaternary epitopes and exposing epitopes hidden in the circulating Env indicated in light green and brown. The CD4 binding site is indicated in purple.

The A204E and G312V mutations were selected to permit interaction with mCD4, and they are critical for allowing Env to mediate entry of a CD4 receptor encoding isoleucine at position 39, as found in the mCD4 molecule [137]. We recently published a study demonstrating that the antigenic changes that accompany adaptation to mCD4 are similar to those that result from adaptation for CD4-independence [145]. The neutralization profiles of the A204E mutants were similar to those of the CD4-independent Env variants derived from one of the Env variants tested in this study (QA255.662M.C), including resistance to quaternary-directed MAbs and increased sensitivity to MAbs targeting CD4-induced epitopes. Thus, the process of adapting to mCD4 may lead to similar functional and structural changes as the process of adapting to low human CD4 receptor levels and CD4-independence. Related to this model, prior studies of SIV observed that Env trimers from a CD4-independent SIV by cryo-electron microscopy exhibited an open conformation highly related to CD4-activated Envs and markedly different from the more compact CD4-dependent SIV Env [165,171]. Of note, the mutations that lead to adaptation to mCD4 also confer the ability to use lower amounts of the human CD4 receptor for entry [137]. Interestingly, previously described variants capable of infecting cells expressing low amounts of CD4 exhibit antigenic properties similar to those identified for A204E and G312V mutants, including resistance to PG9/PG16 and sensitivity to 447-52D [172]. Taken together, the results suggest that HIV-1 Env variants may experience similar selective pressures and resulting conformational changes as they adapt to cells expressing either mCD4 or low amounts of human CD4.

Given the changes in conformation in HIV-1 Env that resulted from adaptation to mCD4-mediated entry *in vitro*, we hypothesized that HIV-1 Env variants encoded by pathogenic SHIVs, which are selected after adaptation for replication in macaques, would show similar disruption of

quaternary antigenic determinants. Of note, these Envs from pathogenic SHIVs encode A at position 204 and G at 312, suggesting that other amino acids are determining the ability of these Envs to mediate entry via the mCD4 receptor. These differences allowed us to probe whether general features of the Env that confer the ability to use the macaque receptor alter its antigenic properties, irrespective of the specific mutations involved.

Two of the Envs tested (AD8-EO and SF162P3) were resistant to PG9/PG16, as reported previously [108], suggesting that they did not form the quaternary epitope required for antibody binding. In the case of AD8-EO, resistance to PG9/PG16 developed after passage of the parental virus (AD8) in animals, an evolution that is similar to that observed for the variants specifically selected for mCD4-mediated entry. Interestingly, Envs AD8-EO and SF162P3 exhibited more limited changes in antigenicity that were characteristic of the G312V mutants, including resistance to PG9/PG16 but not impacting VRC03 and 697-30D or 447-52D recognition. Env 1157ipEL-p had a unique profile exhibiting resistance to VRC03, but none of the other changes associated with either G312V or A204E. Env 1157ipEL-p was neutralized by 17b and HIV-1+ pooled plasma suggesting that epitopes similar to those exposed upon CD4 binding were formed in the native trimer.

Several prior studies have investigated the effect of serial passage in macaques on the antigenic properties of Env from CXCR4-using or dual-tropic SHIVs and also noted increased resistance to some monoclonal antibodies compared to the un-passaged viruses [107,134,173]. In these models, it is unclear what role adaptation to mCD4 plays given that CXCR4-tropic, lab-adapted viruses are able to infect using the mCD4 receptor [137,174,175]. Some studies have indicated a role of the host antibody response in driving escape that resulted in resistance to MAbs and plasma from infected macaques [133-135,176]. Also, this evolution has been

attributed in part to changes in co-receptor affinity or specificity [105,135], and changes in Env that allowed better interaction with the CD4 receptor may have facilitated this co-receptor switch [177]. Our findings suggest that in addition to direct antibody pressure and selection for a change in co-receptor specificity, selection for increased entry via the mCD4 receptor may be an important driver of antigenic variation in SHIVs. It is possible that the SHIVs that advanced through animal testing, which likely represent a small subset of those constructed and tested in cell culture, are the ones that were engineered with the rare HIV-1 Envs that utilized the mCD4 receptor with relatively high efficiency. It is also possible that adaption in macaques further enhanced infectivity. In some cases, such as 1157ipEL-p and SF162P3, the virus was passaged during acute infection [111,178], minimizing competing selection by host neutralizing antibodies. For virus such as AD8-EO, passage was later infection [112], a time when Env was potentially under competing selective pressures to maximize mCD4-mediated entry and at the same time escape from neutralizing antibodies. In this regard, our findings provide a potentially informative approach to identifying Envs that can use the mCD4 receptor, yet have minimal changes in their antigenic profile by adapting Env to mCD4 in the presence of antibodies such as 17b, 697-30D or 447-52D that would limit global structural effect on antibody recognition that lead to increased sensitivity.

The SHIVs analyzed in this study are important tools for assessing the capacity of antibodies to block infection provide therapeutic benefits and have lead to important findings concerning pathogenesis in macaques. Nonetheless, the disruption of key antigenic determinants in HIV-1 variants adapted for replication in macaques potentially limits the utility of this model for fairly assessing the benefit of antibodies directed to quaternary epitopes. PG9 and PG16 are prototype quaternary, glycan-dependent MAbs that target one of the major epitopes on HIV-1

Env, and thus, may be an important component of protective antibody responses elicited by a vaccine [75]. The potency of these MAbs may be underestimated using current SHIV models, although our data suggest that the SHIV 1157ipEL-p may be well suited for studies with these MAbs. Similarly, increased neutralization sensitivity to antibodies directed to epitopes that are more concealed on Env trimers could also be misleading. Therefore, there is need for novel pathogenic SHIVs that maintain quaternary epitopes targeted by antibodies such as PG9/PG16 and are otherwise more representative of the Env structure of viruses circulating in humans. We screened a large panel of HIV-1 Envs isolated recently after transmission and identified a subset of un-adapted Envs that are able to use the mCD4 receptor for entry at levels close to those of human CD4. This subset represents HIV-1 Envs from subtypes A, B and C that exhibit a wide range of sensitivity (IC50 0.003 – 50 μg/ml) to the MAbs PG9/PG16. Identification of HIV-1 Envs that are able to use the mCD4 receptor for entry and maintain quaternary epitopes may facilitate the generation of SHIVs that are more representative of transmitted/founder HIV-1 variants.

Chapter III: Identifying viral determinants of high-level replication capacity of SHIVs

Introduction

In the previous chapter, I described the complications for virus biology of adapting SHIVs to use the macaque CD4 receptor for entry [145]. The mCD4 receptor is, however, just one of many host factors that influence the ability of SHIVs to replicate in macaques. In the last decade, a number of host factors induced by the innate immune response that restrict the replication of lentiviruses have been identified [27,179]. Lentiviruses have evolved antagonists of these host factors allowing them to evade host cell restriction and replicate efficiently. Because HIV and SIV have evolved within their respective hosts, viral antagonists often act in a species-specific manner to counteract these antiviral factors [27,179]. As HIV-1 has adapted for replication in people and evolved the ability specifically to counteract human antiviral proteins, HIV-1 viral antagonists are largely ineffective against factors from other species, including macaques. The importance of evasion of macaque restriction factors for HIV-1 replication *in vitro* and *in vivo* has been clearly demonstrated [123,125,180]. Despite the fact that they encode the SIV antagonists of macaque restriction factors, many SHIVs replicate poorly in macaque cells [131]. The subset of SHIVs that do replicate well *in vitro* in macaque cells require additional animal-animal serial passage in order to establish persistent infection and cause disease [98]. In each case of animal-animal passage, adaptation has resulted in amino acid changes in the HIV-1 *env* gene, but the functional changes associated with adaptation have been poorly defined.

Currently, there are only a handful of pathogenic SHIVs being used in pre-clinical vaccine and therapeutics studies, and there is an urgent need to develop a larger panel of SHIVs that are more representative of HIV-1 variants circulating in people. Despite their limited numbers, pathogenic SHIVs do provide the opportunity to investigate the changes that have been selected for during the multiple rounds of adaption, both *in vitro* and *in vitro*. Some progress has been made in identifying the amino acid changes that contribute to increased transmissibility and pathogenicity of macaque-passaged SHIVs that encode HIV-1 Envs derived from chronic infection. In the case of the pathogenic SHIV SF162P3, amino acid changes in gp120 resulting from macaque-passage were found to increase mucosal transmissibility of the virus compared to the parental SHIV [106]. Another pathogenic virus, SHIV 89.6P, has been studied in detail. A series of studies identified amino acids changes in both gp120 and gp41 that resulted in increased pathogenicity [104,105,135]. Although these studies have provided important information on changes that occur during macaque-passage for these particular viruses, the mechanisms by which these adaptations increase pathogenicity have not been well defined. It also remains unclear whether these changes would have similar affects on replication capacity and pathogenicity in SHIVs that encode HIV-1 sequences that are more representative of variants circulating in people.

Identification of the viral determinants that contribute to the ability of SHIVs to replicate to high levels during acute infection and establish persistent infection with progression to AIDS in macaques would provide important information on the mechanisms of transmission and pathogenesis and might allow for the rational design of pathogenic SHIVs based on HIV-1 variants circulating in people, including T/F viruses. The goals of this chapter were to determine if pathogenic SHIVs differ from SHIV based on circulating HIV-1 variants with respect to

replication capacity and to identify the viral determinants that contribute to the ability of pathogenic SHIVs to replicate to high levels in macaque cells. We tested the replication capacity and IFN-I sensitivity of a panel of nine SHIVs, including three representing pathogenic SHIVs adapted for replication in macaques, two encoding culture-adapted HIV-1 Envs and four encoding HIV-1 Envs representing circulating variants. Our results indicate that adapted SHIVs selected for replication in lab culture or in macaques have higher replication capacity compared to minimally adapted SHIV based on circulating HIV-1 Envs. We identified HIV-1 Env as an important determinant of high replication capacity in macaque cells. Our data also suggest that the amount of HIV-1 Env expressed in infected macaque cells and present in virions contributes to the ability of pathogenic SHIVs to replicate well in macaques. Thus, modulation of HIV-1 Env expression in infected macaque cells and virion-associated Env content may be a novel strategy to increase the replication capacity of SHIVs based on circulating HIV-1 variants.

Materials and Methods

Construction of full-length proviral clones

Full-length proviral SHIV clones encoding the region spanning the *vpu* and *env* open reading frames from different HIV-1 variants were generated using SHIV AD8-EO as a vector [112]. Expression plasmids encoding *vpu* and *env* open reading frames for Q23ENV.17, BG505.W6M.ENV.B1, MG505.W0M.ENV.H3, and QF495.23M.ENV.A3 were amplified using primers to introduce an EcoRI restriction site 5' of the *vpu* start codon and a SalI restriction site

3' of the *env* stop codon [136,137]. Amplicons of ~2.7 kb were digested using EcoRI and SalI and ligated into the SHIV AD8-EO full-length proviral plasmid.

Chimeras between SHIV AD8-EO and SHIV Q23AE were generated by overlap-extension PCR. Fragments from SHIV AD8-EO and SHIV Q23AE were amplified using primers with overlapping overhang segments (Table 3.1). Outer primers containing EcoRI and SalI restriction sites were used to amplify the ~2.7 kb *vpu* and *env* open reading frames. Amplicons encoding chimeric proviral DNA were digested with EcoRI and SalI restriction enzymes and ligated into the SHIV AD8-EO full-length proviral plasmid.

The following full-length proviral plasmids of the parental SHIVs were also used in this study: SHIV AD8 [112] and SHIV SF162 [131].

Production of virus

Full-length, replication-competent virus was generated from proviral molecular clones by transfecting 293T cells with 4 μg of proviral plasmid DNA. For these studies, $2x10^6$ 293T cells were plated in a T-75 flask ~24 hours prior to transfection in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) and 2 mM L-glutamine (complete DMEM). For each transfection, proviral plasmid DNA was mixed with 12 μl of Fugene 6 transfection reagent (Roche). Replication-competent virus was harvested 48 hours post-transfection. Supernatants were passed through a 0.2 μm sterile filter to any contaminants and cell debris and were concentrated using a 100k molecular weight protein concentrator (Amicon). Aliquots of replication-competent virus were stored at -80°C. The viral titer of each transfection supernatant was determined by infecting TZM-bl cells and counting the number of blue cells at 48 hours post-infection after staining for β-galactosidase activity [150].

Replication-competent stocks of SHIV SF162P3 [122] and SHIV 1157ipd3N4 [111] were generated by expanding the virus in immortalized pig-tailed macaque lymphocytes [151]. For each virus, $2x10^6$ cells were infected at an initial multiplicity of infection (MOI) of ~0.02. Virus and cells were spinoculated in a 24-well plate in a total volume of 1 ml at 1200 x g for 90 minutes at room temperature. After spinoculation, the infected cells were washed 1x with 1 ml of Iscove's modified Dulbecco's medium (IMDM) supplemented 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) (complete IMDM). Infected cells were then re-suspended in 2.4 ml of media and plated in a 6-well plate. Every three days, infected cells were harvested into a 15 ml conical tube and pelleted at 650 x g for five minutes at room temperature. The entire infected cell supernatant was harvested, and six aliquots of 300 µl each were made. Aliquots of expanded virus were stored at -80° C.

Viral replication assays

Replication of SHIVs was assessed using immortalized pig-tailed macaque lymphocytes [151], primary pig-tailed macaque (Ptm) peripheral blood mononuclear cells (PBMCs) and rhesus macaque (Rhm) PBMCs. Immortalized Ptm lymphocytes were maintained in complete IMDM supplemented with 100 U/ml of IL-2. $1x10^6$ immortalized Ptm lymphocytes were infected at an estimated MOI of 0.02 in a final volume of 250 µl in a 96-well plate. Virus and cells were spinoculated at 1,200 x g for 90 minutes at room temperature. The infected cells were washed three times in 1.0 ml of complete IMDM and re-suspended into duplicate 600 µl cultures in a 48-well plate. 400 µl of each cell supernatant was harvested, and cultures were replenished

with the appropriate medium every three days. SIV p27 concentrations were determined using a SIV p27 antigen enzyme-linked immunosorbent assay (ELISA) kit (ABL, Rockville, MD).

For viral replication assays in Ptm and Rhm PBMCs, cells were isolated from whole macaque blood. Isolated PBMCs were stimulated for three days prior to infection with IL-2 (20 U/ml) and concanavalin A (5 μg/ml) in RPMI 1640 medium supplemented with 20% FCS and 2 mM L-glutamine. $1x10^6$ Ptm or RhPBMCs were spinoculated and maintained as described for immortalized Ptm lymphocytes.

Viral infectivity assay

The specific infectivity of supernatants harvested from SHIV-infected immortalized Ptm lymphocytes at six and nine days post-infection was determined using the TZM-bl infectivity assay. In order to normalize the cell supernatants for SIV p27 input, the concentration of SIV p27 was determined using a SIV p27 antigen enzyme-linked immunosorbent assay (ELISA) kit (ABL, Rockville, MD). For each sample, 1 ng of SIV p27 was brought up in complete DMEM to a final volume of 200 μl and transferred to a well of a 96-well plate. Then, $1x10^4$ TZM-bl cells in 50 μl of media were added to each well of a 96-well plate in the presence of 10 μg/ml of DEAE-dextran. For each sample, infections were performed in duplicate. Infections were incubated for 48 hours at 37° C, and then β-galactosidase activity was measured using the Galacto-Lite system (Applied Biosystems).

Western blot analysis of HIV-1 envelope content and expression

The amount of HIV-1 envelope (Env) in virions and infected macaque cells was determined by semi-quantitative western blot using the LICOR Odyssey system. For virion-

associated Env content, supernatants from infected immortalized Ptm lymphocyte cultures were pelleted through a 25% sucrose cushion by ultracentrifugation for 90 minutes at 28,000 rpm. Virus pellets were lysed in 70 µl of radioimmunoprecipitation assay buffer (RIPA) for 10 minutes at room temperature (ref for RIPA recipe). The concentration of SIV p27 antigen in the virus lysates was determined ELISA, and virus lysate input was normalized to 5 ηg of p27. Western blotting was performed as described previously [181] using as primary antibodies rabbit polyclonal anti-HIV-1 Env sera [182] and mouse anti-SIV p27 monoclonal antibody (ABL, catalog no. 4323). For western blotting of whole cell lysates, SHIV-infected immortalized Ptm lymphocytes were pelleted by spinning at 650 x g for 5 minutes at room temperature. Cell pellets were washed with 1x PBS and then lysed in 100 µl of RIPA buffer for 10 minutes at room temperature. Western blotting was performed as described for virus lysates.

HIV-1 envelope quantitative reverse transcriptase PCR

The amount of HIV-1 envelope mRNA and unspliced RNA in SHIV-infected macaque cells was measured by quantitative reverse transcriptase PCR (qRT-PCR). Total RNA was isolated from SHIV-infected macaque using the miRNeasy Mini Kit (Qiagen) according to the manufacturers instructions. Total RNA concentration was measured using the Nanodrop 2000 spectrophotometer (Thermo Scientific). For each reaction, 50 ng of total RNA was amplified using the Superscript III Platinum SYBR Green One-Step qRT-PCR kit with ROX (Invitrogen). To amplify unspliced Gag RNA, the forward primer 5'-AGGGACTTGGCAAATGGATTGTAC-3' and reverse primer 5' GTGTAATAGGCCATCTGCCTGCC-3' were used. To amplify splice Vpu/Env mRNA, the forward primer 5'-AGGAACCAACCACGACGGAGTGCTC -3' and reverse primer 5'-

CATTGCCACTGTCTTCTGCTCTTTC-3' were used. To amplify macaque beta actin mRNA, the forward primer 5'-CAACCGCGAGAAGATGACCCAGATCATG-3' and the reverse primer 5'-AGGATGGCATGGGGGAGGGCATAC-3' were used. Samples were run in a 96-well plate using the Applied Biosystems 7900HT. Relative levels of HIV-1 Env mRNA for each virus was determined using the following equation: $2^{-\Delta\Delta} = [(C_T\ env - C_T\ beta\ actin)] - [(C_T\ gag - C_T\ beta\ actin)]$ [183].

SHIV Q23AE-AD8gp160			
Round	Forward/Reverse	Name	Sequence (5' - 3')
1	Forward	AD8P5_Q23AE_gp160_1	CAG AAG ACA GTG GCA ATG AAA GTG AAG
1	Reverse	AD8P5_Q23AE_gp160_2	CTT CAC TTT CAT TGC CAC TGT CTT CTG
2	Forward	SHIV_YE_EcoRI_F	CTG CAT CAA ACA AGT AAG TAG AAT TCG
2	Reverse	SHIV_YE_SalI_R	CCA TGG TCT CGT CGA CTT ATA GCA AAG

SHIV Q23AE-AD8gp120			
Round	Forward/Reverse	Name	Sequence (5' - 3')
1	Forward	AD8P5_Q23AE_gp120_1	GCA GAG AGA AAA AAG AGC AGT TGG AAT AG
1	Reverse	AD8P5_Q23AE_gp120_2	CTA TTC CAA CTG CTC TTT TTT CTC TCT GC
2	Forward	SHIV_YE_EcoRI_F	CTG CAT CAA ACA AGT AAG TAG AAT TCG
2	Reverse	SHIV_YE_SalI_R	CCA TGG TCT CGT CGA CTT ATA GCA AAG

SHIV Q23AE-AD8gp41			
Round	Forward/Reverse	Name	Sequence (5' - 3')
1	Forward	AD8P5_Q23AE_gp41_1	GAG AAA AAA GAG CAG TTG GAG CAA TAG G
1	Reverse	AD8P5_Q23AE_gp41_2	CCT ATT GCT CCA ACT GCT CTT TTT TCT C
2	Forward	SHIV_YE_EcoRI_F	CTG CAT CAA ACA AGT AAG TAG AAT TCG
2	Reverse	SHIV_YE_SalI_R	CCA TGG TCT CGT CGA CTT ATA GCA AAG

SHIV Q23AE-AD8tat			
Round	Forward/Reverse	Name	Sequence (5' - 3')
1	Forward	SHIV Q23AE-AD8tat_1_F	GTCATTTCAGACCCATCTCCCAACCCCGAG
1	Reverse	SHIV Q23AE-AD8tat_1_R	CTCGGGGTTGGGAGATGGGTCTGAAATGAC
2	Forward	SHIV_YE_EcoRI_F	CTG CAT CAA ACA AGT AAG TAG AAT TCG
2	Reverse	SHIV_YE_SalI_R	CCA TGG TCT CGT CGA CTT ATA GCA AAG
3	Forward	SHIV Q23AE-AD8tat_2_F	GATCCGTGCGATTAGTGAGCGGATTCTTAG
3	Reverse	SHIV Q23AE-AD8tat_2_R	CTAAGAATCCGCTCACTAATCGCACGGATC
4	Forward	SHIV_YE_EcoRI_F	CTG CAT CAA ACA AGT AAG TAG AAT TCG
4	Reverse	SHIV_YE_SalI_R	CCA TGG TCT CGT CGA CTT ATA GCA AAG

SHIV Q23AE-ADAleader			
Round	Forward/Reverse	Name	Sequence (5' - 3')
1	Forward	SHIVQ23AE-NL43leader1_F	TCG TAA ATG TAA TGA GAG TGA AGG AGA AGT ATC AG
1	Reverse	SHIVQ23AE-NL43leader1_R	CTG ATA CTT CTC CTT CAC TCT CAT TAC ATT TAC GA
2	Forward	SHIV_YE_EcoRI_F	CTG CAT CAA ACA AGT AAG TAG AAT TCG
2	Reverse	SHIV_YE_SalI_R	CCA TGG TCT CGT CGA CTT ATA GCA AAG
3	Forward	SHIVQ23AE-NL43leader2_F	TGA TGA TCT GTA GTG CTA CAG AAA ACT TGT GGG TTA
3	Reverse	SHIVQ23AE-NL43leader2_R	TAA CCC ACA AGT TTT CTG TAG CAC TAC AGA TCA TCA
4	Forward	SHIV_YE_EcoRI_F	CTG CAT CAA ACA AGT AAG TAG AAT TCG
4	Reverse	SHIV_YE_SalI_R	CCA TGG TCT CGT CGA CTT ATA GCA AAG

SHIV Q23AE-CTdel			
Round	Forward/Reverse	Name	Sequence (5' - 3')
1	Forward	SHIVQ23AE-CTdel2_F	GAA CTT CTG GGA AGA CTG GGG TGG
1	Reverse	SHIVQ23AE-CTdel2_R	CCA CCC CAG TCT TCC CAG AAG TTC
2	Forward	SHIV_YE_EcoRI_F	CTG CAT CAA ACA AGT AAG TAG AAT TCG
2	Reverse	SHIV_YE_SalI_R	CCA TGG TCT CGT CGA CTT ATA GCA AAG

Table 3.1. Primers used to generate SHIVQ23AE-AD8 chimeras

Results

Replication kinetics of SHIVs in macaque cells

We initially assessed the ability of SHIVs representing macaque-passaged, pathogenic SHIVs (AD8-EO), lab-cultured SHIVs (AD8) and SHIVs derived from HIV-1 variants isolated early in infection (QF495AE, Q23AE, MG505GV and BG505AE) to replicate in primary Ptm peripheral blood mononuclear cells (PBMCs). SHIVs that were derived from HIV-1 variants isolated early in infection encode single amino acid changes that allow them to use the macaque CD4 receptor for entry [137]. Otherwise, these SHIVs are unmodified from the original isolate and will be referred to as circulating SHIVs as they are representative of HIV-1 Envs circulating in people. We tested the panel of six SHIVs for replication capacity over a 12-day time course starting with a low initial MOI of 0.02 to allow spreading infection in the culture. Supernatants from infected cells were collected every three days, and the concentration of SIV p27 was determined as a measure of virus levels. As examples, SHIV AD8-EO, a pathogenic, macaque-passaged virus that is currently used pre-clinical macaque studies, replicated to high levels of $>10^6$ pg/ml of SIV p27 by six days post-infection, while a circulating variant, SHIV Q23AE, replicated to much lower levels reaching peak virus levels of $>10^3$ pg/ml of SIV p27 by nine days post-infection (Fig. 3.1a). In addition to primary Ptm PBMCs, we also assessed replication in an immortalized CD4+ Ptm lymphocyte cell line [151]. For the assessment of replication kinetics in the immortalized Ptm lymphocytes, we tested three additional SHIVs, two that were adapted by macaque-passage (SHIV SF162P3 and SHIV 1157ipd3N4) and one adapted by lab-culture (SF162), for a total of nine viruses. SHIV AD8-EO replicated to high levels reaching peak virus levels of $>10^6$ pg/ml of SIV p27 by six days post-infection. In contrast, SHIV Q23AE,

a virus derived from an HIV-1 variant isolated from a patient in the first year of infection, replicated slower reaching peak virus levels of $>10^5$ pg/ml of SIV p27 by nine days post-infection (Fig. 3.1b).

In order to compare the replication kinetics of the panel of SHIVs, we determined a summary measure of replication. Replication kinetics were determined as the slope of a best-fit straight line of the replication curve during the first six days of infection when the virus was expanding exponentially in the culture. Comparing replication slopes of the six viruses tested in primary Ptm PBMCs, SHIVs that were adapted by macaque-passage or lab-culture demonstrated significantly faster replication kinetics compared to circulating SHIVs (0.92 vs. 0.50, $p = 0.01$) (Fig. 3.1c). We also determined the replication slope for the expanded panel of nine SHIVs in the immortalized Ptm lymphocyte cell line. Overall, the adapted SHIVs exhibited very similar replication kinetics in both cell types while the circulating SHIVs exhibited faster replication kinetics in the immortalized Ptm lymphocytes compared to primary Ptm PBMCs. In the immortalized Ptm cell line, we again observed a significant difference when comparing the replication slopes of adapted SHIVs to circulating SHIVs (0.92 vs. 0.75, $p = 0.01$) (Fig. 3.1d). Although the difference in replication kinetics between adapted and circulating SHIVs, in particular between SHIV AD8-EO and SHIV Q23AE, was more pronounced in primary cells, we observed similar patterns of SHIV replication in both Ptm PBMCs and immortalized Ptm lymphocytes. For future studies investigating the mechanisms underlying these differences in replication, we used the immortalized Ptm lymphocytes.

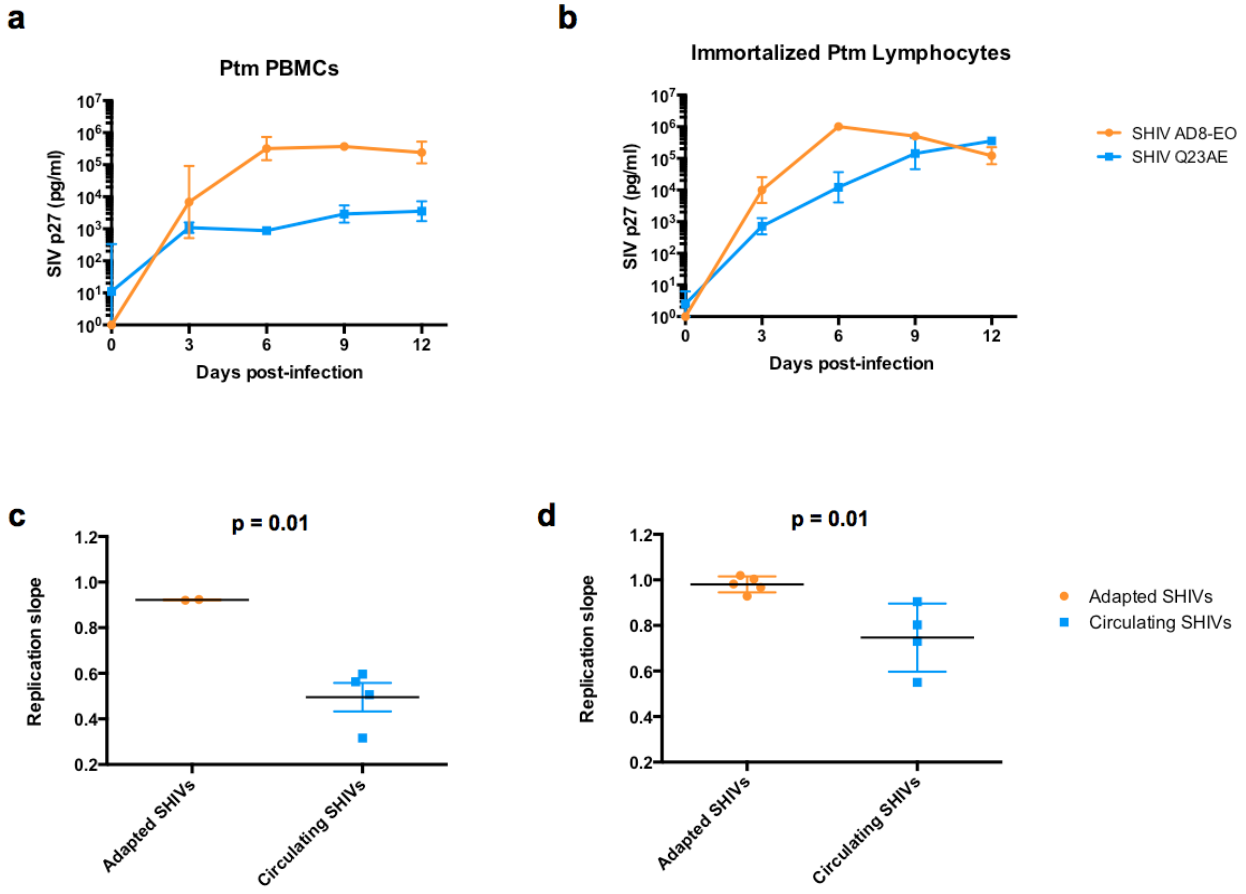

Fig. 3.1 Replication kinetics of SHIVs in macaque cells. (a) Replication kinetics of adapted (orange) and circulating (blue) SHIVs were measured in primary Ptm PBMCs over a 12-day time course measuring levels of SIV p27 in supernatants . (b) Replication kinetics of adapted and circulating SHIVs were also measured in immortalized Ptm lymphocytes. Data points represent the average of at least two independent experiments, and error bars represent the SD. (c) Mean replication slope in Ptm PBMCs of SHIVs adapted either by macaque-passage or in lab-culture was compared with that of circulating SHIVs by two-tailed student's t test. Each data point represents the average replication slope of at least two independent experiments for each SHIV. (d) Mean replication slope of SHIVs in immortalized Ptm lymphocytes was compared by two-tailed student's t test. Each data point represents the average replication slope of at least two independent experiments for each SHIV

Mapping viral determinants of high-level replication capacity

In order to identify the viral determinants that contribute to the differences in replication capacity of macaque-passaged and lab-cultured SHIVs, we generated chimeras between the viruses that demonstrated the greatest difference in replication capacity, SHIV AD8-EO and SHIV Q23AE. We took a gain-of-function approach and introduced partial or full genes from SHIV AD8-EO to SHIV Q23AE and assessed replication kinetics of each chimera in immortalized macaque lymphocytes. Importantly, for the mapping experiments, SHIV Q23AE was generated by cloning full *vpu* and *env* genes from the original Q23.17 molecular clone [184] into a full-length proviral plasmid of SHIV AD8-EO; thus, SHIV Q23AE and SHIV AD8-EO are isogenic except for the HIV-1 genes *vpu, env* and the second exons of *tat/rev*. Introduction of the entire *env* gene from SHIV AD8-EO to SHIV Q23AE resulted in a complete recovery of replication capacity (Fig. 3.2a). Introduction of the gp120 surface subunit of *env* resulted in a modest increase in replication kinetics while introduction of the gp41 trans-membrane subunit of *env* did not result in a detectable increase in replication. Because regions of the *env* gene contain overlapping reading frames with *vpu* and *tat*/*rev*, we also introduced the full *vpu* gene and the second *tat* exon, including a portion of *rev*, from SHIV AD8-EO to SHIV Q23AE. Introduction of neither *vpu* nor the second *tat/rev* exon resulted in a significant increase in replication kinetics (Fig. 3.2b). Introduction of the full *env* gene from SHIV AD8-EO to SHIV Q23AE also restored high-level replication in primary Ptm PBMCs (Fig. 3.2c). Taken together, these results indicate that the HIV-1 *env* gene is an important viral determinant of the ability of SHIVs to replicate in macaque cells and determinants in both the gp120 and gp41 subunits of Env contribute to replication.

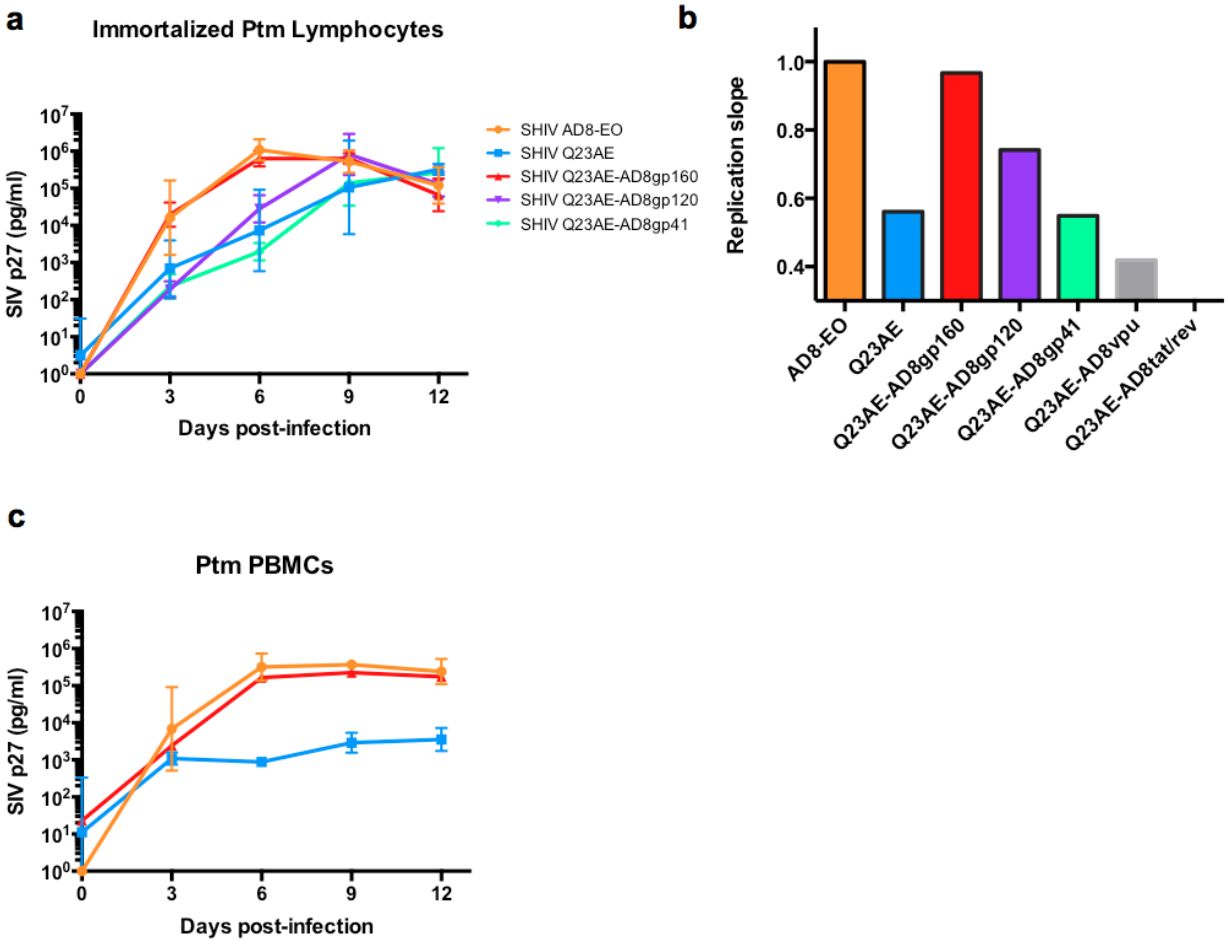

Fig. 3.2. HIV-1 Env is a viral determinant of high-level replication capacity in macaque cells. (a) Replication kinetics of SHIV AD8-EO, SHIV Q23AE and Env chimeras (Q23AE-AD8gp160, Q23AE-gp120 and Q23AE-gp41) were determined by measuring SIV p27 levels over a 12-day time course in immortalized Ptm lymphocytes. The key at the right indicates the color corresponding to each SHIV tested. Data points represent the average of two independent experiments, and error bars represent SD. (b) The slope of the replication curve in the first six days of infection immortalized Ptm lymphocytes of all chimeras generated (Q23AE-AD8gp160, Q23AE-gp120 and Q23AE-gp41, Q23AE-AD8vpu and Q23AE-AD8tat/rev) were compared to those of SHIV AD8-EO and SHIV Q23AE. Bars represent the average replication slope of at least two independent experiments. (c) Replication kinetics of SHIV Q23AE-AD8gp160 was measured with SHIV AD8-EO and SHIV Q23AE in primary Ptm PBMCs.

Virion envelope content is a major viral determinant of high-level replication capacity

The mapping studies indicated that HIV-1 envelope was a critical determinant of replication in macaque cells. Previous studies with SIV suggested that the amount of envelope protein found in virions contributes to infectivity and replication capacity *in vitro* [185,186]. Therefore, we measured the virion-associated envelope content across the panel of nine viruses described in Fig. 3.1d to determine whether the amount of envelope is a predictor of replication kinetics. Immortalized macaque cells were infected at a low MOI (0.02) to allow spreading infection in the culture and harvested cell supernatants every three days. We performed western blot analysis on infected cell supernatants harvested at 6 and 9 days post-infection that were purified using a 25% sucrose cushion. Viral lysates were normalized for SIV p27 input, and the total amount of HIV-1 envelope protein (Env) relative to SIV p27 capsid protein was determined. Polyclonal rabbit sera from animals immunized with a subtype A Env protein [182] was used to probe for HIV-1 Env. HIV-1 Env was clearly detectable for each of the SHIVs adapted by macaque-passage or in lab culture. In contrast, HIV-1 Env was barely detectable, if at all, for the circulating SHIVs. Comparison of HIV-1 Env content relative to SIV p27 between SHIV AD8-EO and SHIV Q23AE revealed a >10-fold difference (Fig. 3.3a). These patterns of virion-associated Env content were nearly identical at 9 days post-infection (Fig. 3.3b). Because the panel of SHIVs encode HIV-1 Envs from diverse subtypes, we probed for Env using another primary antibody, HIVIG (NIH AIDS Reagent Program). We observed the same patterns of virion-associated Env content using either of the primary antibodies indicating that differences in HIV-1 Env detection were not due to differences in antibody recognition of the diverse proteins (Fig. 3.3c). Overall, these results indicated that SHIVs exhibit a range of virion-associated HIV-1

Env content and that SHIVs adapted by lab-culture and/or macaque-passage have higher amounts of Env content compared to circulating SHIVs.

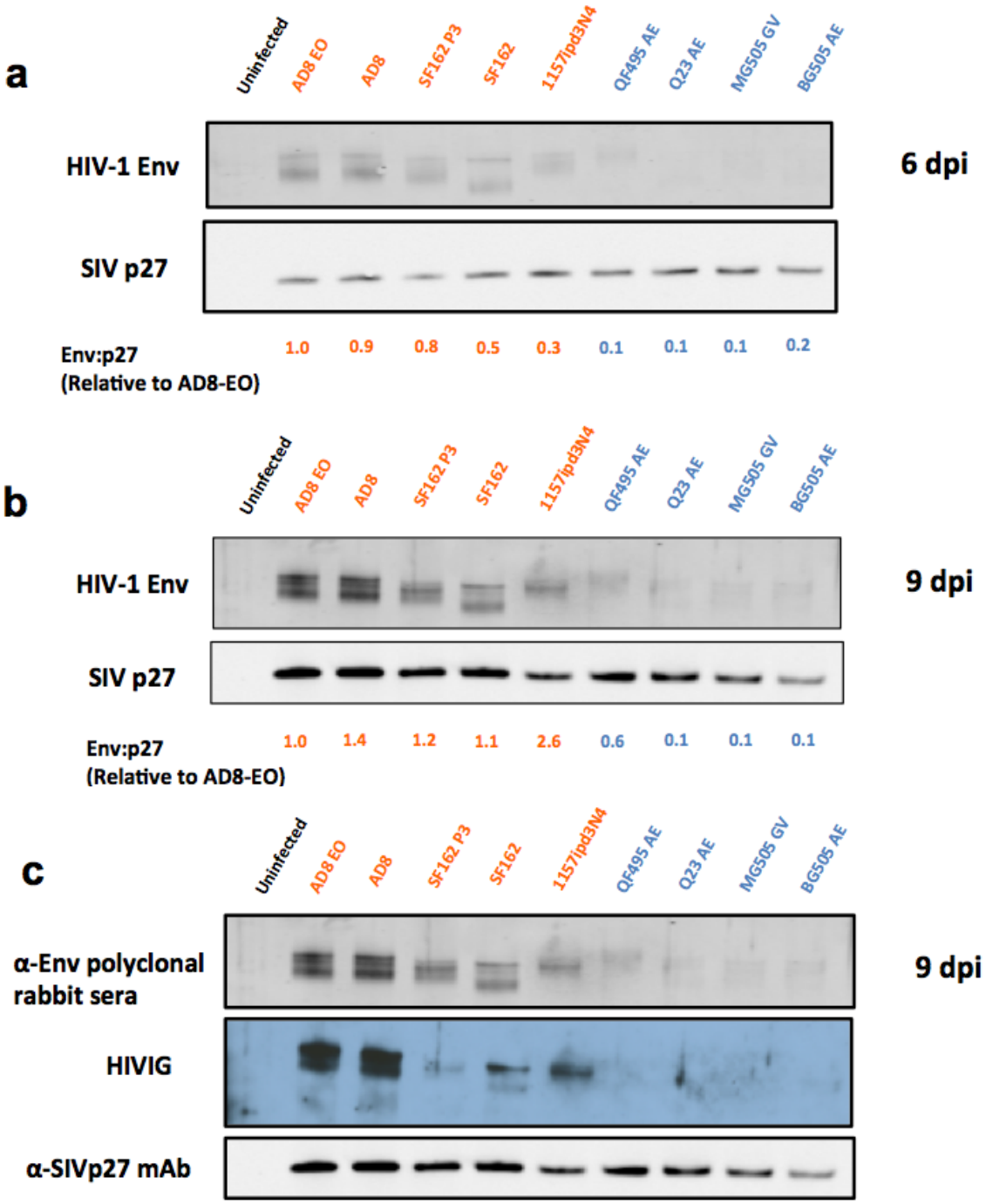

Fig. 3.3. Western blot analysis of virion-associated Env content in cell-free supernatants harvested at six (a) and nine (b) days post-infection. The top panel presents the HIV-1 Env signal probing the blot with α-Env polyclonal rabbit sera (Doria-Rose *et al*. J Virol 2005), and the bottom panel presents SIV p27 capsid probing with a mouse α-SIV p27 monoclonal antibody (ABL). The name of each SHIV being tested is indicated above each lane. Color-coding indicates whether the SHIVs has been adapted by macaque-passage/lab-culture (orange) or represents a circulating HIV-1 variant (blue). For each sample, 5 ng of SIV p27, as measured by ELISA, was loaded into each lane.. The Env:p27 signal relative to SHIV AD8-EO is indicated at the bottom of the panel for each virus. (c) HIV-1 Env was also probed using HIVIG (NIH AIDS Reagent Program) as a primary antibody.

We also determined the virion-associated Env content for the Env chimeras used to map determinants of replication kinetics. Introduction of the full *env* gene from SHIV AD8-EO to SHIV Q23AE resulted in virion-associated Env content similar to that of SHIV AD8-EO. Introduction of only gp120 resulted in a nearly 10-fold increase in Env content compared to the wild-type SHIV Q23AE (0.03 to 0.2) while introduction of gp41 did not result in any detectable increase (Fig. 3.4a). The results for virion-associated Env content mirrored the results for replication kinetics providing further support that the amount of Env in the virions contributes to the ability of the virus to replicate in macaque cells (Fig. 3.4b).

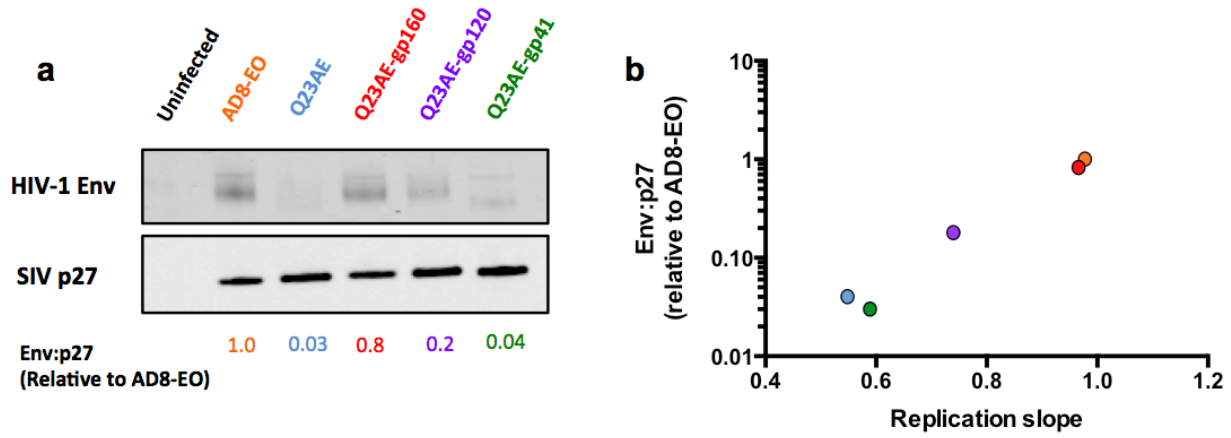

Fig. 3.4. Mapping determinants of virion-associated Env content. (a) Virion-associated HIV-1 Env content of Env chimeras (Q23AE-AD8gp160, Q23AE-AD8p120 and Q23AE-AD8gp41) were analyzed by western blot. The top panel presents the HIV-1 Env signal probing the blot with α-Env polyclonal rabbit sera (Doria-Rose *et al*. J Virol 2005), and the bottom panel presents SIV p27 capsid probing with a mouse α-SIV p27 monoclonal antibody (ABL). For each sample, 5 ng of SIV p27, as measured by ELISA, was loaded into each lane.. The Env:p27 signal relative to SHIV AD8-EO is indicated at the bottom of the panel for each virus. The value represents the average of two independent experiments. (b) Relationship between replication slope and virion-associated HIV-1 Env content of Env chimeras. Each colored dot represents a different SHIV, AD8-EO (orange), Q23AE (blue), Q23AE-AD8gp160 (red), Q23AE-AD8gp120 (purple), Q23AE-AD8gp41 (green). Virion-associated HIV-1 Env content, as determined by western blot, is plotted on the y-axis, and replication slope on the x-axis.

We next addressed the possibility that virion-associated Env content influences replication kinetics of SHIVs in macaque cells. Comparison of virion-associated Env content and replication kinetics in macaque cells revealed a statistically significant positive correlation (Spearman $r = 0.90$, $p = 0.002$) suggesting that the amount of Env in the virions contributes to the ability of adapted SHIVs to replicate in macaque cells (Fig. 3.5a). Given the critical role of Env in very early stages of the viral replication life cycle, we hypothesized that we would also observe differences in viral infectivity between SHIV AD8-EO and SHIV Q23AE. In order to address this hypothesis, we determined the specific infectivity of SHIV AD8-EO and SHIV Q23AE virions harvested at six and nine days post-infection. Infectivity of SHIV AD8-EO virions, normalized by SIV p27 input, was ~4-fold greater than SHIV Q23AE for virus harvested at 6 dpi and ~3-fold greater or virus harvested at 9 dpi (Fig. 3.5b). Thus, the higher Env content of SHIV AD8-EO may result in higher infectivity, which may contribute, in part, to its faster replication kinetics.

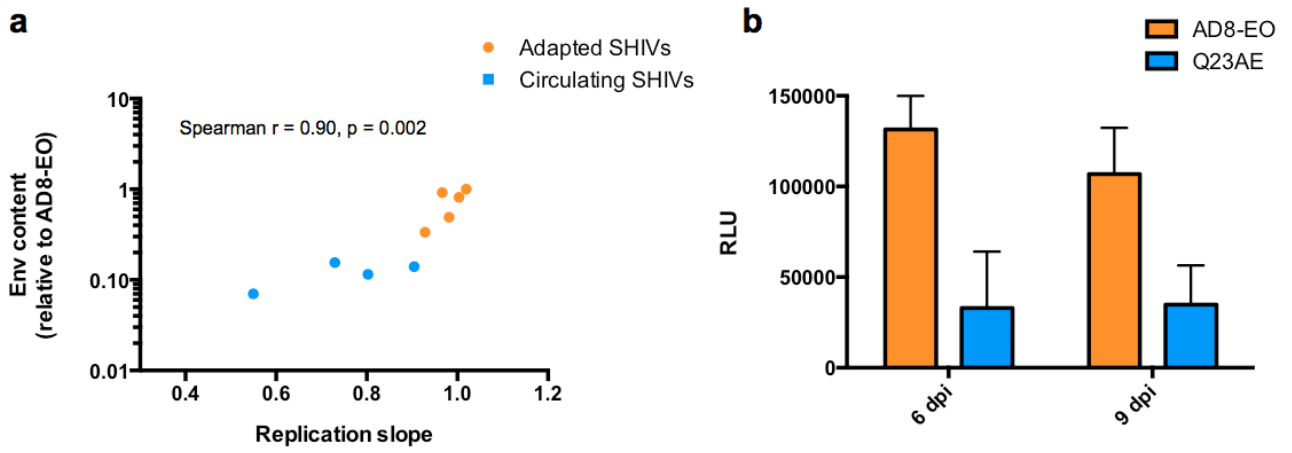

Fig. 3.5. Relationship between virion-associated HIV-1 Env content and viral replication kinetics of SHIVs in macaque cells. (a) The association between the slope of viral replication in the first six days of infection and virion-associated HIV-1 Env content of cell-free virions was determined. SHIVs are color-coded according the whether they have been adapted by macaque-passage/lab-culture (orange) or they represent circulating HIV-1 variants (blue). Spearman r represents the Spearman rank correlation coefficient determined using Prism Graphpad Version 6.0c. (b) Specific infectivity of virions harvested at six and 9 days post-infection (dpi) was determined by the TZM-bl infectivity assay. Viral input was normalized to 1 ng of SIV p27 as measured by ELISA. Relative light units (RLU) as a measure of beta-galactosidase activity are plotted on the y-axis. Bars represent the average of three independent experiments, and error bars indicate the SD.

Levels of HIV-1 Env expression in SHIV-infected macaque cells

We next investigated whether differences in the levels of HIV-1 in SHIV virions was due to variation in synthesis within the cell or to variation in incorporation into newly formed virions. We harvested infected immoratalized macaque cells at 6 and 9 days post-infection and probed for HIV-1 Env using the polyclonal rabbit sera and SIV gag using an anti-SIV p27 monoclonal antibody. The anti-SIV p27 monoclonal was able to detect both precursor (PrGag) and processed (p27) Gag. The amount of HIV-1 Env was determined relative to the total amount of Gag detected (PrGag + p27) and was then normalized to SHIV AD8-EO. At 6 days post-infection, HIV-1 Env was detectable for the adapted SHIVs, but was barely detectable for circulating SHIVs despite clear detection of PrGag at levels comparable to the adapted SHIVs. Comparison of cells infected with SHIV AD8-EO and SHIV Q23AE revealed a >30-fold difference (1.0 vs. 0.03) in expression of HIV-1 Env relative to total Gag (Fig. 3.6a). At 9 days post-infection, HIV-1 Env was detectable for all of the circulating SHIVs, although the levels of expression were lower overall compared to the adapted SHIVs (Fig. 3.6b). Interestingly, 9 days post-infection is also the time point at which the levels of viral replication are similar between adapted and circulating SHIVs (Fig. 3.1b). When we analyzed cells infected with the Env chimeras, we observed patterns of Env expression very similar to those for virion-associated Env content (Fig 3.6c) indicating that there are determinants in both gp120 and gp41 that contribute to Env protein expression in infected macaque cells.

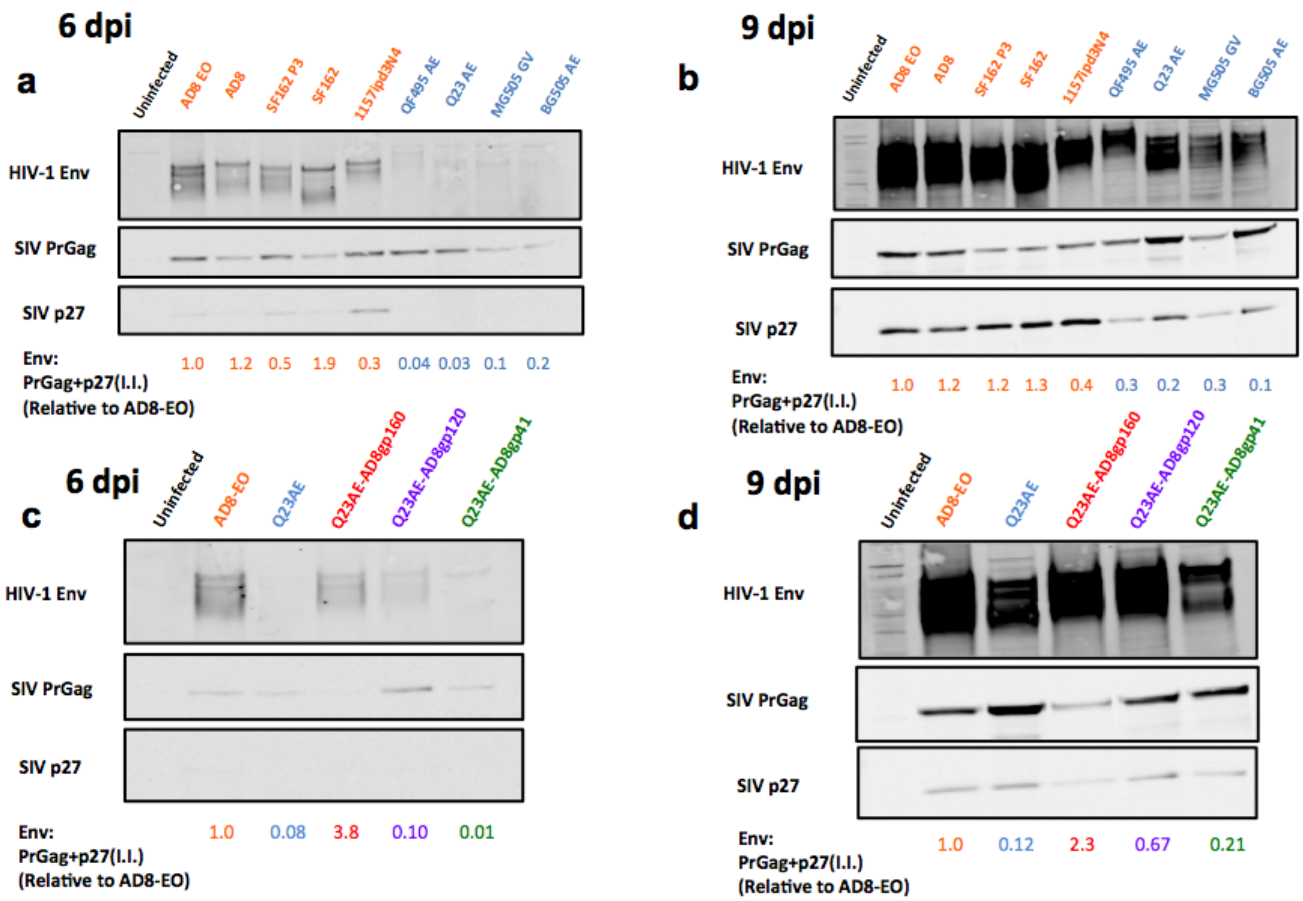

Fig. 3.6. Western blot analysis of cell-associated HIV-1 Env expression in SHIV-infected macaque cells. SHIV-infected macaque cells harvested and lysed at six (a,c) and nine (b,c) days post-infection analyzed for HIV-1 Env expression probing with α-Env polyclonal rabbit sera (Doria-Rose *et al.* J Virol 2005) and SIV PrGag and p27 with a mouse α-SIV p27 monoclonal antibody (ABL). For each whole-cell lysate, 10 µg of total protein as measured by Bradford Assay were loaded into each lane. The top panel presents HIV-1 Env signal while the bottom two panels present SIV PrGag and p27. The values below the bottom panel indicate the ratio of HIV-1 Env signal to the sum of PrGag and p27 (Env: PrGag + p27) and represent the average of two independent experiments. For panels (a) and (b), the orange color-coding indicates that the SHIV has been adapted either by lab-culture and/or macaque-passage while blue indicates that the SHIV is derived from a circulating HIV-1 variant. For panels (c) and (d), each SHIV is color-coded according to the identify of the Env chimera, Q23AE-AD8gp160 (red), Q23AE-AD8gp120 (purple) and Q23AE-AD8gp41 (green).

For the nine SHIVs tested, there was a strong positive correlation between the relative cell-associated Env expression and the relative virion-associated Env content (Spearman $r = 0.87$, $p = 0.005$) suggesting that the amount of Env expressed by the infected cells largely determines how much envelope can be detected in cell-free virions (Fig. 3.7a). We cannot, however, rule out the possibility that differences in incorporation might contribute to the amount of Env detected in virions. Because there is so little Env being expressed by cells infected with circulating SHIVs, it would be difficult to detect differences in incorporation. The dramatic differences in Env expression could be due to differences in transcription and splicing of vpu/env mRNA. To address this possibility, we measured spliced Vpu/Env mRNA and unspliced viral RNA by quantitative reverse-transcriptase PCR (qRT-PCR) for SHIV AD8-EO and SHIV Q23AE to determine if there were any differences between adapted and un-adapted SHIVs. We did not observe a statistically significant difference between the SHIV AD8-EO and SHIV Q23AE with respect to the amount of spliced Vpu/Env mRNA relative to unspliced viral RNA at any of the three time points tested (Fig. 3.7b). These results suggested that the differences in levels of Env expression are due to post-transcriptional events in SHIV-infected macaque cells.

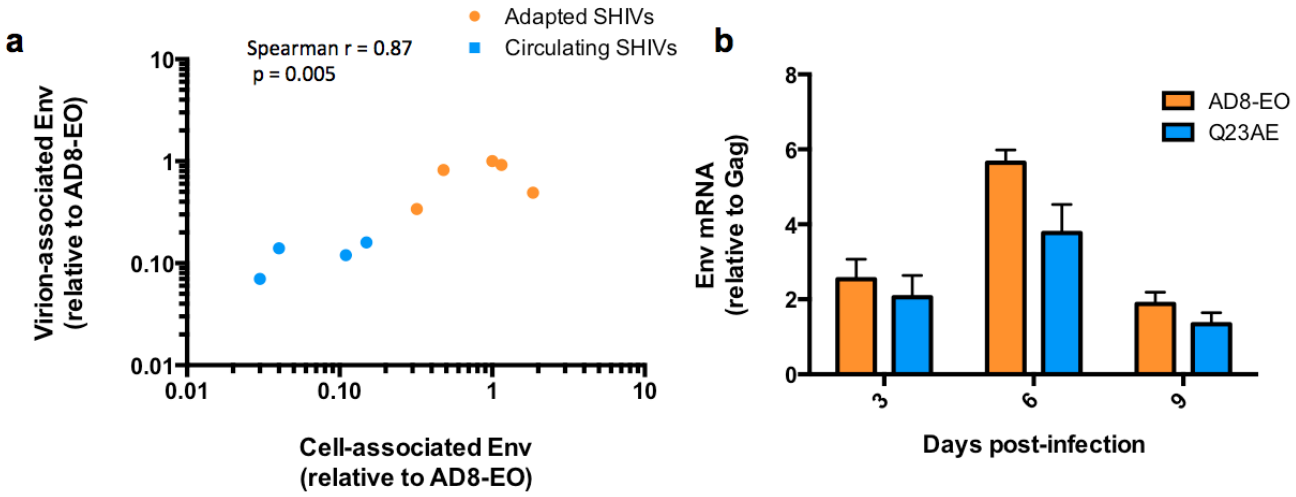

Fig. 3.7. Relationship between virion-associated HIV-1 Env content and cell-associated expression. (a) The association between virion-associated Env content and cell-associated Env expression was determined for the panel of nine SHIVs. Each dot represents a different SHIV variant and is color-coded according the whether they have been adapted by macaque-passage/lab-culture (orange) or they represent circulating HIV-1 variants (blue). Spearman r represents the Spearman rank correlation coefficient determined using Prism Graphpad Version 6.0c. (b) The amount of Env mRNA relative to Gag mRNA was determined by quantitative reverse transcription PCR (qRT-PCR) at 3, 6 and 9 dpi. Ratio of Env mRNA relative to Gag mRNA is presented on the y-axis and was determined using the ΔΔCT method (Schmittgen and Livak Nat Prot 2008). The bars represent the average of two independent experiments and error bars represent the SD.

Discussion

SHIVs that are able to establish persistent infection and cause AIDS in macaques are critical tools for pre-clinical HIV-1 vaccine and therapeutic studies and have provided important information on transmission and pathogenesis of lentiviruses. Recently, there has been an effort to generate pathogenic SHIVs that are more representative of HIV-1 variants that are transmitted (T/F) and circulating in people. There has been limited success in generating SHIVs that encode HIV-1 Envs representing circulating variants as there are a number of important species-specific factors that inhibit viral replication. These factors include antiviral restriction factors that actively inhibit replication and species-specific differences in required host proteins, such as the CD4 receptor used for entry [98,137]. Two recent studies identified a small subset of SHIVs that encode T/F HIV-1 Envs from a larger pool that were able to cause disease in macaques without animal-animal passage [126,127]. The fact that only one or two viruses from the larger pool were able to establish persistent infection in the macaques suggests that these viruses were selected because of unique characteristics. The viral determinants that allowed these select SHIVs to persist in the macaques have not been identified.

Here we provide evidence that HIV-1 Env is an important determinant of the ability of SHIVs to replicate in macaque cells. In addition, our results suggest that the amount of HIV-1 Env expressed in SHIV-infected macaque cells and present in cell-free virions contribute to replication kinetics. Among a panel of nine SHIVs, viruses that were adapted by lab-culture and/or macaque-passage demonstrated high replication capacity and higher virion-associated Env content compared to viruses derived from HIV-1 variants isolated from patients early in infection. Our results suggest that pathogenic SHIVs may have been selected for high Env

content and replication kinetics in order to replicate to high levels and establish persistent infection in macaques.

In the present study, we compared the replication kinetics of SHIVs encoding HIV-1 Envs representing macaque-passaged and lab-cultured variants (adapted SHIVs) and representing variants circulating in people (circulating SHIVs). Overall, we found that SHIVs encoding adapted Envs, either by lab-culture and/or macaque-passage, had higher replication capacity compared to circulating SHIVs. Importantly, the SHIVs encoding lab-cultured HIV-1 variants (SHIV SF162 and SHIV AD8) are the parental viruses of two of the macaque-passaged, pathogenic viruses (SHIV SF162P3 and SHIV AD8-EO) [128,178]. Thus, SHIVs that were selected for further development may have already had high replication capacity, which allowed them to infect macaques and then to be serially passaged from animal to animal to enhance their pathogenic potential. Although we were unable to detect statistically significant differences between the parental and macaque-passaged SHIVs with respect to the replication slope, the process of serial passage may have further increased replication kinetics.

By generating chimeras between the SHIVs with the largest differences in replication kinetics, we identified HIV-1 Env as a critical determinant of high-level replication. Introduction of gp160 from SHIV AD8-EO to SHIV Q23AE resulted in a complete recovery of replication kinetics in macaque cells. Introduction of the gp120 from SHIV AD8-EO to SHIV Q23AE resulted in an intermediate phenotype with a modest increase in replication kinetics while introduction of gp41 did not result in any observable increase. Thus, there are likely determinants in both gp120 and gp41 that contribute to high-level replication. Given that our results suggested that HIV-1 Env is a critical determinant of replication kinetics, we next tested the hypothesis that the amount of virion-associated Env content contributes to the ability of SHIVs to replicate in

macaque cells. Studies of SIV have indicated that increased Env content in virions can result in increased infectivity and replication capacity [185,186]. Western blot analysis of virion-associated Env content revealed a dramatic difference in the Env content of adapted SHIVs compared to circulating SHIVs (Fig. 3.3). In addition, we demonstrated a significant positive correlation between virion-associated Env content and replication kinetics. The correlation between virion-associated Env content and replication kinetics may be, in part, due to improved infectivity of virions with higher Env content. We compared the infectivity a virus with high Env content and fast replication kinetics (SHIV AD8-EO) to a virus with low Env content and slow replication kinetics and observed an ~4-fold difference between the two viruses in a single round of infection. The 4-fold difference observed in a single round of infection could be amplified over multiple rounds of infection, or there could be additional mechanisms at play at other stages of the viral life cycle that contribute to the observed differences in replication kinetics for adapted and circulating SHIVs in macaque cells.

In addition to differences in virion-associated Env content, we observed a similar difference between adapted and circulating SHIVs with respect to the amount of intracellular HIV-1 Env expressed in SHIV-infected macaque cells. For example, we observed an approximately 30-fold difference between SHIV AD8-EO and SHIV Q23AE in the amount of HIV-1 Env relative to the amount of Gag expressed in the macaque cells. Consistent with the replication kinetics results, circulating SHIVs demonstrated an increase in Env expression from 6 to 9 days post-infection. Because macaque cells infected with circulating SHIVs expressed such low amounts of Env, we were unable to determine if these viruses exhibited a deficit in the ability to incorporate HIV-1 Env into virions. Given that we observed a very modest difference in the amount of Env mRNA relative to unspliced viral RNA, events occurring after transcription

are likely responsible for the low levels of Env expression for circulating SHIVs in macaque cells, and, presumably, adapted SHIVs are not effected by these events. An intriguing hypothesis is that the high levels of HIV-1 Env expression observed for adapted SHIVs compared to circulating SHIVs contribute to improved cell-cell transmission and fast replication kinetics. In the context of infection *in vivo*, efficient cell-cell spread very early after transmission may allow SHIVs to replicate to high levels during acute infection and establish persistent infection in macaques [187]. The post-transcriptional differences in expression of HIV-1 Envs representing circulating variants could be due to protein stability, a species-specific difference in host proteins required for Env synthesis or targeted degradation of the protein by a host factor. Given that our results suggest that both the gp120 and gp41 subunits are important, it is possible that gp120 and gp41 interact to allow efficient expression, or that multiple mechanisms are at play that target each subunit independently. Defining these mechanisms could provide important information on the interaction of HIV-1 Env with host cell factors.

Overall, our results describe a critical role of HIV-1 Env in the ability of SHIVs to replicate to high-levels in macaque cells. Moreover, the amount of Env expressed in infected cells and present in virions contributes to the ability of SHIVs to replicate *in vitro* and may contribute to their ability to establish persistent infection *in vivo*. The finding that high Env expression/content is common among pathogenic SHIVs has implications for the rational design of circulating SHIVs that typically do not replicate well *in vivo*. Modulation of HIV-1 Env content may allow un-adapted SHIVs that are more representative of transmitted or circulating HIV-1 variants to replicate to high levels and establish persistent infection in macaques.

Chapter IV: Determining the sensitivity of SIV/HIV chimeric viruses to type-I interferon

Introduction

In the previous chapter, I investigated the viral determinants that contribute to the ability of pathogenic SHIVs to replicate to high-levels in macaque cells. As described in Chapter I, in the context of infection *in vivo*, the host rapidly responds to the presence of virus. Immune cells are able to sense the presence of viral infection and respond by producing type-I interferon (IFN-I), which, in turn, leads to the up-regulation of hundreds of host genes with antiviral potential [188,189]. The IFN-I response acts as one of the first lines of defense against viral infection. Notably, all of the known restriction and resistance factors that inhibit lentiviral replication are induced by IFN-I [27]. The interaction of HIV-1 with the IFN-I response remains a very active area of research. A number of lines of evidence indicate that overcoming the IFN-I response early in infection is important for establishing persistent infection in a host. Initial infection with HIV-1 in people and SIV in non-human primates induces a robust IFN-I response within days of infection [48,49]. A recent experimental study of SIV infection in rhesus macaques demonstrated that blocking the IFN-I response resulted in decreased host restriction factor expression, increased viral reservoir size and faster progression to AIDS [45]. Studies of the effect of IFN-I treatment in HIV-1-infected people also indicate that administration of IFN-I results in increased restriction factor expression and a decrease in HIV-1 viremia during chronic infection [44,190,191]. Interestingly, not all patients respond to IFN-I treatment, and those that fail to respond exhibit higher baseline expression of IFN-stimulated genes and poor IFN induction

[191]. Taken together, these results suggest that the IFN-I response has the ability to limit viral replication *in vivo* and presents an important potential barrier to the establishment of infection.

There is also evidence that the innate immune response selects for HIV-1 variants that are relatively resistant to IFN-I during transmission. Two recent studies demonstrated that transmitted/founder (T/F) HIV-1 variants that established initial infection in people are more resistant to IFN-I treatment compared to variants isolated from the same individuals at six months of chronic infection [94,95]. This finding, however, has yet to be demonstrated in other cohorts, and there is still some debate as to whether T/F founder variants are more generally resistant to IFN-I. Nonetheless, these studies do suggest that the IFN-I response may contribute to the severe genetic bottleneck observed during HIV-1 transmission and select for viruses with the ability to replicate well in the presence of the innate immune response. These findings have important implications for the development of improved animal models of HIV-1 transmission and infection. Ideally, challenge viruses used in pre-clinical vaccine and therapeutics studies would capture these characteristics of T/F viruses and otherwise be more representative of HIV-1 variants circulating in people.

As mentioned in chapter III, SHIVs that are currently used in macaque studies have undergone animal-animal serial passage in order to acquire pathogenic properties. SHIVs require these additional rounds of adaption despite the fact that they encode species-specific viral antagonists of know restriction factors, including SIV CA, SIV Vif, SIV Nef and SIV Vpx [27,98] A number of SHIVs have undergone rapid serial passage within the first two weeks of infection, a time during which there is a robust IFN-I response [49,109,111,178]. Given the robust IFN-I response induced by infection, I hypothesized that the process of adapting SHIVs for replication in macaques selects for viruses that are resistant to the macaque IFN-response.

In order to test this hypothesis, I assessed the IFN-I sensitivity of the panel of nine SHIVs described in Chapter III that represent macaque-passaged, lab-cultured and circulating HIV-1 variants in target cells from multiple species, including pig-tailed macaques, rhesus macaques and humans. Here, I provide evidence that SHIVs adapted either by macaque-passage or in lab-culture are more resistant to IFN-I treatment compared to circulating SHIVs. I also provide evidence that replication kinetics in untreated macaque cells predicts that ability of SHIVs to overcome the macaque IFN-I response and that HIV-1 Env is an important determinant of IFN-I resistance. These findings provide new information on the mechanisms by which lentiviruses are able to evade or antagonize the IFN-I response and have important implications for the development of improved challenge viruses for macaque models of HIV-1 transmission and infection.

Materials and Methods

Viral replication assays

Replication of SHIVs was assessed using immortalized pig-tailed macaque lymphocytes [151], primary pig-tailed macaque (Ptm) peripheral blood mononuclear cells (PBMCs) and rhesus macaque (Rhm) PBMCs. Immortalized Ptm lymphocytes were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented 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) (complete IMDM). For assays in which cells were pre-treated with IFN-I, $1x10^6$ immortalized Ptm lymphocytes were plated 24 hours prior to infection in a 48-well plate in complete IMDM containing recombinant human IFNα-2a (PBL Interferon Source) at a final

concentration of 1,000 U/ml. Cells were infected at an estimated multiplicity of infection (MOI) of 0.02 in a final volume of 250 µl in a 96-well plate. The virus and cells were spinoculated at 1,200 x g for 90 minutes at room temperature. The infected cells were washed three times in 1.0 ml of complete IMDM and re-suspended into duplicate 600 µl cultures in a 48-well plate. For the viral replication assays with IFN-I treatment after infection, IFNα-2a was added to the appropriate wells at a final concentration of 1,000 U/ml five hours after the initial infection. 400 µl of each cell supernatant was harvested, and cultures were replenished with the appropriate medium every three days. For the IFN-I-treated cultures, IFNα-2a was maintained throughout the time course. SIV p27 concentrations were determined using a SIV p27 antigen enzyme-linked immunosorbent assay (ELISA) kit (ABL, Rockville, MD).

For viral replication assays in Ptm and Rhm PBMCs, cells were isolated from whole macaque blood. Isolated PBMCs were stimulated for three days prior to infection with IL-2 (20 U/ml) and concanavalin A (5 µg/ml) in RPMI 1640 medium supplemented with 20% FCS and 2 mM L-glutamine. $1x10^6$ Ptm or RhPBMCs were spinoculated and maintained as described for immortalized Ptm lymphocytes.

SHIV IFN-I sensitivity assay

IFN-I sensitivity of SHIVs was determined using immortalized Ptm lymphocytes. For each SHIV, $4.25x10^6$ Ptm lymphocytes were infected at an estimated MOI of 0.02 in a final volume of 1.4 ml of complete IMDM supplemented with IL-2 (100 U/ml) in a 24-well plate. The infections were spinoculated for 90 minutes at 1,200 x g at room temperature. After spinoculation, the infections were washed two times with 2 ml complete IMDM and then re-suspended in 3.4 ml of complete IMDM supplemented with IL-2. Approximately $2.5x10^5$

infected cells in 200 µl of media were plated in each well of a 96-well plate containing 50 µl of media containing IFNα-2a. For each condition, the infection cultures were setup in duplicate. Cell supernatants were harvested at 3 and 7 days post-infection. Two-thirds of the supernatant were carefully removed from each culture and transferred to a fresh 96-well plate. Cultures were then replenished with the appropriate media. The amount of virus in each supernatant was determined by titering on TZM-bl cells. Four 1:10 serial dilutions of each cell supernatant were performed leaving 50 µl of diluted supernatant in each well. $1x10^4$ TZM-bl cells in 100 µl of complete DMEM were added to each well. The infections were incubated at 37 degrees C for 48 hours. β-galactosidase activity was measured using the Galacto-Lite system (Applied Biosystems). For the data analysis, all values were plotted and statistical analyses performed using Prism version 6.0c (GraphPad Software). Percent viral replication was determined by dividing the amount of β-galactosidase activity in the IFN-I treated sample by the untreated sample. The concentration of IFN-I at which 50% viral inhibition was achieved was interpolated from a best-fit curve. The amount residual viral replication at the highest concentration of IFN-I (5000 U/ml) was also determined.

Results

IFN-I sensitivity of SHIVs in macaque cells

We first assessed the IFN-I sensitivity of SHIVs in macaque cells using an IFN-I replication time course assay. For this assay, immortalized Ptm lymphocytes were infected at a low initial MOI of 0.02 to allow spreading infection in the culture. Five hours after the initial infection, infected-cells were treated with an IFN-I concentration similar to that observed in

natural infection (1000 U/ml), and this concentration was maintained throughout the 12-day time course [49]. In order to compare SHIVs quantitatively, IFN-I sensitivity was measured as the ratio of the area-under-curve (AUC) of the replication curve in the IFN-I-treated cells divided by AUC of the replication curve in the untreated cells. The panel of SHIVs demonstrated a range of IFN-I sensitivities (Fig. 4.1). For example, SHIV AD8-EO, a pathogenic SHIVs, replicated in the presence of IFN-I nearly as well as the untreated cells and had an AUC ratio (IFN+/IFN-) of 0.96. In contrast, SHIV Q23AE, circulating SHIV, exhibited a pronounced IFN-I-induced inhibition of viral replication corresponding to an ~100-fold reduction in SIV p27 levels at 9 days post-infection, and had an AUC ratio of 0.68. Overall, SHIVs that were adapted by macaque-passage or lab-culture were significantly more resistant to IFN-I treatment compared to circulating SHIVs (0.92 vs. 0.79, $p = 0.02$) (Fig. 4.2a). As described in Chapter III, adapted SHIVs also exhibited significantly faster replication kinetics in macaque cells compared to circulating SHIVs when the replication slope during the first six days of infection was analyzed. We observed a significant positive correlation between replication slope and AUC ratio (IFN+/IFN-) for the panel of nine SHIVs (Spearman $r = 0.88$, $p = 0.003$) indicating that replication kinetics in the untreated macaque cells predicts the ability of SHIVs to overcome the macaque IFN-I response (Fig. 4.2b).

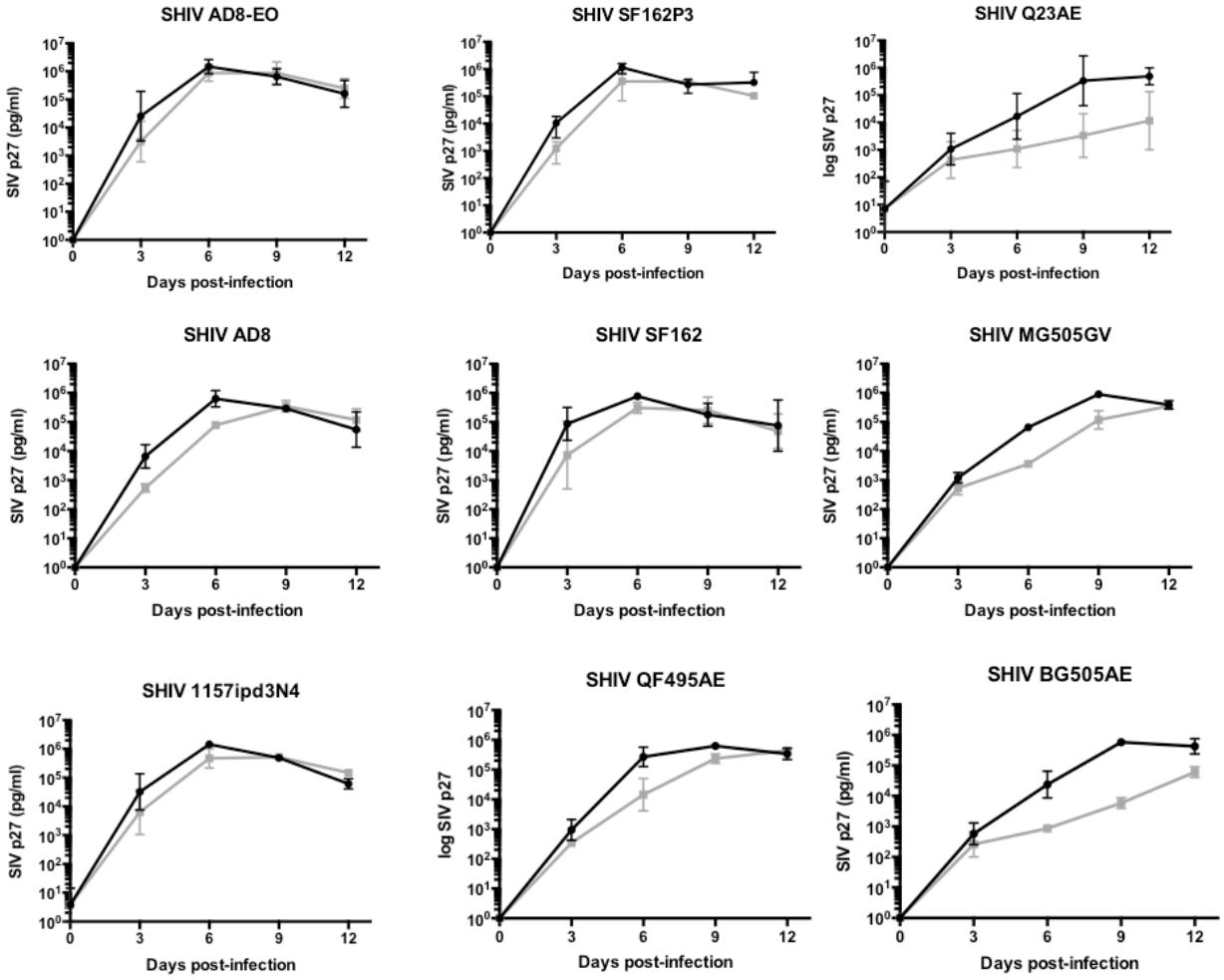

Fig. 4.1. Effect of IFN-I treatment on viral replication kinetics of SHIVs in immortalized Ptm lymphocytes. Each chart presents the viral replication kinetics of a SHIV in the presence of 1000 U/ml of IFNα-2a (gray lines) or absence of treatment (black lines). The identity of each SHIV is indicated above the chart. SIV p27 concentration in infected cell supernatants, as measured by ELISA, are plotted on the y-axis and days post-infection on the x-axis. Data points represent the average of at least two independent experiments and error bars indicate SD.

The positive correlation between replication slope and AUC ratio suggested that SHIVs might overcome the IFN-I response in macaque cells by saturating IFN-I-induced host factors. To further explore this hypothesis, we tested the effect of increasing and decreasing the initial MOI on the AUC ratio for SHIV AD8-EO (IFN-I resistant) and SHIV Q23AE (IFN-I sensitive). When the initial MOI was decreased by 10-fold to 0.002, both SHIV AD8-EO and SHIV Q23AE exhibited greater sensitivity to IFN-I treatment. In contrast, when the initial MOI was increased by 10-fold to 0.2, the AUC ratio for SHIV Q23AE increased from 0.60 to 0.78 while the AUC ratio for SHIV AD8-EO remained > 0.90 (Fig. 4.2c). These findings support the hypothesis that the IFN-I response can be saturated in macaque cells.

In addition to treatment with IFN-I five hours after initial infection as described above, we also tested the effect of pre-treating macaque cells with IFN-I 24 hours before infection. For these experiments, immortalized Ptm lymphocytes were plated 24 hours prior to infection and treated with 1000 U/ml of IFNα-2a or cells were left untreated. After infection by spinoculation, the IFN-I treated and the untreated cells were both divided into two groups. For the pre-treated cells, one group was continuously treated with IFN-I throughout the time course, and the other group of cells was left untreated. For the cells that were not pre-treated with IFN-I, one group received continuous IFN-I treatment starting at five hours after the initial infection, and the other group of cells was never treated with IFN-I. We observed little, if any, difference between the replication curves for SHIVs in cells that were pre-treated with IFN-I and treated throughout the time course (PreIFN+/IFN+) and those in cells that were not pre-treated but were treated throughout the time course (Untreated/IFN+). In addition, we observed no difference between the replication curves of SHIVs that were pre-treated with IFN-I and then left untreated (PreIFN+/Untreated) and those in cells that were untreated before and after infection

(Untreated/Untreated) (Fig.4.2d). Thus, our results indicate that treatment of cells with IFN-I prior to infection does not impact the replication kinetics of SHIVs in macaque cells.

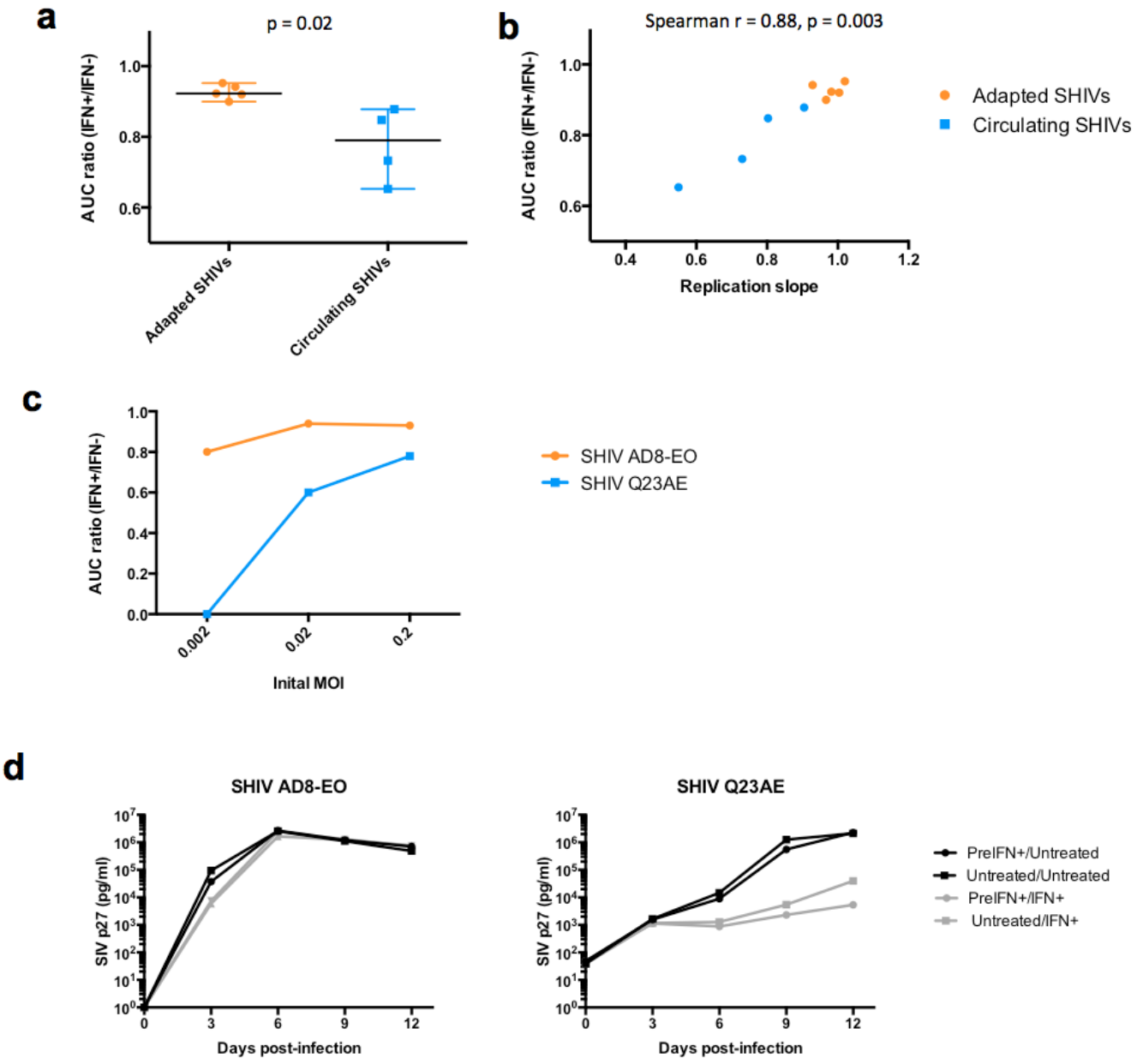

Fig. 4.2. Comparison of IFN-I sensitivity of adapted and circulating SHIVs in immortalized Ptm lymphocytes. (a) The area-under-curve ratio (IFN+/IFN-) is plotted on the y-axis for adapted (orange) and circulating (blue) SHIVs. Each dot represents a different SHIV variant. (b) The relationship between the AUC ratio (IFN+/IFN-) and the replication slope across the panel of nine SHIVs is presented. Spearman r represents the Spearman rank correlation coefficient as determined by Prism Graphpad Version 6.0c. (c) Effect of MOI on observed AUC ratio (IFN+/IFN-). The AUC ratio is plotted on the y-axis and the intial multiplicity of infection (MOI) on the x-axis for SHIV AD8-EO (orange) and SHIV Q23AE (blue). (d) Effect of IFN-I treatment prior to infection on the replication of SHIV AD8-EO and SHIV Q23AE in immortalized Ptm lymphocytes. The key at the right indicates the sequence of IFN-I treatment.

In order to validate our findings in immortalized Ptm lymphocytes, we tested a subset of SHIVs (SHIV AD8-EO and SHIV Q23AE) for IFN-I sensitivity in primary Ptm PBMCs. SHIV AD8-EO demonstrated modest sensitivity to IFN-I treatment in the primary Ptm PBMCs compared to the immortalized macaque lymphocytes according to the AUC ratio (0.83 vs. 0.95). Although it replicated to lower levels in the primary Ptm PBMCs compared to immortalized macaque lymphocytes, SHIV Q23AE exhibited very similar AUC ratios in the two cells (0.63 vs. 0.65) (Fig. 4.3). Thus, in both immortalized Ptm lymphocytes and in Ptm PBMCs, SHIV AD8-EO was less sensitive to IFN-I treatment compared to SHIV Q23AE.

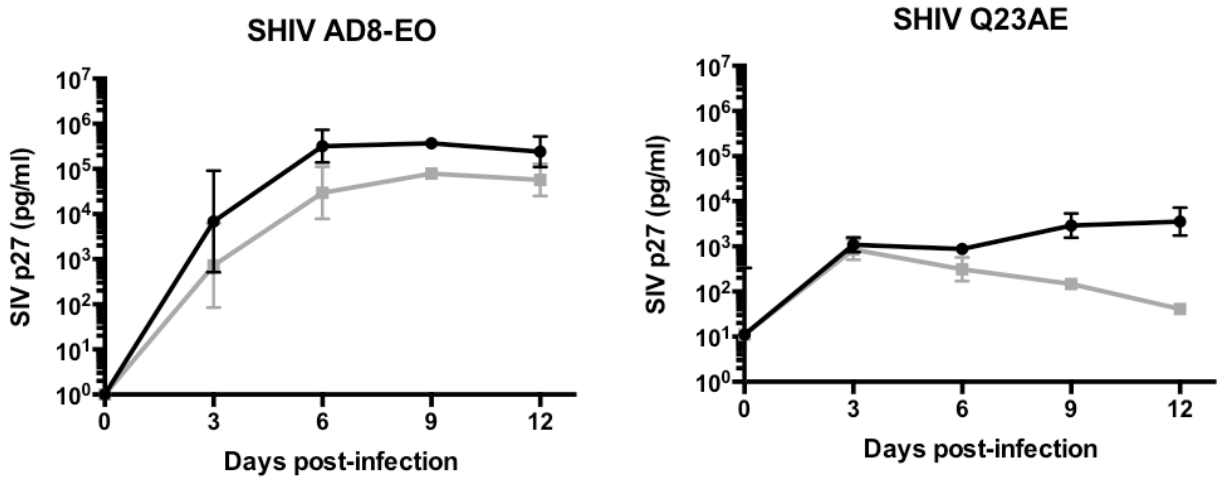

Fig. 4.3. Effect of IFN-I treatment on viral replication kinetics of SHIVs in primary Ptm PBMCs. Each chart presents the viral replication kinetics of a SHIV in the presence of 1000 U/ml of IFNα-2a (gray lines) or absence of treatment (black lines). The identity of each SHIV is indicated above the chart. SIV p27 concentration in infected cell supernatants, as measured by ELISA, are plotted on the y-axis and days post-infection on the x-axis. Data points represent the average of two independent experiments and error bars indicate SD.

HIV-1 Env is a viral determinant of IFN-I resistance of SHIVs

We previously mapped the determinants of high-level replication of SHIVs in macaque cells to the HIV-1 *env* gene using SHIV AD8-EO and SHIV Q23AE, and our results suggested that determinants map to gp160 with both the gp120 and gp41 subunits likely contributing to viral replication. We next tested the chimeras used to map the determinants of high-level replication for IFN-sensitivity in immortalized macaque lymphocytes and primary Ptm PBMCs. Introduction of the entire HIV-1 *env* gene from SHIV AD8-EO to SHIV Q23AE resulted in a nearly complete recovery of IFN-I-resistance (Fig. 4.4a). The gp120 Env chimeras exhibited a modest increase in the IFN-I resistance, while the gp41 chimera did not demonstrate any detectable increase in IFN-I resistance compared to SHIV Q23AE. Importantly, neither introduction of the full *vpu* gene nor the second *tat/rev* exon resulted in an increase in IFN-I resistance. The gp160 Env chimera was also tested in primary Ptm PBMCs. The gp160 Env chimera, encoding AD8-EO Env gp160, demonstrated an AUC ratio nearly identical to that of SHIV AD8-EO (Fig. 4.4b). Thus, as was observed for results mapping the viral determinants of high-level replication, HIV-1 Env is a major determinant of resistance to IFN-I treatment in macaque cells.

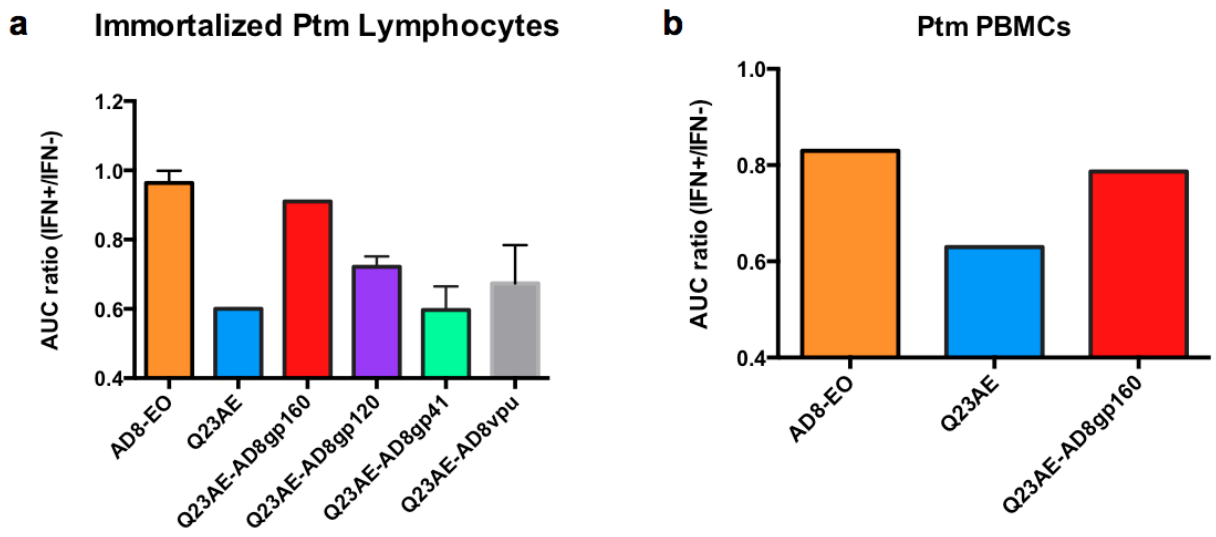

Fig. 4.4. Mapping the viral determinants of IFN-I resistance in immortalized Ptm lymphocytes (a) and primary Ptm PBMCs (b). The area-under-curve (AUC) ratio was determined by dividing the AUC of the replication curve in IFN-I treated cells by the AUC in untreated cells. AUC ratio (IFN+/IFN-) is plotted on the y-axis for each of the indicated chimeras. Data represent the average of at least two independent experiments, and error bars indicate the SD.

Analysis of IFN-I sensitivity by IC50 and Vres

Although the replication time courses provided summary measures of both replication kinetics and IFN-I sensitivity that were useful in comparing the SHIVs of interest, the dynamic range of the AUC ratio measure was not sensitive enough to make comparisons of IFN-I sensitivity within the group of adapted SHIVs. Recently, Fenton-May *et al*. described a sensitive assay to measure IFN-I sensitivity [95]. In this assay, cells were infected in the presence of varying concentrations of IFN-I, and the concentration of IFN-I required to inhibit viral replication by 50% was determined (IC50). The residual viral replication at the highest concentration of IFN-I (Vres) was also determined. We adapted this assay for SHIV infection of immortalized Ptm lymphocytes in order to determine IFN IC50 and Vres values for the panel of nine SHIVs of interest. As described for the replication time courses, macaque cells were infected at a low initial MOI of 0.02 and cell supernatants were harvested at three and seven days post-infection. The nine SHIVs exhibited a range of IFN-I IC50 values (2.3 – 5,000 U/ml). For example, SHIV Q23AE was highly sensitive to IFN-I treatment and exhibited a clear dose-dependent inhibition of viral replication with an IC50 value of 2.3 U/ml. In contrast, SHIV AD8-EO was completely IFN-I resistant and did not exhibit inhibition of viral replication at any of the concentrations tested (Fig. 4.5a). Four of the five adapted SHIVs did not reach 50% viral replication with IFN-I treatment. For these viruses, the IC50 value was reported as 5,000 U/ml for the purposes of statistical analysis. Comparing IC50 values, SHIVs adapted by macaque-passage or lab culture were significantly more resistant to IFN-I treatment in macaque cells compared to circulating SHIVs (4,500 vs. 62, $p = 0.0001$) (Fig. 4.5b). Comparison of the percent residual viral replication (% Vres) at the highest concentration of IFN-I tested (5,000 U/ml) also revealed a significant difference with respect to IFN-I sensitivity (83% vs. 3.5%, $p = 0.01$) (Fig.

4.5c). Thus, the results from the IFN IC50 assay confirmed the result from the IFN replication time courses that adapted SHIVs are more resistant to IFN-I treatment than circulating SHIVs.

The increased sensitivity of the IFN IC50 assay also allowed us to make comparisons within the groups of IFN-I-resistant (adapted) and -sensitive (circulating) SHIVs. We hypothesized that the process of adapting SHIVs by serial animal-animal passage in macaques increases the IFN-I resistance of SHIVs as serial animal-animal passage has been demonstrated to increase the replication capacity of SHIVs both *in vitro* and *in vivo* [109,111,128,178]. For SHIVs SF162P3 and 1157ipd3N4, rapid animal-animal passage within the first two weeks of infection, a time during which macaque mount a robust IFN-I response to infection [49], was performed [109,111,178]. Therefore, we compared the IFN-I sensitivity of the parental, un-passaged SHIVs with the corresponding macaque-passaged variants. Both the parental and passaged variants for each SHIV pair (SHIV AD8 and SHIV SF162) were relatively resistant to IFN-I compared to circulating SHIVs and did not reach 50% viral replication at the highest concentration of IFN-I tested (5,000 U/ml). We did, however, observe a difference between parental and passaged SHIVs with respect to % Vres. In each case, viral replication for the passaged SHIV was at or above 100% at the highest concentration of IFN-I. In contrast, both the parental SHIVs exhibited a significant decrease in viral replication to ~50% (Fig. 4.5d). These results indicated that the parental SHIVs were more sensitive to high doses of IFN-I compared the corresponding macaque-passaged variants.

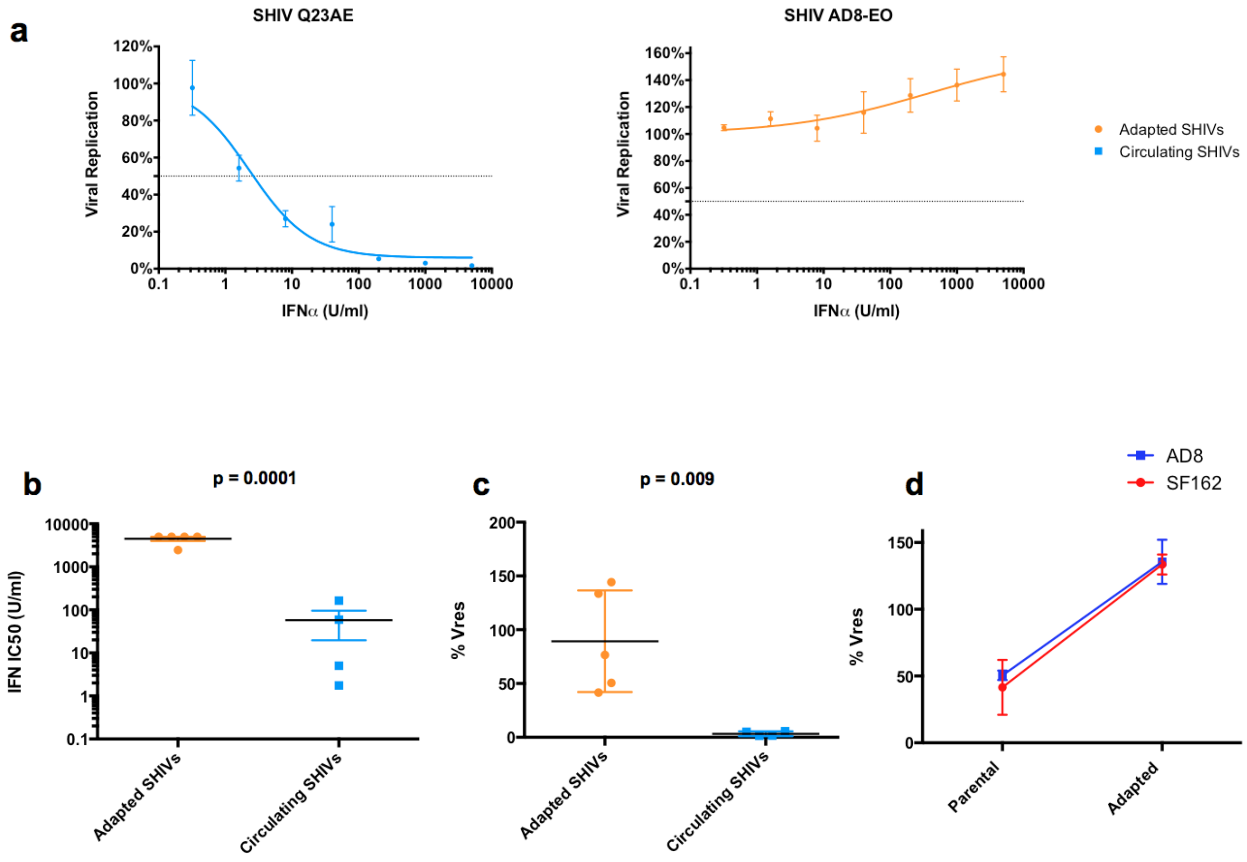

Fig. 4.5. Assessment of IFN-I sensitivity by IFN IC50 assay. (a) Dose-dependent inhibition of viral replication by IFN-I. Data for SHIVs representing circulating (SHIV Q23AE) and adapted (SHIV AD8-EO) variants are presented in the charts. Viral replication as a percentage of replication in untreated macaque cells is plotted on the y axis. Concentration (U/ml) of IFNα-2a is plotted on a log scale on the x-axis. Each data point represents the average of at least two independent experiments and error bars indicate SD. Data are fitted to a non-linear curve using Prism Graphpad version 6.0c. The dashed grey line indicates 50% inhibition of viral replication used to determine the IC50 value. (b) Comparison of IFN-I IC50 values between adapted and circulating SHIVs. IC50 value is plotted on the log scale on the y-axis. Each data point represents the average IC50 value for one SHIV variant. The key at the right indicates the color-coding for SHIV variants, adapted (orange) and circulating (blue). (c) Comparison of % residual viral replication (Vres) at the highest concentration of IFNα-2a (5000 U/ml) between adapted and circulating SHIVs. (d) Comparison of Vres values between parental and adapted SHIV pairs. Data points are color-coded according to the identify of the SHIVs pair, AD8 (blue) or SF612 (red) and indicate the average of two experiments with error bars indicating SD..

Virion-associated HIV-1 Env content correlates with IFN-I resistance

In chapter III, we reported that SHIVs adapted by macaque-passage or lab-culture exhibited higher amounts of virion-associated HIV-1 Env content and higher replication kinetics compared to circulating SHIVs. Given that adapted SHIVs are also more resistant to IFN-I treatment, we compared the virion-associated Env content and IFN-I sensitivity across the panel of SHIVs. Virion-associated Env content demonstrated a positive correlation with both the AUC ratio (Spearman $r = 0.77$, $p = 0.02$) and the IFN IC50 value (Spearman $r = 0.89$, $p = 0.003$) (Fig. 4.6a,b). We also observed significant positive correlations between the cell-associated Env content and AUC ratio (Spearman $r = 0.72$, $p = 0.04$) and IFN IC50 value (Spearman $r = 0.91$, $p = 0.002$) (Fig. 4.6c,d). Thus, the amount of HIV-1 Env expressed in SHIV-infected macaque cells and the amount of virion-associated Env content are both strong predictors of the ability to overcome the macaque IFN-I response.

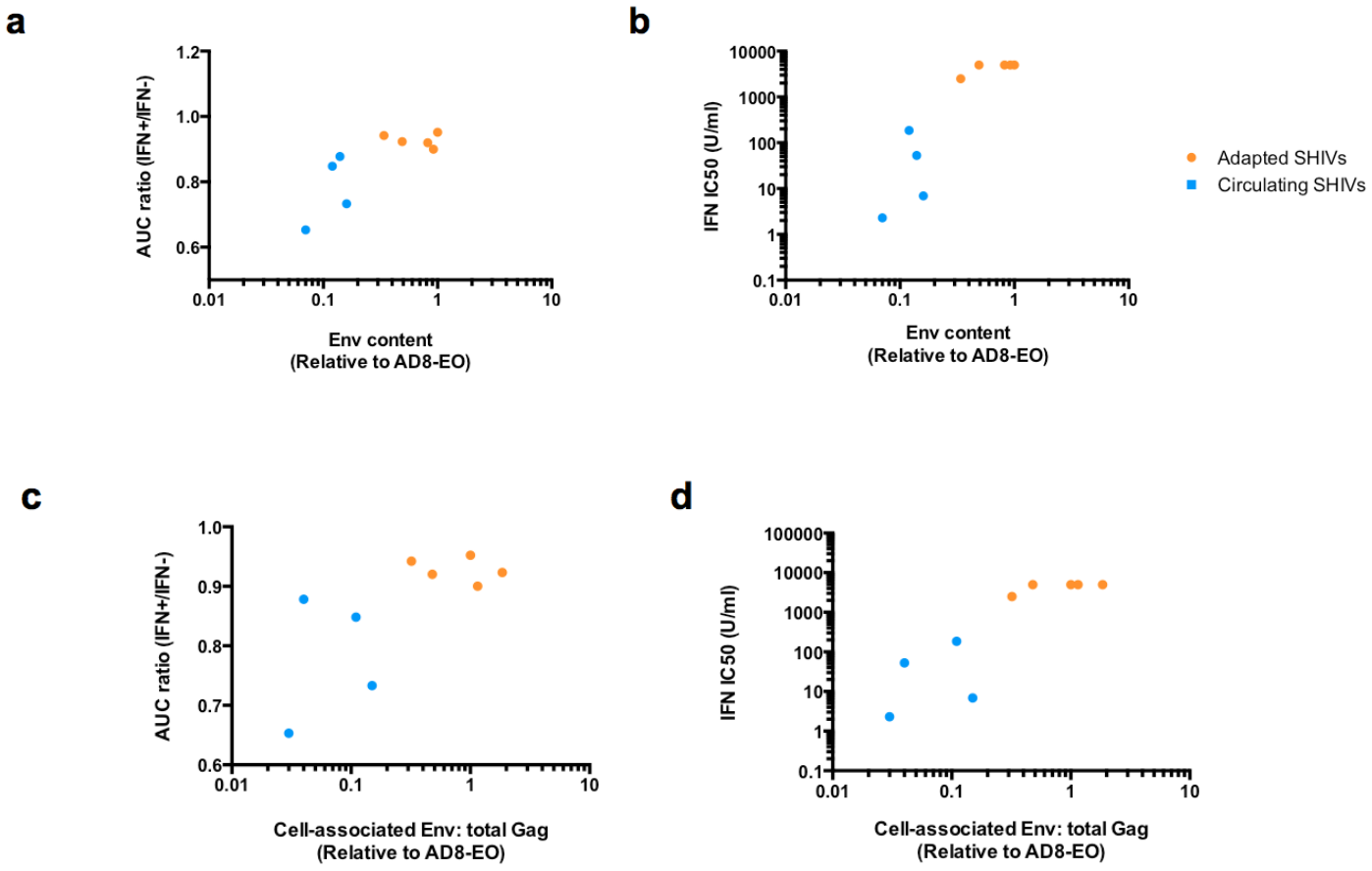

Fig. 4.6. Relationship between virion-associated Env content and cell-associated Env expression with IFN-I sensitivity. (a) Correlation between virion-associated Env content and IFN-I sensitivity as measured by AUC ratio (IFN+/IFN-). AUC ratio (IFN+/IFN-) over a 12-day replication time course is plotted on the y-axis, and virion-associated Env content of virus harvested at 6 days post-infection relative to SHIV AD8-EO is plotted on the x-axis on a log scale. (b) Correlation between virion-associated Env content and IFN-I IC50 value. IFN IC50 value (U/ml) represents the concentration of IFNα-2a at which 50% inhibition of viral replication is achieved and is plotted on the y-axis. Virion-associated Env content is plotted on the x-axis on the log scale. (c) Correlation between cell-associated Env expression and IFN-I sensitivity as measured by AUC ration (IFN+/IFN-). AUC ratio (IFN+/IFN-) is plotted on the y-axis, and cell-associated Env expression in plotted on the x-axis. Cell-associated Env expression is normalized to the total amount of Gag protein (PrGag + p27) as measured by western blot and is reported normalized to SHIV AD8-EO. (d) Correlation between cell-associated Env expression and IFN-I IC50 value. IFN-I IC50 value (U/ml) is plotted on the y-axis on the log scale while cell-associated Env expression is plotted on the x-axis on the log scale. Each data point represents a single SHIV variant and is the average of two independent experiments. The key at the right indicates the color-coding of SHIV variants, adapted (orange) and circulating (blue).

Effect of modulation of Env content of SHIVs

Take together our results suggested that modifications that increase Env expression may improve the replication capacity and IFN resistance of circulating SHIVs. Amino acid signatures in the Env leader peptide, which is involved in transporting the nascent Env protein to the endoplasmic reticulum, have been shown to have dramatic effects on Env expression in human cells [192]. In addition, a number of studies have demonstrated that truncations of the cytoplasmic tail result in increased Env incorporation and may also increase Env expression in cells [193]. Alignment of Env sequences from macaque-passaged, lab-cultured and circulating variants revealed diversity in both the leader peptide and the cytoplasmic tail. Of interest, the leader peptide sequence was very similar among macaque-passaged and lab-cultured variants but distinct from circulating variants (Fig. 4.7a). Also of interest, a seven amino acid deletion in the cytoplasmic tail was common between two pathogenic SHIVs (SHIV AD8-EO and SHIV SF162P3) but was not present in circulating SHIVs (Fig. 4.7b). We introduced the Env leader peptide from the HIV-1 ADA variant (the parental Env of SHIV AD8-EO) to SHIV Q23AE. Independently, we made the seven amino acid truncation of the cytoplasmic tail in the context of SHIV Q23AE. We then tested the Env leader peptide mutant and cytoplasmic tail mutant for replication capacity and IFN-I sensitivity in immortalized Ptm lymphocytes. Compared to the original SHIV Q23AE, the Env leader peptide mutant did not demonstrated any observable increase in replication kinetics or IFN-I resistance. The cytoplasmic tail mutant did not replicate well in either the untreated or IFN-I-treated macaque cells. Thus, modifications in the leader peptide and the cytoplasmic tail of HIV-1 do not appear to account for the differences in IFN-I sensitivity.

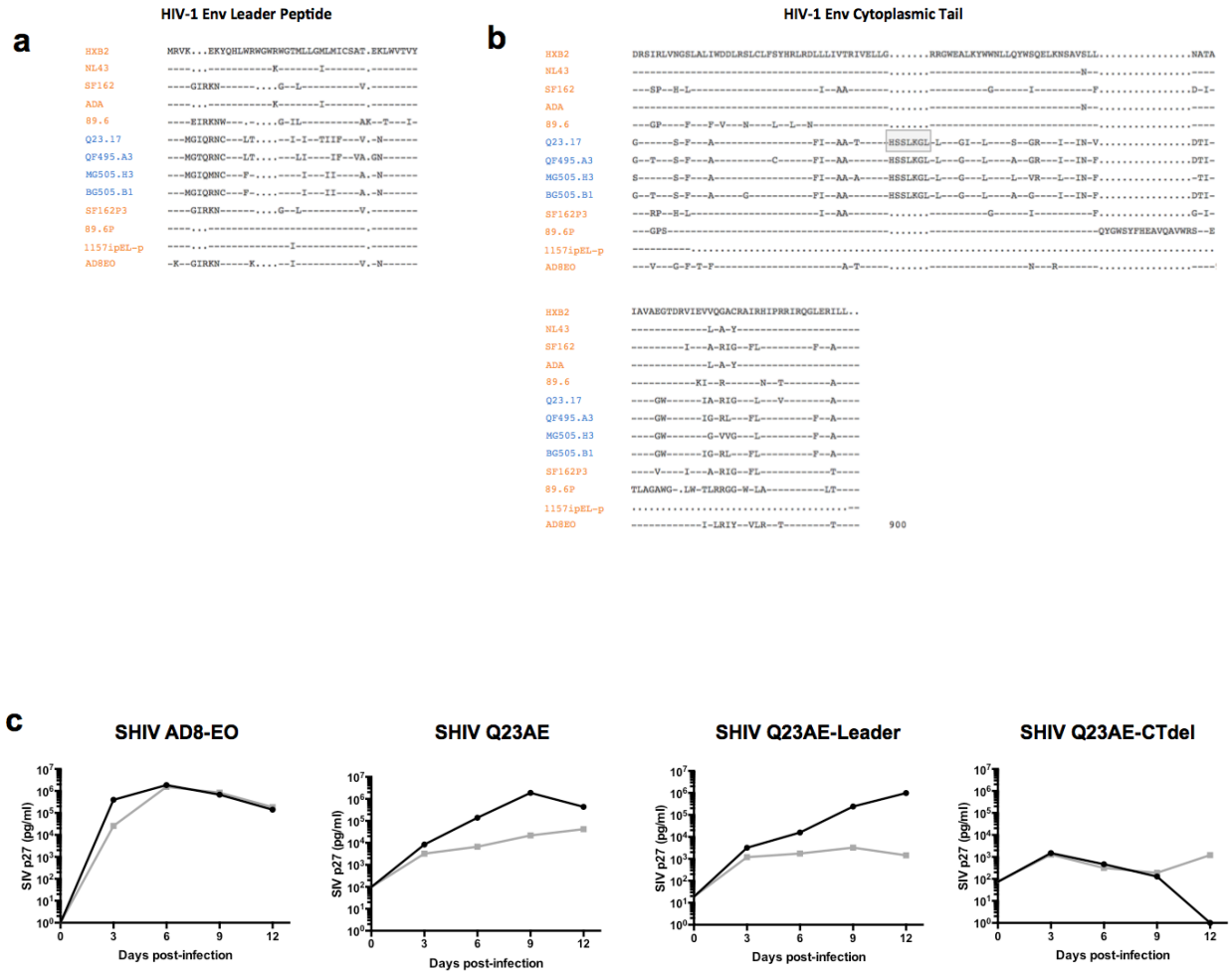

Fig. 4.7. Effect of modifications in HIV-1 Env leader peptide and cytoplasmic tail. (a) Alignment of HIV-1 Env leader amino acid sequences from lab-adapted, macaque-passaged and circulating HIV-1 variants. Identity of variants is indicated at the left. (b) Alignment of HIV-1 Env cytoplasmic tail amino acid sequences. The seven amino acid region that was deleted from SHIV Q23AE is highlighted in gray. Color-coding indicates whether the Env represents a lab-cultured or macaque-passaged variant (orange) or a circulating variant (blue). (c) Replication kinetics of Env leader and cytoplasmic tail chimeras in immortalized Ptm lymphocytes in the absence (black lines) or presence (gray lines) of 1000 U/ml of IFNα-2a.

IFN-I sensitivity of SHIVs in primary cells from different species

Considering the species-specific of the viral antagonists of known IFN-I-induced restriction factors, we tested the IFN-I sensitivity of a subset of SHIVs in both human and rhesus (Rh) macaque PBMCs (Fig. 4.8). SHIV AD8-EO replicated to high levels in Rh PBMCs reaching a peak of $>10^5$ pg/ml of SIV p27 at 6 dpi. In contrast, SHIV Q23AE replicated to low levels reaching a peak of $\sim10^3$ pg/ml of SIV p27 at 9 dpi. The replication results in Rh PBMCs for SHIV AD8-EO and SHIV Q23AE were nearly identical to the results in Ptm PBMCS. As was described for Ptm PBMCs, SHIV AD8-EO was largely resistant to treatment with IFN-I. Overall, SHIV Q23AE replicated poorly in both untreated and IFN-I treated Rh PBMCs. At the later time points (9 and 12 dpi), SIV p27 was barely detectable ($\leq 10^2$ pg/ml) in the IFN-I-treated cells while it was easily detectable ($\geq10^3$ pg/ml) in the untreated cells. The replication results for SHIV AD8-EO and SHIV Q23AE in untreated human PBMCs were very similar to the results in untreated macaque PBMCs, either pig-tailed or rhesus with SHIV AD8-EO replicating to higher levels than SHIV Q23AE. Interestingly, SHIV AD8-EO was more sensitive to IFN-I treatment in human PBMCs (AUC ratio 0.70) compared to rhesus PBMCs (AUC ratio 0.90) or pig-tailed PBMCs (AUC ratio 0.83) despite similar replication kinetics in untreated cells. SHIV Q23AE did not replicate well enough in human PBMCs to assess quantitatively IFN-I sensitivity although the replication curves do suggest IFN-I-induced inhibition of replication as the measured SIV p27 levels are ~10-fold lower in the IFN-treated cells at 9 and 12 dpi. The results indicate that there is no difference with respect to replication kinetics or IFN-I sensitivity for this subset of SHIVs between different species of primary macaques. However, SHIV AD8-EO is more sensitive to IFN-I treatment in human cells compared to macaque cells perhaps reflecting

species-specificity of an IFN-I induced host factor. These results also suggest that IFN-I-induced host factor(s) that target circulating SHIVs is species-specific.

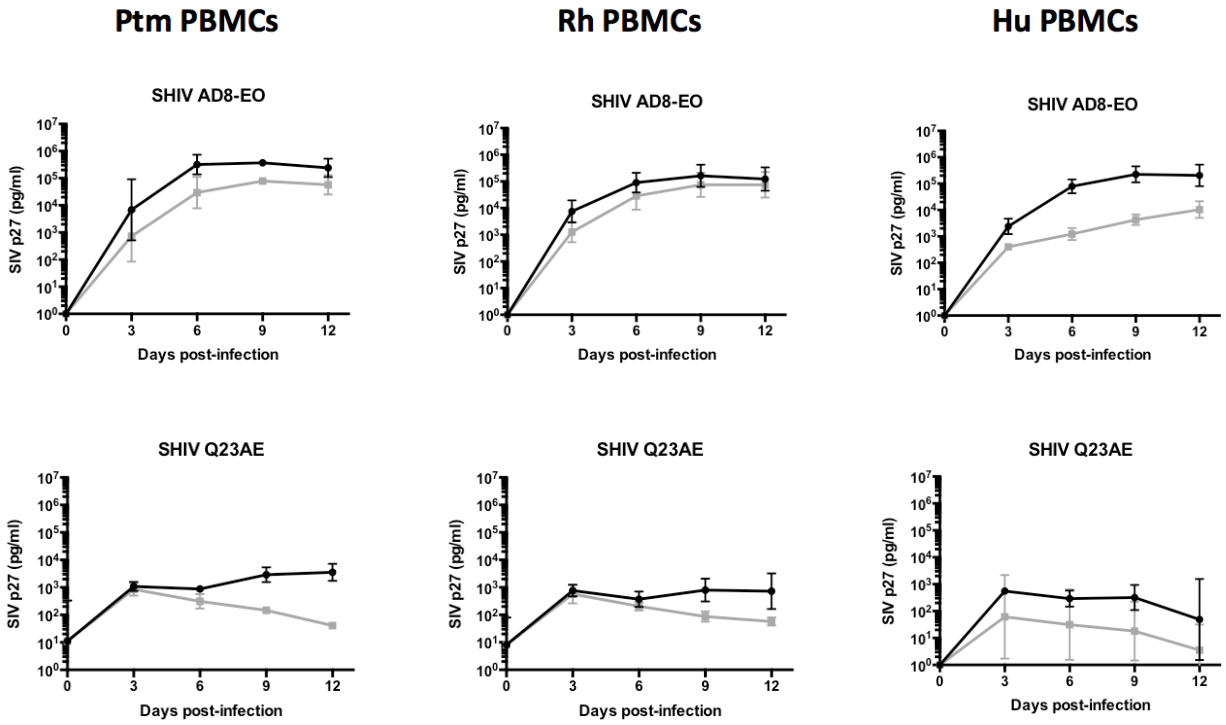

Fig. 4.8. IFN-I sensitivity of SHIVs in primary cells from different species. Replication kinetics of SHIVs in the absence (gray lines) and presence (black lines) of IFN-I treatment was determined over a 12-day time course in pig-tailed macaque (Ptm) PBMCs, rhesus macaque (Rh) PBMCs and human (Hu) PBMCs. SIV p27 concentration (pg/ml) as a measure of viral replication is plotted on the y-axis, and days post-infection on the x-axis. Data points represent the average of three independent experiments using PBMCs from different donors. Error bars represent the SD.

Discussion

Pathogenic SHIVs that have been adapted for replication in macaques are able to replicate to high levels during acute infection in the face of a robust innate immune response [45,49]. The viral determinants that allow pathogenic SHIVs to replicate so well in macaques and allow these viruses to evade or antagonize the innate immune response have not been clearly defined. Identification of such viral determinants would provide important information on the basic biology of lentiviral pathogenesis and would inform the rational design of novel challenge viruses for macaque models of HIV-1 transmission and infection. Here we provide evidence that HIV-1 Env is an important determinant of the ability of SHIVs to overcome the type-I interferon response and that the amount of HIV-1 Env expressed in SHIV-infected macaque cells and/or present in cell-free virions contribute to IFN-I resistance. Among a panel of nine SHIVs, viruses that were adapted by macaque-passage and/or lab-culture demonstrated high replication capacity, greater IFN-I resistance and higher virion-associated Env content compared to viruses derived from HIV-1 variants isolated from patients early in infection. Our results suggest that through multiple rounds of selection and adaptation in lab-culture and by macaque-passage pathogenic SHIVs may have acquired high Env content and faster replication kinetics allowing them to overcome the IFN-I response and establish persistent infection in macaques.

SHIVs that are able to establish persistent infection and cause AIDS in macaques are critical tools for pre-clinical HIV-1 vaccine and therapeutic studies and have provided important information on transmission and pathogenesis of lentiviruses. Recently, there has been an effort to generate pathogenic SHIVs that are more representative of HIV-1 variants that are transmitted and circulating in people. There has been limited success in generating SHIVs that encode HIV-1 Envs representing circulating variants for reasons that are unclear given that the SIV portions of

SHIVs encoded known viral antagonists of species-specific restriction factors. These factors include antiviral restriction factors that actively inhibit replication and species-specific differences in required host proteins, such as the CD4 receptor used for entry [98,137]. Two recent studies identified a small subset of SHIVs that encode T/F HIV-1 Envs from a larger pool that were able to cause disease in macaques without animal-animal passage [126,127]. The fact that only one or two viruses from the larger pool were able to establish persistent infection in the macaques suggests that these viruses were selected during the infection because of unique characteristics.

In the present study, we compared the IFN-I sensitivity of SHIVs encoding HIV-1 Envs representing macaque-passaged and lab-cultured variants (adapted SHIVs) and representing variants circulating in people (circulating SHIVs). Overall, we found that SHIVs encoding adapted Envs, either by macaque-passage or lab-culture, had higher replication capacity and higher IFN-I resistance compared to circulating SHIVs. Importantly, the SHIVs encoding lab-cultured HIV-1 variants (SHIV SF162 and SHIV AD8) are the parental viruses of two of the macaque-passaged, pathogenic viruses (SHIV SF162P3 and SHIV AD8-EO) [128,178]. Thus, SHIVs that progressed through the initial process of adaption *in vitro* may have already had high IFN resistance, which allowed them to infect macaques and to be serially passaged from animal to animal. At high concentrations of IFN-I (5000 U/ml), we were able to detect statistically significant differences between the parental and macaque-passaged SHIVs suggesting that animal-animal serial passage may increase the ability of these viruses to evade or antagonize the innate immune response and contribute to their increased pathogenesis. In future studies, it will be interesting to investigate the effect of rapid serial passage in macaques on IFN-I sensitivity using additional sequential isolates. Recently, Ren et al. reported the generation of a pathogenic

SHIV encoding a subtype C HIV-1 Env isolated early in infection (SHIVC109P3) by rapid serial passage within the first two weeks of infection in macaques [109]. With each of the first three serial passages, peak viral levels during acute infection, measured in viral RNA copies/ml, increased by ~ 1 log. Testing isolates from each passage would allow for a more robust test of the hypothesis that animal-animal serial passage adapts SHIVs to resist the IFN-I response.

By generating chimeras between the SHIVs with the largest differences in IFN-I sensitivity, we identified HIV-1 Env as a critical determinant of IFN resistance. Introduction of the gp120 from SHIV AD8-EO to SHIV Q23AE resulted in an intermediate phenotype with modest increases in replication kinetics and IFN resistance while introduction of gp41 did not result in any observable increases in either of the two measures. Thus, there are likely determinants in both gp120 and gp41. Our results are similar to a recent study from Thippeshappa *et al.* that found that gp120 of HIV-1 Env is a partial determinant of IFN resistance in macaque cells [194]. In this study, we also provide evidence that the amount of HIV-1 Env expressed in SHIV-infected cells and present in cell-free virions contribute to IFN-I resistance. Among the SHIVs tested, we demonstrated a strong correlation between IFN-I resistance and both the amount of Env expressed in cells and present in virions.

The finding that Env expression in cells and virion-associated Env content correlated with IFN resistance was somewhat surprising given that a mechanism by which HIV-1 Env evades or antagonizes an IFN-induced host factor has not yet been described [27]. The strong positive correlation between virion-associated Env content and replication kinetics could suggest that virion-associated Env content leads to IFN resistance by allowing the virus to rapidly replicate in cell culture and saturate IFN-induced restriction factors in target cells. Numerous studies have demonstrated that lentiviral restriction factors can be saturated *in vitro* [195]. In

support of this explanation, we observe a decrease in inhibition of viral replication of IFN-I-sensitive SHIVs when we infect at a high (>0.1) initial MOI. In addition, at later time points (~12 dpi) of the replication time courses, we observe increases in viral replication for circulating SHIVs. When cell-free virus levels reach ~10^4 pg/ml of SIV p27, circulating SHIVs can reach levels of viral replication similar to those observed in untreated cells.

It is also possible that HIV-1 Env antagonizes or evades a host restriction factor by a direct mechanism that remains to be identified. For example, the increased Env content in virions may allow SHIVs to evade inhibition at the stage of entry by the recently described IFITM proteins [42,43]. Interestingly, for IFN-I sensitive variants such as SHIV Q23AE, we observe a delay in the IFN-I induced inhibition. This delay may suggest that an IFN-I-induced factor is packaged into virions and has its effect in the subsequent round of infection. Recently, Compton *et al.* demonstrated that IFITM proteins are packaged into HIV-1 virions [43]. The role of IFITMs in inhibiting replication of circulating SHIVs is a promising area of research. Another recent study described an as yet unidentified restriction factor expressed in macrophages that specifically degrades HIV-1 Env via a lysosomal pathway [196]. This finding is particularly interesting given that our data indicate that post-transcriptional events are responsible for the low levels of HIV-1 Env expression for circulating SHIVs. The nature of these post-transcriptional events and the potential role of an IFN-induced restriction factor in this process warrant further investigation.

As mentioned in Chapter III, one possible explanation for the relationship between viral replication kinetics in cell culture and HIV-1 Env content is that high levels of Env expression in infected cells permits efficient cell-cell transmission in the culture. Cell-cell transmission facilitated by high Env expression may allow SHIVs to overcome the IFN-I response by

saturating IFN-I-induced factors. In addition, if the IFN-I-induced factors act early at the stage of entry, efficient cell-cell transmission may allow SHIVs to bypass this inhibition. The possible role that cell-cell transmission plays in overcoming the IFN-I response would be an interesting area for future study.

Our viral replication and IFN-I sensitivity results in Ptm, Rh macaque and human PBMCs indicate that there may be species-specificity to the host factor(s) responsible for the observed IFN-I-induced inhibition of viral replication. In immortalized Ptm lymphocytes, we demonstrated that viral replication kinetics in untreated cells predict IFN-I resistance. Despite nearly identical replication kinetics in untreated Ptm, Rh or human PBMCs, SHIV AD8-EO was more sensitive to IFN-I treatment in human cells. One possible explanation for the increased IFN-I sensitivity of SHIV AD8-EO in human PBMCS is that a previously described species-specific restriction factor, such as TRIM5α, is inhibiting viral replication by interacting with the SIV capsid, although data suggest that human TRIM5α is largely ineffective at restricting SIVmac when artificially expressed in human cells lines [197]. Another possibility is that through multiple rounds of selection SHIV AD8-EO has adapted to a novel restriction factor in macaques but remains sensitive to the human version. Identification of the IFN-I-induced host factor(s) contributing to the observed inhibition of viral replication will be critical for the development of challenge viruses that can antagonize or evade its activity.

The findings presented in this study also have implications for the development of SHIVs based on transmitted and/or circulating HIV-1 variants. Several recent studies have provided evidence that T/F HIV-1 variants from acute infection are more resistant to IFN-I treatment compared to variants isolated at six months after initial infection [94,95]. Interestingly, one of these studies found that T/F viruses had more virion-associated Env content compared to chronic

viruses [94]. The circulating SHIVs investigated in this study encode HIV-1 variants isolated very early in infection (<50 dpi) and others isolated later in infection (~ 1 year). Therefore, it remains unclear how the timing of the isolation of an HIV-1 variants affects IFN-I sensitivity in the context of a SHIV. In contrast to previous studies that investigated IFN-I sensitivity of HIV-1 variants in human cells, we explored IFN-I sensitivity of SHIVs encoding different HIV-1 Env variants in macaque cells. Thus, species-specific differences in IFN-I-induced host factors may contribute to the differences observed with respect to the IFN-I sensitivity of viruses encoding HIV-1 Envs isolated early in infection. For the purpose of SHIV development, the species-specificity of IFN-I resistance warrants further investigation.

In conclusion, the findings presented here highlight the critical role that HIV-1 Env plays in the viral replication and IFN-I sensitivity of SHIVs encoding macaque-passaged, lab-cultured and circulating HIV-1 variants. Modulation of HIV-1 Env content may provide a strategy to increase replication kinetics and IFN-I resistance of SHIVs encoding HIV-1 Envs representative of variants circulating in people. Given the effect that macaque-passage has on the antigenic properties of SHIVs, modulation of Env content may increase replication capacity in macaques whiles maintaining key biological properties of circulating HIV-1 variants.

Chapter V: Implications for the development of improved challenges viruses for macaque models of HIV-1 infection

The development of animal models that capture the most important aspects of HIV-1 infection in people has proven to be a difficult task despite decades of effort. As discussed, HIV-1 does not productively infect most non-human primates, including macaques, which are the main model for HIV-1 infection. Challenge viruses that encode genes derived from HIV-1 require extensive adaptation in order to establish persistent infection in macaques. The basic biology of the process of adapting HIV-1 for replication in macaques is not well understood. The interactions of lentiviral proteins with host factors that either promote or inhibit viral replication are the basis of this adaptation process. Although much progress has been made in recent years in understanding the mechanisms of some of these virus-host interactions, a systemic approach to uncovering the most important interactions contributing to adaptation of HIV-1 for replication in non-human primates has not been taken. The results presented in this thesis provide insight into the process of adapting HIV-1 for replication in macaques and, thus, have implications for the development of SHIVs that are more representative of HIV-1 variants that are circulating in people. HIV-1 faces a number of selective pressures, including species-specific differences in the CD4 receptor and host factors induced by the innate immune response, as it adapts for replication in macaques. In this thesis, I demonstrate that the highly variable Env protein of HIV-1 variants circulating in people undergoes dramatic conformational changes as it adapts to the macaque CD4 receptor, and these changes affect the antigenic properties of the virus. In addition to its structure and conformation, the amount of HIV-1 Env protein expressed in infected cells and present in virions is an important determinant of the ability of SHIVs to replicate in macaque

cells. SHIVs that have been selected for replication in macaques have high amounts of Env and replicate to high levels in macaque cells. These viruses are also able to rapidly overcome the type-I interferon (IFN-I) response, a critical component of the innate immune system. The results presented here highlight promising areas of future study related to the interaction of Env with the IFN-I response and have implications for the development of more relevant challenge viruses for macaque models of HIV-1 infection. Also, in this chapter, I speculate on how understanding of host-virus protein interactions might be exploited for therapeutics and provide my thoughts on important vaccine strategies that could be pursued in the near future.

Development of SHIVs based on their ability to use macaque CD4

The results from Chapter II indicate that there are antigenic consequences of adapting circulating HIV-1 Envs to efficiently use the macaque CD4 receptor. However, screening a large panel of HIV-1 Envs isolated from patients early in infection, we identified a small subset of variants that are able to use the mCD4 receptor comparable to levels for human CD4 and still maintain key epitopes that are characteristic of transmitted HIV-1 variants. Specifically, the subtype A HIV-1 Env variant Q842d16, which was isolated ~50 days post-infection, uses mCD4 nearly as well as huCD4 and has a neutralization profile characteristics of transmitted variants, including sensitivity to broadly neutralizing antibodies that target quaternary epitopes [147]. It will be interesting to test the ability of a SHIVs encoding this Env to replicate in macaque cells, and if those results look promising, in the context of an *in vivo* infection.

It remains unclear whether HIV-1 Env undergoes further adaptation to the mCD4 receptor during the course of infection within an individual macaque or during serial passage

between macaques. Studies of co-receptor switching of HIV-1 Env in macaques suggests that conformational changes similar to those observed during adaptation to mCD4 do occur at late stages of infection *in vivo* [125,177]. In addition, a very recent study investigating the stoichiometry of HIV-1 Env trimers and infection of cells expressing huCD4 suggests that a macaque-passaged Env (SF162P3N) requires fewer trimers to infect target cells compared to the parental Env (SF162) [198]. The process of macaque-passage may have resulted in the lower stoichiometry and may contribute to the pathogenicity of the virus. It would be interesting to compare the stoichiometry of parental and adapted SHIVs using target cells expressing the mCD4 receptor. The results presented in Chapter II of this thesis and in other studies [136,137] indicate that the parental variants of pathogenic SHIVs already had the ability to use mCD4 before adaption by macaque passage. However, a more sensitive assay such as the one described by Brandenberg *et al*. may allow for detection of differences between the parental and adapted variants.

Identification of additional mutations that allow circulating HIV-1 Env to use macaque CD4 for entry

The results in Chapter II focused on two independent point mutations (A204E and G312V) that allow circulating HIV-1 Envs to use mCD4 for entry. These mutations were identified by serial passage of a single HIV-1 Env variant (Q23.17) in macaque cells [136]. The selection of these independent mutations likely depended on the context of amino acid residues in close proximity within the Env trimer as these mutations affected the quaternary conformation of the protein. These specific point mutations are shared with only one other Env variant that

replicates in macaques suggesting that there are many other mutations that allow HIV-1 Env to use the mCD4 receptor for entry [125]. Identification of additional point mutations that allow for efficient use of mCD4 in the context of other Env trimers would provide important insight into the adaptation of HIV-1 Env to mCD4 and would inform the development of SHIVs based on circulating HIV-1 variants. One approach to the identification of such point mutations would be to passage a panel of diverse HIV-1 Env variants isolated early in infection in macaque cells using a protocol similar to that described by Humes *et al.* [136]. An alternative approach to identifying adaptive mutations would be to utilize mutational scanning to introduce mutations at each residue in the Env trimer and then selective for adaptive mutations by infection of macaque cells. This technique introduces every possible amino acid change at each codon position of the protein by PCR and generates a library of mutants from which adaptive mutations can be selected [199] and has been demonstrated to work well for influenza [200]. The characterization of the spectrum of mutations that allow HIV-1 Env to use mCD4 would provide important information on the structural requirements for efficient use of mCD4 by HIV-1 Env for entry and a new set of mutations that could facilitate the development of improved SHIVs.

It is possible that adaptation of HIV-1 to use the macaque CD4 receptor for entry is necessarily accompanied by antigenic changes in Env. If this is the case, then the adaptation process poses a limitation to the ability to fairly assess the efficacy of an HIV-1 vaccine in macaques, specifically vaccine strategies that target epitopes that undergo changes. An alternative to modifying the virus for replication in macaques would be to identify different non-human primate species that are more amenable to infection with HIV-1. Recently, a species of new world monkey, the owl monkey, was identified as having a CD4 receptor that is compatible with HIV-1 Env variants isolated early in infection [201]. It remains to be seen if owl monkeys

can be infected with HIV-1 or if there are additional restrictions, such as IFN-induced host factors, that limit HIV-1 replication. Whether or not owl monkeys are ultimately capable of supporting HIV-1 infection, this study highlights the possibility of improving animal models of HIV-1 infection with a rational approach based upon what is known about the basic biology of retroviruses and their interactions with hosts. Methods for sequencing entire genomes are becoming faster and cheaper, and abundant genetic information about a wide variety of animal species could become a means to identify animal hosts that may be genetically susceptible to HIV-1 infection.

Characterizing the restriction of circulating HIV-1 Env synthesis in macaque cells

The difficulty in generating pathogenic SHIVs that are representative of circulating HIV-1 variants reflects a lack of a deep understanding of host-virus interactions, both advantageous and detrimental to viral replication, in macaque cells. The results presented in Chapter III of this thesis suggest that there are previously un-described interactions between HIV-1 Env macaque host factors that influence viral replication. Specifically, there are events that likely occur after transcription that inhibit the synthesis of Env from circulating variants in macaque cells, and variants that have been adapted in lab-culture or by macaque-passage are able to overcome this inhibtion. The species-specific activity of host proteins, both co-factors that promote and restriction factors that restrict viral replication, has been described by many previous studies [98]. It is unclear if the post-transcriptional inhibition of Env synthesis is species-specific. Given that the SHIVs tested in this thesis replicate to lower levels overall in human cells, Env expression in infected human cells in the context of an HIV-1 provirus could address this question. Determining if the mechanism underlying low Env expression of circulating HIV-1 variants is species-specific might also inform the mechanistic basis for the

observed inhibition.

It will also be important to identify the stage of the viral life cycle at which the inhibition of HIV-1 Env synthesis in macaque cells occurs. Inefficient translation, low protein stability and targeted degradation of Env by a host factor are just three potential mechanisms that could lead to low levels of Env in macaque cells. The inefficiency of translation of HIV-1 Env mRNA based on codon usage has been previously described [202]. In addition, a host factor, schlafen11, which inhibits viral protein synthesis, was recently identified [38] suggesting that a host factor could interfere with viral protein synthesis and result in low Env expression. Post-translational events could also affect HIV-1 Env expression in macaque cells. Each of the circulating SHIVs studied encodes either the A204E or G312V mutation allowing them to use mCD4 for entry, and each of these mutations increases the sensitivity to neutralization by soluble CD4 (sCD4) [136,137]. Reactivity to sCD4 may reflect decreased overall stability of the protein. In addition to being inherently less stable, the circulating HIV-1 Env proteins could also be specifically degraded by a host factor through a proteosomal or lysosomal degradation pathway. Identifying which of these mechanisms, or perhaps others not mentioned here, will be critical to developing circulating SHIVs that are able to efficiently replicate in macaque cells.

Interactions between HIV-1 Env and the IFN-I response

In recent years, there has been exciting progress made in understanding how retroviruses interact with the innate immune response. The presence of retroviral infection is sensed through a number of different mechanisms leading to the production of IFN-I and induction of an anti-viral state in target cells [203-205]. The mechanisms by which the downstream effectors induced by IFN-I inhibit viral replication are a very active area of research. Many of the accessory

proteins of HIV-1 have been implicated in antagonism or evasion IFN-I induced factors [27]. However, the role that HIV-1 Env plays in evading or antagonizing the IFN-I response has not been previously described. The results from Chapter IV of this thesis suggest that HIV-1 Env plays an important role in overcoming the IFN-I response in macaque cells. It is unclear whether this occurs through an indirect mechanism by which HIV-1 Env leads to increased viral replication and saturation of IFN-I induced factors, or a direct mechanism by which HIV-1 interacts directly with these factors by protein-protein interactions. Direct protein-protein interaction between HIV-1 Env and IFN-I induced macaque factors would allow for the mapping of important viral and host determinants for these interactions. Identification of these determinants would provide important information on the mechanisms of antagonism or evasion and would provide the basis for modifying these determinants for improved viral replication.

The results from Chapter IV also indicate that the amount of HIV-1 protein is important. The role that the amount of HIV-1 Env plays in replicating in the presence of the innate immune response has largely been uninvestigated. One recent study examining the phenotypic properties of transmitted/founder (T/F) HIV-1 variants reported that T/F variants were more resistant to IFN-I treatment and had higher virion-associated Env content compared to variants isolated during chronic infection [94]. Taken together with our data indicating that virion-associated Env content predicts replication kinetics and IFN-I resistance, these results suggest that HIV-1 variants that establish infection in a host may be selected for higher Env content, which increases replicative fitness in the presence of the IFN-I response. These results also suggest that different immune pressures within a host may influence Env content over the course of infection. For example, high Env content may be advantageous during acute infection when there is a robust IFN-I response, but lower Env content may be beneficial during chronic infection when antigenic

stimulation by Env induces adaptive immune responses. Analysis of virion-associated Env content of variants isolated longitudinally throughout the course of infection would provide important information on modulation of Env content in the face of different selective immune pressures. Also, it would be interesting to directly test the hypothesis that higher Env content is selected for during transmission and establishment of infection in a host by infecting macaques with SHIVs that have varying amounts of virion-associated Env content

Identifying IFN-I-induced host factors that inhibit replication of circulating SHIVs

The results presented in this thesis focus on the viral determinants of SHIV replication and IFN-I resistance and highlight the importance of HIV-1 Env. It will also be of interest to identify the host factors induced by IFN-I treatment in macaque cells that inhibit viral replication of circulating SHIVs. A number of recent large-scale screens have identified hundreds of IFN-I-induced genes with antiviral potential [188,189,206]. The species-specificity of these potentially antiviral proteins has not been investigated. There are a number of high-throughput methods available for studying the effects of host genes on infectivity and viral replication, including overexpression of select genes, siRNA libraries, and more recently CRISPR libraries. A genome-wide CRISPR screen in immortalized pig-tailed macaque (Ptm) lymphocytes is a particularly attractive approach to identifying factors that influence SHIV replication [207]. A genome-wide approach would limit the biases of selective a subset of genes of interest while the "knock-out" approach of the CRISPR/Cas9 system would ensure that low-level expression of a particular protein was not confounding the ability to identify an inhibitor factor. One potential limitation of this approach is the availability of a CRISPR guide RNA library specific for macaques. An

alternative approach would be to overexpress candidate genes in immortalized Ptm lymphocytes. For this strategy, a genome-wide screen likely would not be feasible considering the significant amount of work that would be required to clone individual macaque genes into expression vectors. Therefore, a select number of IFN-I-induced candidate genes would be tested. Regardless of the strategy employed, a more systemic approach to identifying host factors that limit SHIV replication, in particular circulating SHIVs, in macaque cells will provide important information on the species-specific restriction of retroviral replication and will inform the rational design of challenge viruses for macaque models of HIV-1 infection.

Role of IFN-I-induced factors in natural infection and therapeutic potential

The dynamics between HIV-1 infection and the interferon response has received much attention in the last several years. Over a decade of studies has elucidated the mechanisms by which downstream resistance and restriction factors induced by IFN-I inhibit viral replication. More recently, there has been interest in identifying the upstream mechanisms by which cells of the innate immune system sense the presence of HIV-1 infection and initiate production of interferons. As we come to understand the complex interactions between HIV-1 and the innate immune system, it will be important to determine which interactions are most relevant to natural infection. HIV-1 infection in people is complex and dynamic. One or a few viral variants are selected from a much larger genetically diverse pool during the process of transmission. Upon successful infection, HIV-1 rapidly establishes persistent infection and diversifies within an individual. During acute and chronic infection the virus is evolving in response to both innate and adaptive immune responses that affect the course of infection. With respect to the IFN-I

response, the very early events of natural infection, including transmission and the first few days of acute infection during which viral infection disseminates, would be most interesting to study. These initial events set the stage for the ensuing persistent infection, and viral determinants and components of the innate immune response likely have a major impact on the course of disease. Data from this thesis suggest that HIV-1 Env plays a critical role in overcoming the IFN-I response. When the IFN-I response is viewed as an antiviral state inducing the expression of hundreds of host factors with the potential to inhibit viral replication, increasing replication kinetics via Env may be a strategy to saturate these factors and minimize their effect on viral replication. Given that the restriction factor field is relatively young (~12 years), there are likely many other host factors yet to be identified, which have the potential to inhibit viral replication.

In addition to the replication kinetics of HIV-1 within a target cell, the dynamics of replication within different compartments of a host would likely influence the ability of the IFN-I response to limit viral replication. For most studies including those presented in this thesis, the interaction between HIV-1 and the IFN-I response has been investigated in target cells *in vitro*. It is unclear whether the kinetics observed in cell culture is relevant to natural infection. For example, the data presented in Chapter IV indicate that viruses that exhibit fast replication kinetics are quickly able to overcome the IFN-I response in immortalized CD4+ cells *in vitro*. Replication kinetics may depend on the route of transmission and the compartments in which the virus is replicating. Both of these factors could also influence the innate immune responses induced in the host. Related to this idea, it is possible that target cells that are present in different compartments, for example genital mucosa, gastro-intestinal mucosa or draining lymph nodes, could exhibit different levels of restriction/resistance factor expression. Although the IFN-I response is often described as leading to an "anti-viral state" with hundreds of ISGs induced, the

IFN-I response is likely nuanced and modifiable with different factors such as the infecting virus and route of infection.

Understanding the dynamics of the interactions between the HIV-1 and the IFN-I response provides the opportunity to exploit these interactions for preventive or therapeutic interventions. An ideal restriction/resistance factor to target for such interventions would be one that is induced rapidly upon viral sensing, acts early in the viral life cycle and is expressed in target cells at the site of initial infection. I envision an effective therapeutic as a small molecule designed to interfere with the interaction of a viral antagonist with a corresponding restriction factor. As with any therapeutic drug against a highly mutable virus, there are always concerns about the development of resistance Thus, effective therapeutic interventions would likely require targeting multiple different viral antagonist-restriction factor interactions. Investigation of innate immune responses to HIV-1 infection is a relatively young field. There is much to be learned about both the mechanisms of sensing of HIV-1 infection and the host antiviral proteins that directly interfere with viral replication.

Current vaccine strategies

During the last three decades, many different vaccine strategies to prevent HIV-1 infection have been proposed and tested in either pre-clinical or clinical vaccine trials. For pre-clinical trials, the SHIV/macaque model, despite it's limitations, has been the gatekeeper of promising vaccine strategies. These strategies have included both T-cell-based platforms designed to elicit cellular immune responses that kill HIV-1 infected cells and antibody-based platforms designed to elicit neutralizing antibody responses that provide sterilizing immunity and

prevent infection. Both strategies have had limited success in pre-clinical studies, and perhaps, even more limited success in human vaccine trials. The biology of HIV-1 infection presents unique challenges to T-cell and antibody-based vaccine strategies. First, the pool of HIV-1 variants circulating in people is highly diverse. This diversity presents a challenge to both general vaccine strategies because in theory a single platform that elicits immune responses against most circulating variants would be required. Another important challenge is that HIV-1 integrates into the genome of infected cells and establishes persistent infection with a latent reservoir of infected cells. Recent studies in macaques indicate that the reservoir of HIV-1 infected cells is established very early within three days of initial infection [46]. This early establishment of a viral reservoir sets a very high bar for an effective vaccine and will likely require potent cellular immune responses to eliminate the virally infected cells. Given these important challenges, and many others not discussed in this chapter, I envision a safe and effective vaccine against HIV-1 requiring a combination of both T-cell- and antibody-based strategies in a single platform.

There are two vaccine strategies, one T-cell- and one antibody-based, that show particular promise and if combined could have the potential to elicit robust immune responses capable of providing sterilizing immunity. Recently, the development of a cytomegalovirus (CMV) vector SIV vaccine capable of inducing robust T-cell responses in macaques was reported [208]. In follow-up studies, the authors demonstrated that ~50% of the animals that become infected later cleared SIV infection with no virus detectable after biopsies of multiple tissues [209]. Although the safety of CMV-based vectors in people still needs to be carefully investigated, this platform shows great promise for eliminating cells infected with HIV-1 in the context of natural infection. Thus, even if the viral reservoir is rapidly seeded within the first few

days of infection, T-cell responses elicited by a CMV-based vector vaccine may be able to eliminate those infected cells.

Despite the challenges of developing an antibody-based vaccine, the isolation of broad and potent neutralizing antibodies from HIV-1+ individuals and the finding that these antibodies do provide sterilizing immunity when passively transferred to macaques do suggest eliciting protective neutralizing antibodies is possible [210]. With respect to antibody-based vaccines, one promising strategy for eliciting broad and potent neutralizing antibodies is immunization with native-like soluble HIV-1 Env trimers. In order to elicit antibodies that target epitopes on the closed, pre-fusion conformation of the trimer and effectively neutralize the virus, an immunogen will likely have to present these epitopes in a native-like conformation. In support of this idea, a recent study investigating neutralizing antibody responses to immunization with soluble, stabilized SOSIP Env trimer in rabbits and macaques reported elicitation of antibodies capable of neutralizing a resistant, sequence-matched HIV-1 variant (BG505) [211,212]. Recent studies have sought to characterize the initial events that stimulate B cells that go on to produce broadly neutralizing antibodies and their developmental pathways [213,214]. Such studies will inform optimization of the vaccination strategy using native-like, soluble trimers. For example, sequential immunization with different HIV-1 Env variants may help guide the development of broadly neutralizing antibodies. In addition, the schedule and route of vaccination will also be important parameters to optimize.

Given the promising initial results of the two vaccination strategies described above, it would be interesting to prime the immune system with a CMV vector-based HIV-1 vaccine and then boost with native-like, soluble HIV-1 Env trimers. Ideally, the protein boost would induce neutralizing antibodies that are able to neutralize most HIV-1 variants to which an individual is

exposed. In the event that viral variants are able to establish infection despite the presence of neutralizing antibodies, CD8+ T-cell responses induced by the CMV-vector component of the vaccine would be present to kill target cells that do get infected.

For both strategies, it will be critical to develop both autologous and heterologous challenge viruses to test protective efficacy in the macaque model of infection. Challenge of immunized macaque with a sequence-matched, autologous virus would be important to demonstrate that the vaccine strategy is capable of eliciting strain-specific, protective neutralizing antibodies responses. Challenge with heterologous virus would test the ability of the vaccine to protect against diverse variants. Ideally, a panel of diverse SHIV challenge viruses that represent the antigenic properties of HIV-1 variants circulating in people would be available so that challenge viruses could identified to address specific research questions. As has been discussed throughout this thesis, the development of such viruses that capture the most important aspects of HIV-1 infection in people has proven to be a very difficult task. Studies designed to identify the most important barriers to HIV-1 infection of macaques will help develop a blueprint for the rational design of a diverse panel of SHIV challenge viruses. As the name implies, the SHIV/macaque model is a "model" of HIV-1 infection and necessarily involves some level of selection from the vast genetic diversity of HIV-1.

Conclusion

SIV/HIV chimeric viruses play a key role as challenge viruses for macaque models of HIV-1 infection that serve as pre-clinical gatekeepers for vaccine candidates and potential therapeutic interventions. Overall, the results presented in this thesis have identified key antigenic changes and viral determinants that have been selected during the process of adapting SHIVs for replication in macaques. Many selective pressures, including but not limited to species-specific differences in the CD4 receptor and the innate immune response, have shaped the properties of challenge viruses that are capable of causing persistent infection in macaques, and the HIV-1 envelope protein plays a critical role in overcoming potential barriers to infection. Given that HIV-1 plays an important role in the adaption process and is the target of the adaptive immune responses, development of improved challenge viruses will likely require balancing changes that increase replication capacity in macaques with those that affect the antigenic properties of the virus. A systematic approach to the identification of changes that allow increase replication capacity of SHIVs in macaques and of host factors that promote or inhibit viral replication will provide a blueprint for the rational design of improved challenge viruses. Improved understanding of the basic biology of HIV-1 replication in macaques will inform the mechanisms of cross-species transmissions of retroviruses and has direct application to the development of more clinically relevant animal models of HIV-1 infection.

References

1. Hahn BH, Shaw GM, De Cock KM, Sharp PM. AIDS as a zoonosis: scientific and public health implications. Science. 2000;287: 607–614.

2. D'arc M, Ayouba A, Esteban A, Learn GH, Boué V, Liegeois F, et al. Origin of the HIV-1 group O epidemic in western lowland gorillas. Proc Natl Acad Sci USA. 2015;112: E1343–52. doi:10.1073/pnas.1502022112

3. Sharp PM, hahn BH. Origins of HIV and the AIDS pandemic. Cold Spring Harb Perspect Med. 2011;1: a006841. doi:10.1101/cshperspect.a006841

4. Zhu T, Korber BT, Nahmias AJ, Hooper E, Sharp PM, Ho DD. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. Nature. 1998;391: 594–597. doi:10.1038/35400

5. Gao F, Bailes E, Robertson DL, Chen Y, Rodenburg CM, Michael SF, et al. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature. 1999;397: 436–441. doi:10.1038/17130

6. Hemelaar J, Gouws E, Ghys PD, Osmanov S, WHO-UNAIDS Network for HIV Isolation and Characterisation. Global trends in molecular epidemiology of HIV-1 during 2000-2007. AIDS. 2011;25: 679–689. doi:10.1097/QAD.0b013e328342ff93

7. Beer BE, Bailes E, Sharp PM, Hirsch VM. Diversity and Evolution of Primate Lentiviruses. Kuiken CL, Foley B, Hahn B, Korber B, McCutchan F, Marx PA, et al., editors. Human Retroviruses and AIDS 1999. Los Alamos, NM; 1999;: 460–474.

8. Lemey P, Pybus OG, Wang B, Saksena NK, Salemi M, Vandamme A-M. Tracing the origin and history of the HIV-2 epidemic. Proc Natl Acad Sci USA. 2003;100: 6588–6592. doi:10.1073/pnas.0936469100

9. Foley B, Leiter T, Apetrei C, hahn BH, Mizrachi I, Mullins J, et al. HIV Sequence Compendium 2014. Los Alamos National Laboratory, Theoretical Biology and Biophysics. Los Alams, New Mexico; 2014;LA-UR-14-26717: 1–145.

10. Freed EO. HIV-1 replication. Somat Cell Mol Genet. 2001;26: 13–33.

11. Checkley MA, Luttge BG, Freed EO. HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. J Mol Biol. 2011;410: 582–608. doi:10.1016/j.jmb.2011.04.042

12. Wyatt R, Kwong PD, Desjardins E, Sweet RW, Robinson J, Hendrickson WA, et al. The antigenic structure of the HIV gp120 envelope glycoprotein. Nature. 1998;393: 705–711. doi:10.1038/31514

13. Yamashita M, Emerman M. Retroviral infection of non-dividing cells: old and new

perspectives. Virology. 2006;344: 88–93. doi:10.1016/j.virol.2005.09.012

14. Ayinde D, Maudet C, Transy C, Margottin-Goguet F. Limelight on two HIV/SIV accessory proteins in macrophage infection: is Vpx overshadowing Vpr? Retrovirology. 2010;7: 35. doi:10.1186/1742-4690-7-35

15. Chen B, Vogan EM, Gong H, Skehel JJ, Wiley DC, Harrison SC. Structure of an unliganded simian immunodeficiency virus gp120 core. Nature. 2005;433: 834–841. doi:10.1038/nature03327

16. Rizzuto CD, Wyatt R, Hernández-Ramos N, Sun Y, Kwong PD, Hendrickson WA, et al. A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding. Science. 1998;280: 1949–1953.

17. Tran EEH, Borgnia MJ, Kuybeda O, Schauder DM, Bartesaghi A, Frank GA, et al. Structural mechanism of trimeric HIV-1 envelope glycoprotein activation. PLoS Pathog. 2012;8: e1002797. doi:10.1371/journal.ppat.1002797

18. Arhel N. Revisiting HIV-1 uncoating. Retrovirology. 2010;7: 96. doi:10.1186/1742-4690-7-96

19. Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature. 1995;373: 123–126. doi:10.1038/373123a0

20. Suzuki Y, Craigie R. The road to chromatin — nuclear entry of retroviruses. Nature Publishing Group. 2007;5: 187–196. doi:10.1038/nrmicro1579

21. Llano M, Morrison J, Poeschla EM. Virological and cellular roles of the transcriptional coactivator LEDGF/p75. Curr Top Microbiol Immunol. 2009;339: 125–146. doi:10.1007/978-3-642-02175-6_7

22. Craigie R, Bushman FD. HIV DNA integration. Cold Spring Harb Perspect Med. 2012;2: a006890. doi:10.1101/cshperspect.a006890

23. Nabel G, Baltimore D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. Nature. 1987;326: 711–713. doi:10.1038/326711a0

24. Wei P, Garber ME, Fang SM, Fischer WH, Jones KA. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell. 1998;92: 451–462.

25. Purcell DF, Martin MA. Alternative splicing of human immunodeficiency virus type 1 mRNA modulates viral protein expression, replication, and infectivity. Journal of Virology. 1993;67: 6365–6378.

26. Bell NM, Lever AML. HIV Gag polyprotein: processing and early viral particle assembly. Trends in Microbiology. 2013;21: 136–144. doi:10.1016/j.tim.2012.11.006

27. Doyle T, Goujon C, Malim MH. HIV-1 and interferons: who's interfering with whom? Nature Publishing Group. 2015. doi:10.1038/nrmicro3449

28. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. Nature. 2004;427: 848–853. doi:10.1038/nature02343

29. Sheehy AM, Gaddis NC, Choi JD, Malim MH. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature. 2002;418: 646–650. doi:10.1038/nature00939

30. Bishop KN, Holmes RK, Sheehy AM, Malim MH. APOBEC-mediated editing of viral RNA. Science. 2004;305: 645. doi:10.1126/science.1100658

31. Laguette N, Sobhian B, Casartelli N, Ringeard M, Chable-Bessia C, Ségéral E, et al. SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. Nature. 2011;474: 654–657. doi:10.1038/nature10117

32. Goldstone DC, Ennis-Adeniran V, Hedden JJ, Groom HCT, Rice GI, Christodoulou E, et al. HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase. Nature. Nature Publishing Group; 2011;480: 379–382. doi:10.1038/nature10623

33. Neil SJD, Zang T, Bieniasz PD. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature. 2008;451: 425–430. doi:10.1038/nature06553

34. Lim ES, Fregoso OI, McCoy CO, Matsen FA, Malik HS, Emerman M. The ability of primate lentiviruses to degrade the monocyte restriction factor SAMHD1 preceded the birth of the viral accessory protein Vpx. Cell Host and Microbe. 2012;11: 194–204. doi:10.1016/j.chom.2012.01.004

35. Fregoso OI, Ahn J, Wang C, Mehrens J, Skowronski J, Emerman M. Evolutionary Toggling of Vpx/Vpr Specificity Results in Divergent Recognition of the Restriction Factor SAMHD1. PLoS Pathog. 2013;9: e1003496. doi:10.1371/journal.ppat.1003496

36. Zhang F, Wilson SJ, Landford WC, Virgen B, Gregory D, Johnson MC, et al. Nef Proteins from Simian Immunodeficiency Viruses Are Tetherin Antagonists. Cell Host and Microbe. Elsevier Inc; 2009;6: 54–67. doi:10.1016/j.chom.2009.05.008

37. Sauter D, Schindler M, Specht A, Landford WN, MUnch J, Kim K-A, et al. Tetherin-Driven Adaptation of Vpu and Nef Function and the Evolution of Pandemic and Nonpandemic HIV-1 Strains. Cell Host and Microbe. Elsevier Ltd; 2009;6: 409–421. doi:10.1016/j.chom.2009.10.004

38. Li M, Kao E, Gao X, Sandig H, Limmer K, Pavon-Eternod M, et al. Codon-usage-based inhibition of HIV protein synthesis by human schlafen 11. Nature. 2012;491: 125–128. doi:10.1038/nature11433

39. Goujon C, Moncorgé O, Bauby H, Doyle T, Ward CC, Schaller T, et al. Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. Nature. 2013;502: 559–562. doi:10.1038/nature12542

40. Kane M, Yadav SS, Bitzegeio J, Kutluay SB, Zang T, Wilson SJ, et al. MX2 is an interferon-induced inhibitor of HIV-1 infection. Nature. 2013. doi:10.1038/nature12653

41. Liu Z, Pan Q, Ding S, Qian J, Xu F, Zhou J, et al. The interferon-inducible MxB protein inhibits HIV-1 infection. Cell Host and Microbe. 2013;14: 398–410. doi:10.1016/j.chom.2013.08.015

42. Lu J, Pan Q, Rong L, He W, Liu S-L, Liang C. The IFITM proteins inhibit HIV-1 infection. Journal of Virology. 2011;85: 2126–2137. doi:10.1128/JVI.01531-10

43. Compton AA, Bruel T, Porrot F, Mallet A, Sachse M, Euvrard M, et al. IFITM Proteins Incorporated into HIV-1 Virions Impair Viral Fusion and Spread. Cell Host and Microbe. 2014. doi:10.1016/j.chom.2014.11.001

44. Pillai SK, Abdel-Mohsen M, Guatelli J, Skasko M, Monto A, Fujimoto K, et al. Role of retroviral restriction factors in the interferon-α-mediated suppression of HIV-1 in vivo. Proc Natl Acad Sci USA. 2012;109: 3035–3040. doi:10.1073/pnas.1111573109

45. Sandler NG, Bosinger SE, Estes JD, Zhu RTR, Tharp GK, Boritz E, et al. Type I interferon responses in rhesus macaques prevent SIV infection and slow disease progression. Nature. 2014;511: 601–605. doi:10.1038/nature13554

46. Whitney JB, Hill AL, Sanisetty S, Penaloza-MacMaster P, Liu J, Shetty M, et al. Rapid seeding of the viral reservoir prior to SIV viraemia in rhesus monkeys. Nature. 2014;512: 74–77. doi:10.1038/nature13594

47. Little SJ, McLean AR, Spina CA, Richman DD, Havlir DV. Viral dynamics of acute HIV-1 infection. J Exp Med. 1999;190: 841–850.

48. Stacey AR, Norris PJ, Qin L, Haygreen EA, Taylor E, Heitman J, et al. Induction of a Striking Systemic Cytokine Cascade prior to Peak Viremia in Acute Human Immunodeficiency Virus Type 1 Infection, in Contrast to More Modest and Delayed Responses in Acute Hepatitis B and C Virus Infections. Journal of Virology. 2009;83: 3719–3733. doi:10.1128/JVI.01844-08

49. Jacquelin B, Mayau V, Targat B, Liovat A-S, Kunkel D, Petitjean G, et al. Nonpathogenic SIV infection of African green monkeys induces a strong but rapidly controlled type I IFN response. J Clin Invest. 2009. doi:10.1172/JCI40093DS1

50. Graziosi C, Pantaleo G, Butini L, Demarest JF, Saag MS, Shaw GM, et al. Kinetics of human immunodeficiency virus type 1 (HIV-1) DNA and RNA synthesis during primary HIV-1 infection. Proc Natl Acad Sci USA. 1993;90: 6405–6409.

51. Borrow P, Lewicki H, hahn BH, Shaw GM, Oldstone MB. Virus-specific CD8+

cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. Journal of Virology. 1994;68: 6103–6110.

52. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, Borkowsky W, et al. Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. Journal of Virology. 1994;68: 4650–4655.

53. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, Blanchard J, et al. Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med. 1999;189: 991–998.

54. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, Lifton MA, et al. Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science. 1999;283: 857–860.

55. Forthal D, Hope TJ, Alter G. New paradigms for functional HIV-specific nonneutralizing antibodies. Current Opinion in HIV and AIDS. 2013;8: 392–400. doi:10.1097/COH.0b013e328363d486

56. Albert J, Abrahamsson B, Nagy K, Aurelius E, Gaines H, Nyström G, et al. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. AIDS. 1990;4: 107–112.

57. Richman DD, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci USA. 2003;100: 4144–4149. doi:10.1073/pnas.0630530100

58. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, et al. Antibody neutralization and escape by HIV-1. Nature. 2003;422: 307–312. doi:10.1038/nature01470

59. Davis KL, Gray ES, Moore PL, Decker JM, Salomon A, Montefiori DC, et al. High titer HIV-1 V3-specific antibodies with broad reactivity but low neutralizing potency in acute infection and following vaccination. Virology. 2009;387: 414–426. doi:10.1016/j.virol.2009.02.022

60. Munro JB, Gorman J, Ma X, Zhou Z, Arthos J, Burton DR, et al. Conformational dynamics of single HIV-1 envelope trimers on the surface of native virions. Science. 2014. doi:10.1126/science.1254426

61. Goo L, Milligan C, Simonich CA, Nduati R, Overbaugh J. Neutralizing antibody escape during HIV-1 mother-to-child transmission involves conformational masking of distal epitopes in envelope. Journal of Virology. 2012;86: 9566–9582. doi:10.1128/JVI.00953-12

62. Leonard CK, Spellman MW, Riddle L, Harris RJ, Thomas JN, Gregory TJ. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the

type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J Biol Chem. 1990;265: 10373–10382.

63. Doores KJ, Burton DR. Variable loop glycan dependency of the broad and potent HIV-1-neutralizing antibodies PG9 and PG16. Journal of Virology. 2010;84: 10510–10521. doi:10.1128/JVI.00552-10

64. Mouquet H, Scharf L, Euler Z, Liu Y, Eden C, Scheid JF, et al. Complex-type N-glycan recognition by potent broadly neutralizing HIV antibodies. Proc Natl Acad Sci USA. 2012;109: E3268–77. doi:10.1073/pnas.1217207109

65. Sather DN, Armann J, Ching LK, Mavrantoni A, Sellhorn G, Caldwell Z, et al. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. Journal of Virology. 2009;83: 757–769. doi:10.1128/JVI.02036-08

66. Simek MD, Rida W, Priddy FH, Pung P, Carrow E, Laufer DS, et al. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. Journal of Virology. 2009;83: 7337–7348. doi:10.1128/JVI.00110-09

67. Doria-Rose NA, Klein RM, Daniels MG, O'Dell S, Nason M, Lapedes A, et al. Breadth of human immunodeficiency virus-specific neutralizing activity in sera: clustering analysis and association with clinical variables. Journal of Virology. 2010;84: 1631–1636. doi:10.1128/JVI.01482-09

68. Gray ES, Madiga MC, Hermanus T, Moore PL, Wibmer CK, Tumba NL, et al. The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. Journal of Virology. 2011;85: 4828–4840. doi:10.1128/JVI.00198-11

69. Mikell I, Sather DN, Kalams SA, Altfeld M, Alter G, Stamatatos L. Characteristics of the earliest cross-neutralizing antibody response to HIV-1. PLoS Pathog. 2011;7: e1001251. doi:10.1371/journal.ppat.1001251

70. Hessell AJ, Poignard P, Hunter M, Hangartner L, Tehrani DM, Bleeker WK, et al. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nat Med. 2009;15: 951–954. doi:10.1038/nm.1974

71. Hessell AJ, Rakasz EG, Poignard P, Hangartner L, Landucci G, Forthal DN, et al. Broadly Neutralizing Human Anti-HIV Antibody 2G12 Is Effective in Protection against Mucosal SHIV Challenge Even at Low Serum Neutralizing Titers. Farzan M, editor. PLoS Pathog. 2009;5: e1000433. doi:10.1371/journal.ppat.1000433.t004

72. Shingai M, Donau OK, Plishka RJ, Buckler-White A, Mascola JR, Nabel GJ, et al. Passive transfer of modest titers of potent and broadly neutralizing anti-HIV monoclonal antibodies block SHIV infection in macaques. J Exp Med. 2014;211: 2061–2074.

doi:10.1084/jem.20132494

73. Barouch DH, Whitney JB, Moldt B, Klein F, Oliveira TY, Liu J, et al. Therapeutic efficacy of potent neutralizing HIV-1-specific monoclonal antibodies in SHIV-infected rhesus monkeys. Nature. 2013. doi:10.1038/nature12744

74. Shingai M, Nishimura Y, Klein F, Mouquet H, Donau OK, Plishka R, et al. Antibody-mediated immunotherapy of macaques chronically infected with SHIV suppresses viraemia. Nature. 2013. doi:10.1038/nature12746

75. West AP, Scharf L, Scheid JF, Klein F, Bjorkman PJ, Nussenzweig MC. Structural insights on the role of antibodies in HIV-1 vaccine and therapy. Cell. 2014;156: 633–648. doi:10.1016/j.cell.2014.01.052

76. Royce RA, Seña A, Cates W, Cohen MS. Sexual transmission of HIV. N Engl J Med. 1997;336: 1072–1078. doi:10.1056/NEJM199704103361507

77. Lehman DA, Farquhar C. Biological mechanisms of vertical human immunodeficiency virus (HIV-1) transmission. Rev Med Virol. 2007;17: 381–403. doi:10.1002/rmv.543

78. Wolfs TF, Zwart G, Bakker M, Goudsmit J. HIV-1 genomic RNA diversification following sexual and parenteral virus transmission. Virology. 1992;189: 103–110.

79. Wolinsky SM, Wike CM, Korber BT, Hutto C, Parks WP, Rosenblum LL, et al. Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. Science. 1992;255: 1134–1137.

80. Zhu T, Mo H, Wang N, Nam DS, Cao Y, Koup RA, et al. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. Science. 1993;261: 1179–1181.

81. Poss M, Martin HL, Kreiss JK, Granville L, Chohan B, Nyange P, et al. Diversity in virus populations from genital secretions and peripheral blood from women recently infected with human immunodeficiency virus type 1. Journal of Virology. 1995;69: 8118–8122.

82. Long EM, Martin HL, Kreiss JK, Rainwater SM, Lavreys L, Jackson DJ, et al. Gender differences in HIV-1 diversity at time of infection. Nat Med. 2000;6: 71–75. doi:10.1038/71563

83. Sagar M, Lavreys L, Baeten JM, Richardson BA, Mandaliya K, Ndinya-Achola JO, et al. Identification of modifiable factors that affect the genetic diversity of the transmitted HIV-1 population. AIDS. 2004;18: 615–619.

84. Sagar M, Kirkegaard E, Long EM, Celum C, Buchbinder S, Daar ES, et al. Human immunodeficiency virus type 1 (HIV-1) diversity at time of infection is not restricted to certain risk groups or specific HIV-1 subtypes. Journal of Virology. 2004;78: 7279–7283. doi:10.1128/JVI.78.13.7279-7283.2004

85. Haaland RE, Hawkins PA, Salazar-Gonzalez J, Johnson A, Tichacek A, Karita E, et al. Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1. PLoS Pathog. 2009;5: e1000274. doi:10.1371/journal.ppat.1000274

86. Derdeyn CA, Decker JM, Bibollet-Ruche F, Mokili JL, Muldoon M, Denham SA, et al. Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. Science. 2004;303: 2019–2022. doi:10.1126/science.1093137

87. Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, et al. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc Natl Acad Sci USA. 2008;105: 7552–7557. doi:10.1073/pnas.0802203105

88. Long EM, Rainwater SMJ, Lavreys L, Mandaliya K, Overbaugh J. HIV type 1 variants transmitted to women in Kenya require the CCR5 coreceptor for entry, regardless of the genetic complexity of the infecting virus. AIDS Res Hum Retroviruses. 2002;18: 567–576. doi:10.1089/088922202753747914

89. Hu Q, Huang X, Shattock RJ. C-C chemokine receptor type 5 (CCR5) utilization of transmitted and early founder human immunodeficiency virus type 1 envelopes and sensitivity to small-molecule CCR5 inhibitors. J Gen Virol. 2010;91: 2965–2973. doi:10.1099/vir.0.025270-0

90. Parker ZF, Iyer SS, Wilen CB, Parrish NF, Chikere KC, Lee F-H, et al. Transmitted/founder and chronic HIV-1 envelope proteins are distinguished by differential utilization of CCR5. Journal of Virology. 2013;87: 2401–2411. doi:10.1128/JVI.02964-12

91. Chohan B, Lang D, Sagar M, Korber B, Lavreys L, Richardson B, et al. Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels. Journal of Virology. 2005;79: 6528–6531. doi:10.1128/JVI.79.10.6528-6531.2005

92. Go EP, Hewawasam G, Liao H-X, Chen H, Ping L-H, Anderson JA, et al. Characterization of glycosylation profiles of HIV-1 transmitted/founder envelopes by mass spectrometry. Journal of Virology. 2011;85: 8270–8284. doi:10.1128/JVI.05053-11

93. Ochsenbauer C, Edmonds TG, Ding H, Keele BF, Decker J, Salazar MG, et al. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. Journal of Virology. 2012;86: 2715–2728. doi:10.1128/JVI.06157-11

94. Parrish NF, Gao F, Li H, Giorgi EE, Barbian HJ, Parrish EH, et al. Phenotypic properties of transmitted founder HIV-1. Proc Natl Acad Sci USA. 2013;110: 6626–6633.

doi:10.1073/pnas.1304288110

95. Fenton-May AE, Dibben O, Emmerich T, Ding H, Pfafferott K, Aasa-Chapman MM, et al. Relative resistance of HIV-1 founder viruses to control by interferon-alpha. Retrovirology. 2013;10: 146. doi:10.1186/1742-4690-10-146

96. Chahroudi A, Bosinger SE, Vanderford TH, Paiardini M, Silvestri G. Natural SIV hosts: showing AIDS the door. Science. 2012;335: 1188–1193. doi:10.1126/science.1217550

97. Kestler H, Kodama T, Ringler D, Marthas M, Pedersen N, Lackner A, et al. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science. 1990;248: 1109–1112.

98. Hatziioannou T, Evans DT. Animal models for HIV/AIDS research. Nature Publishing Group. 2012;10: 852–867. doi:10.1038/nrmicro2911

99. Brennan G, Kozyrev Y, Kodama T, Hu SL. Novel TRIM5 Isoforms Expressed by Macaca nemestrina. Journal of Virology. 2007;81: 12210–12217. doi:10.1128/JVI.02499-06

100. Brennan G, Kozyrev Y, Hu S-L. TRIMCyp expression in Old World primates Macaca nemestrina and Macaca fascicularis. Proc Natl Acad Sci USA. 2008;105: 3569–3574. doi:10.1073/pnas.0709511105

101. Pollom E, Dang KK, Potter EL, Gorelick RJ, Burch CL, Weeks KM, et al. Comparison of SIV and HIV-1 genomic RNA structures reveals impact of sequence evolution on conserved and non-conserved structural motifs. PLoS Pathog. 2013;9: e1003294. doi:10.1371/journal.ppat.1003294

102. Choe H, Martin KA, Farzan M, Sodroski J, Gerard NP, Gerard C. Structural interactions between chemokine receptors, gp120 Env and CD4. Semin Immunol. 1998;10: 249–257.

103. Sina ST, Ren W, Cheng-Mayer C. Coreceptor use in nonhuman primate models of HIV infection. J Transl Med. 2011;9 Suppl 1: S7. doi:10.1186/1479-5876-9-S1-S7

104. Karlsson GB, Halloran M, Li J, Park IW, Gomila R, Reimann KA, et al. Characterization of molecularly cloned simian-human immunodeficiency viruses causing rapid CD4+ lymphocyte depletion in rhesus monkeys. Journal of Virology. 1997;71: 4218–4225.

105. Karlsson GB, Halloran M, Schenten D, Lee J, Racz P, Tenner-Racz K, et al. The envelope glycoprotein ectodomains determine the efficiency of CD4+ T lymphocyte depletion in simian-human immunodeficiency virus-infected macaques. J Exp Med. 1998;188: 1159–1171.

106. Hsu M, Harouse JM, Gettie A, Buckner C, Blanchard J, Cheng-Mayer C. Increased Mucosal Transmission but Not Enhanced Pathogenicity of the CCR5-Tropic, Simian AIDS-Inducing Simian/Human Immunodeficiency Virus SHIVSF162P3 Maps to

Envelope gp120. Journal of Virology. 2003;77: 989–998. doi:10.1128/JVI.77.2.989-998.2003

107. Etemad-Moghadam B, Sun Y, Nicholson EK, Karlsson GB, Schenten D, Sodroski J. Determinants of neutralization resistance in the envelope glycoproteins of a simian-human immunodeficiency virus passaged in vivo. Journal of Virology. 1999;73: 8873–8879.

108. Gautam R, Nishimura Y, Lee WR, Donau O, Buckler-White A, Shingai M, et al. Pathogenicity and mucosal transmissibility of the R5-tropic simian/human immunodeficiency virus SHIV(AD8) in rhesus macaques: implications for use in vaccine studies. Journal of Virology. 2012;86: 8516–8526. doi:10.1128/JVI.00644-12

109. Ren W, Mumbauer A, Gettie A, Seaman MS, Russell-Lodrigue K, Blanchard J, et al. Generation of Lineage-Related, Mucosally Transmissible Subtype C R5 Simian-Human Immunodeficiency Viruses Capable of AIDS Development, Induction of Neurological Disease, and Coreceptor Switching in Rhesus Macaques. Journal of Virology. 2013;87: 6137–6149. doi:10.1128/JVI.00178-13

110. Harouse JM. Distinct Pathogenic Sequela in Rhesus Macaques Infected with CCR5 or CXCR4 Utilizing SHIVs. Science. 1999;284: 816–819. doi:10.1126/science.284.5415.816

111. Song RJ, Chenine AL, Rasmussen RA, Ruprecht CR, Mirshahidi S, Grisson RD, et al. Molecularly Cloned SHIV-1157ipd3N4: a Highly Replication- Competent, Mucosally Transmissible R5 Simian-Human Immunodeficiency Virus Encoding HIV Clade C env. Journal of Virology. 2006;80: 8729–8738. doi:10.1128/JVI.00558-06

112. Shingai M, Donau OK, Schmidt SD, Gautam R, Plishka RJ, Buckler-White A, et al. Most rhesus macaques infected with the CCR5-tropic SHIV(AD8) generate cross-reactive antibodies that neutralize multiple HIV-1 strains. Proc Natl Acad Sci USA. 2012;109: 19769–19774. doi:10.1073/pnas.1217443109

113. Moore JP, Kitchen SG, Pugach P, Zack JA. The CCR5 and CXCR4 coreceptors--central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses. 2004;20: 111–126. doi:10.1089/088922204322749567

114. Collman R, Balliet JW, Gregory SA, Friedman H, Kolson DL, Nathanson N, et al. An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. Journal of Virology. 1992;66: 7517–7521.

115. Reimann KA, Li JT, Voss G, Lekutis C, Tenner-Racz K, Racz P, et al. An env gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. Journal of Virology. 1996;70: 3198–3206.

116. Reimann KA, Li JT, Veazey R, Halloran M, Park IW, Karlsson GB, et al. A chimeric

simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. Journal of Virology. 1996;70: 6922–6928.

117. Barouch DH, Santra S, Schmitz JE, Kuroda MJ, Fu TM, Wagner W, et al. Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science. 2000;290: 486–492.

118. Shiver JW, Fu T-M, Chen L, Casimiro DR, Davies M-E, Evans RK, et al. Replication-incompetent adenoviral vaccine vector elicits effective anti-immunodeficiency-virus immunity. Nature. 2002;415: 331–335. doi:10.1038/415331a

119. Fauci AS, Johnston MI, Dieffenbach CW, Burton DR, Hammer SM, Hoxie JA, et al. HIV vaccine research: the way forward. Science. 2008;321: 530–532. doi:10.1126/science.1161000

120. Cheng-Mayer C, Weiss C, Seto D, Levy JA. Isolates of human immunodeficiency virus type 1 from the brain may constitute a special group of the AIDS virus. Proc Natl Acad Sci USA. 1989;86: 8575–8579.

121. Seaman MS, Janes H, Hawkins N, Grandpre LE, Devoy C, Giri A, et al. Tiered Categorization of a Diverse Panel of HIV-1 Env Pseudoviruses for Assessment of Neutralizing Antibodies. Journal of Virology. 2010;84: 1439–1452. doi:10.1128/JVI.02108-09

122. Harouse JM, Gettie A, Eshetu T, Tan RCH, Bohm R, Blanchard J, et al. Mucosal Transmission and Induction of Simian AIDS by CCR5-Specific Simian/Human Immunodeficiency Virus SHIVSF162P3. Journal of Virology. 2001;75: 1990–1995. doi:10.1128/JVI.75.4.1990-1995.2001

123. Hatziioannou T, Ambrose Z, Chung NPY, Piatak M, Yuan F, Trubey CM, et al. A macaque model of HIV-1 infection. Proc Natl Acad Sci USA. 2009;106: 4425–4429. doi:10.1073/pnas.0812587106

124. Thippeshappa R, Polacino P, Yu Kimata MT, Siwak EB, Anderson D, Wang W, et al. Vif substitution enables persistent infection of pig-tailed macaques by human immunodeficiency virus type 1. Journal of Virology. 2011;85: 3767–3779. doi:10.1128/JVI.02438-10

125. Hatziioannou T, Del Prete GQ, Keele BF, Estes JD, McNatt MW, Bitzegeio J, et al. HIV-1-induced AIDS in monkeys. Science. 2014;344: 1401–1405. doi:10.1126/science.1250761

126. Del Prete GQ, Ailers B, Moldt B, Keele BF, Estes JD, Rodriguez A, et al. Selection of Unadapted, Pathogenic SHIVsEncoding Newly Transmitted HIV-1 Envelope Proteins. Cell Host and Microbe. Elsevier Inc; 2014;16: 412–418. doi:10.1016/j.chom.2014.08.003

127. Asmal M, Luedemann C, Lavine CL, Mach LV, Balachandran H, Brinkley C, et al. Infection of monkeys by simian-human immunodeficiency viruses with transmitted/founder clade C HIV-1 envelopes. Virology. 2014;475C: 37–45. doi:10.1016/j.virol.2014.10.032

128. Nishimura Y, Shingai M, Willey R, Sadjadpour R, Lee WR, Brown CR, et al. Generation of the Pathogenic R5-Tropic Simian/Human Immunodeficiency Virus SHIVAD8 by Serial Passaging in Rhesus Macaques. Journal of Virology. 2010;84: 4769–4781. doi:10.1128/JVI.02279-09

129. Joag SV, Li Z, Foresman L, Stephens EB, Zhao LJ, Adany I, et al. Chimeric simian/human immunodeficiency virus that causes progressive loss of CD4+ T cells and AIDS in pig-tailed macaques. Journal of Virology. 1996;70: 3189–3197.

130. Li JT, Halloran M, Lord CI, Watson A, Ranchalis J, Fung M, et al. Persistent infection of macaques with simian-human immunodeficiency viruses. Journal of Virology. 1995;69: 7061–7067.

131. Luciw PA, Pratt-Lowe E, Shaw KE, Levy JA, Cheng-Mayer C. Persistent infection of rhesus macaques with T-cell-line-tropic and macrophage-tropic clones of simian/human immunodeficiency viruses (SHIV). Proc Natl Acad Sci USA. 1995;92: 7490–7494.

132. Humbert M, Rasmussen RA, Song R, Ong H, Sharma P, Chenine AL, et al. SHIV-1157i and passaged progeny viruses encoding R5 HIV-1 clade C env cause AIDS in rhesus monkeys. Retrovirology. 2008;5: 94. doi:10.1186/1742-4690-5-94

133. Etemad-Moghadam B, Karlsson GB, Halloran M, Sun Y, Schenten D, Fernandes M, et al. Characterization of simian-human immunodeficiency virus envelope glycoprotein epitopes recognized by neutralizing antibodies from infected monkeys. Journal of Virology. 1998;72: 8437–8445.

134. Cayabyab M, Karlsson GB, Etemad-Moghadam BA, Hofmann W, Steenbeke T, Halloran M, et al. Changes in human immunodeficiency virus type 1 envelope glycoproteins responsible for the pathogenicity of a multiply passaged simian-human immunodeficiency virus (SHIV-HXBc2). Journal of Virology. 1999;73: 976–984.

135. Etemad-Moghadam B, Sun Y, Nicholson EK, Fernandes M, Liou K, Gomila R, et al. Envelope glycoprotein determinants of increased fusogenicity in a pathogenic simian-human immunodeficiency virus (SHIV-KB9) passaged in vivo. Journal of Virology. 2000;74: 4433–4440.

136. Humes D, Overbaugh J. Adaptation of Subtype A Human Immunodeficiency Virus Type 1 Envelope to Pig-Tailed Macaque Cells. Journal of Virology. 2011;85: 4409–4420. doi:10.1128/JVI.02244-10

137. Humes D, Emery S, Laws E, Overbaugh J. A Species-Specific Amino Acid Difference in the Macaque CD4 Receptor Restricts Replication by Global Circulating HIV-1 Variants Representing Viruses from Recent Infection. Journal of Virology. 2012;86:

12472–12483. doi:10.1128/JVI.02176-12

138. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien J-P, et al. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature. Nature Publishing Group; 2011;477: 466–470. doi:10.1038/nature10373

139. Wu X, Yang ZY, Li Y, Hogerkorp CM, Schief WR, Seaman MS, et al. Rational Design of Envelope Identifies Broadly Neutralizing Human Monoclonal Antibodies to HIV-1. Science. 2010;329: 856–861. doi:10.1126/science.1187659

140. Walker LM, Phogat SK, Chan-Hui PY, Wagner D, Phung P, Goss JL, et al. Broad and Potent Neutralizing Antibodies from an African Donor Reveal a New HIV-1 Vaccine Target. Science. 2009;326: 285–289. doi:10.1126/science.1178746

141. Huang J, Ofek G, Laub L, Louder MK, Doria-Rose NA, Longo NS, et al. Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. Nature. Nature Publishing Group; 2012;491: 406–412. doi:10.1038/nature11544

142. Lyumkis D, Julien J-P, de Val N, Cupo A, Potter CS, Klasse PJ, et al. Cryo-EM Structure of a Fully Glycosylated Soluble Cleaved HIV-1 Envelope Trimer. Science. 2013. doi:10.1126/science.1245627

143. Julien J-P, Lee JH, Cupo A, Murin CD, Derking R, Hoffenberg S, et al. Asymmetric recognition of the HIV-1 trimer by broadly neutralizing antibody PG9. Proc Natl Acad Sci USA. 2013;110: 4351–4356. doi:10.1073/pnas.1217537110

144. Bosch KA, Rainwater S, Jaoko W, Overbaugh J. Temporal analysis of HIV envelope sequence evolution and antibody escape in a subtype A-infected individual with a broad neutralizing antibody response. Virology. 2010;398: 115–124. doi:10.1016/j.virol.2009.11.032

145. Boyd DF, Peterson D, Haggarty BS, Jordan APO, Hogan MJ, Goo L, et al. Mutations in HIV-1 Envelope That Enhance Entry with the Macaque CD4 Receptor Alter Antibody Recognition by Disrupting Quaternary Interactions within the Trimer. Journal of Virology. 2015;89: 894–907. doi:10.1128/JVI.02680-14

146. Blish CA, Jalalian-Lechak Z, Rainwater S, Nguyen M-A, Dogan OC, Overbaugh J. Cross-subtype neutralization sensitivity despite monoclonal antibody resistance among early subtype A, C, and D envelope variants of human immunodeficiency virus type 1. Journal of Virology. 2009;83: 7783–7788. doi:10.1128/JVI.00673-09

147. Goo L, Jalalian-Lechak Z, Richardson BA, Overbaugh J. A combination of broadly neutralizing HIV-1 monoclonal antibodies targeting distinct epitopes effectively neutralizes variants found in early infection. Journal of Virology. 2012;86: 10857–10861. doi:10.1128/JVI.01414-12

148. Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, et al. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for

standardized assessments of vaccine-elicited neutralizing antibodies. Journal of Virology. 2005;79: 10108–10125. doi:10.1128/JVI.79.16.10108-10125.2005

149. Li M, Salazar-Gonzalez JF, Derdeyn CA, Morris L, Williamson C, Robinson JE, et al. Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular env clones from acute and early heterosexually acquired infections in Southern Africa. Journal of Virology. 2006;80: 11776–11790. doi:10.1128/JVI.01730-06

150. Wu X, Parast AB, Richardson BA, Nduati R, John-Stewart G, Mbori-Ngacha D, et al. Neutralization escape variants of human immunodeficiency virus type 1 are transmitted from mother to infant. Journal of Virology. 2006;80: 835–844. doi:10.1128/JVI.80.2.835-844.2006

151. Muñoz NM, Trobridge GD, Kiem H-P. Ex vivoexpansion and lentiviral transduction of Macaca nemestrinaCD4 +T cells. Journal of Medical Primatology. 2009;38: 438–443. doi:10.1111/j.1600-0684.2009.00383.x

152. Gorny MK, Xu JY, Karwowska S, Buchbinder A, Zolla-Pazner S. Repertoire of neutralizing human monoclonal antibodies specific for the V3 domain of HIV-1 gp120. J Immunol. 1993;150: 635–643.

153. Gorny MK, Moore JP, Conley AJ, Karwowska S, Sodroski J, Williams C, et al. Human anti-V2 monoclonal antibody that neutralizes primary but not laboratory isolates of human immunodeficiency virus type 1. Journal of Virology. 1994;68: 8312–8320.

154. Gorny MK, Williams C, Volsky B, Revesz K, Cohen S, Polonis VR, et al. Human monoclonal antibodies specific for conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. Journal of Virology. 2002;76: 9035–9045.

155. Gorny MK, Williams C, Volsky B, Revesz K, Wang X-H, Burda S, et al. Cross-clade neutralizing activity of human anti-V3 monoclonal antibodies derived from the cells of individuals infected with non-B clades of human immunodeficiency virus type 1. Journal of Virology. 2006;80: 6865–6872. doi:10.1128/JVI.02202-05

156. Gorny MK, Wang X-H, Williams C, Volsky B, Revesz K, Witover B, et al. Preferential use of the VH5-51 gene segment by the human immune response to code for antibodies against the V3 domain of HIV-1. Molecular Immunology. 2009;46: 917–926. doi:10.1016/j.molimm.2008.09.005

157. Gorny MK, Revesz K, Williams C, Volsky B, Louder MK, Anyangwe CA, et al. The v3 loop is accessible on the surface of most human immunodeficiency virus type 1 primary isolates and serves as a neutralization epitope. Journal of Virology. 2004;78: 2394–2404.

158. Mouquet H, Klein F, Scheid JF, Warncke M, Pietzsch J, Oliveira TYK, et al. Memory B cell antibodies to HIV-1 gp140 cloned from individuals infected with clade A and B viruses. PLoS ONE. 2011;6: e24078. doi:10.1371/journal.pone.0024078

159. Scheid JF, Mouquet H, Feldhahn N, Seaman MS, Velinzon K, Pietzsch J, et al. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. Nature. 2009;458: 636–640. doi:10.1038/nature07930

160. Thali M, Moore JP, Furman C, Charles M, Ho DD, Robinson J, et al. Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. Journal of Virology. 1993;67: 3978–3988.

161. Blish CA, Nedellec R, Mandaliya K, Mosier DE, Overbaugh J. HIV-1 subtype A envelope variants from early in infection have variable sensitivity to neutralization and to inhibitors of viral entry. AIDS. 2007;21: 693–702. doi:10.1097/QAD.0b013e32805e8727

162. Rusert P, Krarup A, Magnus C, Brandenberg OF, Weber J, Ehlert A-K, et al. Interaction of the gp120 V1V2 loop with a neighboring gp120 unit shields the HIV envelope trimer against cross-neutralizing antibodies. J Exp Med. 2011;208: 1419–1433. doi:10.1084/jem.20110196

163. Julien J-P, Cupo A, Sok D, Stanfield RL, Lyumkis D, Deller MC, et al. Crystal Structure of a Soluble Cleaved HIV-1 Envelope Trimer. Science. 2013. doi:10.1126/science.1245625

164. McLellan JS, Pancera M, Carrico C, Gorman J, Julien J-P, Khayat R, et al. Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. Nature. Nature Publishing Group; 2011;480: 336–343. doi:10.1038/nature10696

165. White TA, Bartesaghi A, Borgnia MJ, Meyerson JR, la Cruz de MJV, Bess JW, et al. Molecular Architectures of Trimeric SIV and HIV-1 Envelope Glycoproteins on Intact Viruses: Strain-Dependent Variation in Quaternary Structure. Rey FA, editor. PLoS Pathog. 2010;6: e1001249. doi:10.1371/journal.ppat.1001249.g008

166. Yang X, Kurteva S, Lee S, Sodroski J. Stoichiometry of antibody neutralization of human immunodeficiency virus type 1. Journal of Virology. 2005;79: 3500–3508. doi:10.1128/JVI.79.6.3500-3508.2005

167. Pinter A, Honnen WJ, He Y, Gorny MK, Zolla-Pazner S, Kayman SC. The V1/V2 domain of gp120 is a global regulator of the sensitivity of primary human immunodeficiency virus type 1 isolates to neutralization by antibodies commonly induced upon infection. Journal of Virology. 2004;78: 5205–5215.

168. Krachmarov CP, Honnen WJ, Kayman SC, Gorny MK, Zolla-Pazner S, Pinter A. Factors determining the breadth and potency of neutralization by V3-specific human monoclonal antibodies derived from subjects infected with clade A or clade B strains of human immunodeficiency virus type 1. Journal of Virology. 2006;80: 7127–7135. doi:10.1128/JVI.02619-05

169. Kwon YD, Finzi A, Wu X, Dogo-Isonagie C, Lee LK, Moore LR, et al. Unliganded HIV-1 gp120 core structures assume the CD4-bound conformation with regulation by

quaternary interactions and variable loops. Proc Natl Acad Sci USA. 2012;109: 5663–5668. doi:10.1073/pnas.1112391109

170. Guttman M, Kahn M, Garcia NK, Hu S-L, Lee KK. Solution structure, conformational dynamics, and CD4-induced activation in full-length, glycosylated, monomeric HIV gp120. Journal of Virology. 2012;86: 8750–8764. doi:10.1128/JVI.07224-11

171. White TA, Bartesaghi A, Borgnia MJ, la Cruz de MJV, Nandwani R, Hoxie JA, et al. Three-dimensional structures of soluble CD4-bound states of trimeric simian immunodeficiency virus envelope glycoproteins determined by using cryo-electron tomography. Journal of Virology. 2011;85: 12114–12123. doi:10.1128/JVI.05297-11

172. O'Connell O, Repik A, Reeves JD, Gonzalez-Perez MP, Quitadamo B, Anton ED, et al. Efficiency of bridging-sheet recruitment explains HIV-1 R5 envelope glycoprotein sensitivity to soluble CD4 and macrophage tropism. Journal of Virology. 2013;87: 187–198. doi:10.1128/JVI.01834-12

173. Si Z, Cayabyab M, Sodroski J. Envelope Glycoprotein Determinants of Neutralization Resistance in a Simian-Human Immunodeficiency Virus (SHIV-HXBc2P 3.2) Derived by Passage in Monkeys. Journal of Virology. 2001;75: 4208–4218. doi:10.1128/JVI.75.9.4208-4218.2001

174. Fomsgaard A, Johnson PR, Nielsen C, Novembre FJ, Hansen J, Goldstein S, et al. Receptor function of CD4 structures from African green monkey and pig-tail macaque for simian immunodeficiency virus, SIVsm, SIVagm, and human immunodeficiency virus type-1. Viral Immunol. 1995;8: 121–133.

175. Chen Z, Zhou P, Ho DD, Landau NR, Marx PA. Genetically divergent strains of simian immunodeficiency virus use CCR5 as a coreceptor for entry. Journal of Virology. 1997;71: 2705–2714.

176. Ye Y, Si ZH, Moore JP, Sodroski J. Association of structural changes in the V2 and V3 loops of the gp120 envelope glycoprotein with acquisition of neutralization resistance in a simian-human immunodeficiency virus passaged in vivo. Journal of Virology. 2000;74: 11955–11962.

177. Zhuang K, Finzi A, Tasca S, Shakirzyanova M, Knight H, Westmoreland S, et al. Adoption of an “open” envelope conformation facilitating CD4 binding and structural remodeling precedes coreceptor switch in R5 SHIV-infected macaques. PLoS ONE. 2011;6: e21350. doi:10.1371/journal.pone.0021350

178. Tan RC, Harouse JM, Gettie A, Cheng-Mayer C. In vivo adaptation of SHIV(SF162): chimeric virus expressing a NSI, CCR5-specific envelope protein. Journal of Medical Primatology. 1999;28: 164–168.

179. Malim MH, Bieniasz PD. HIV Restriction Factors and Mechanisms of Evasion. Cold Spring Harb Perspect Med. 2012;2: a006940. doi:10.1101/cshperspect.a006940

180. Hatziioannou T, Princiotta M, Piatak M, Yuan F, Zhang F, Lifson JD, et al. Generation of Simian-Tropic HIV-1 by Restriction Factor Evasion. Science. 2006;314: 95–95. doi:10.1126/science.1130994

181. Lovelace E, Xu H, Blish CA, Strong R, Overbaugh J. The role of amino acid changes in the human immunodeficiency virus type 1 transmembrane domain in antibody binding and neutralization. Virology. 2011;421: 235–244. doi:10.1016/j.virol.2011.09.032

182. Doria-Rose NA, Learn GH, Rodrigo AG, Nickle DC, Li F, Mahalanabis M, et al. Human immunodeficiency virus type 1 subtype B ancestral envelope protein is functional and elicits neutralizing antibodies in rabbits similar to those elicited by a circulating subtype B envelope. Journal of Virology. 2005;79: 11214–11224. doi:10.1128/JVI.79.17.11214-11224.2005

183. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nat Protoc. 2008;3: 1101–1108. doi:10.1038/nprot.2008.73

184. Poss M, Overbaugh J. Variants from the diverse virus population identified at seroconversion of a clade A human immunodeficiency virus type 1-infected woman have distinct biological properties. Journal of Virology. 1999;73: 5255–5264.

185. Yuste E, Reeves JD, Doms RW, Desrosiers RC. Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity. Journal of Virology. 2004;78: 6775–6785. doi:10.1128/JVI.78.13.6775-6785.2004

186. Yuste E, Johnson W, Pavlakis GN, Desrosiers RC. Virion envelope content, infectivity, and neutralization sensitivity of simian immunodeficiency virus. Journal of Virology. 2005;79: 12455–12463. doi:10.1128/JVI.79.19.12455-12463.2005

187. Agosto LM, Uchil PD, Mothes W. HIV cell-to-cell transmission: effects on pathogenesis and antiretroviral therapy. Trends in Microbiology. Elsevier Ltd; 2015;23: 289–295. doi:10.1016/j.tim.2015.02.003

188. Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, et al. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature. 2011;472: 481–485. doi:10.1038/nature09907

189. Liu S-Y, Sanchez DJ, Aliyari R, Lu S, Cheng G. Systematic identification of type I and type II interferon-induced antiviral factors. Proc Natl Acad Sci USA. 2012;109: 4239–4244. doi:10.1073/pnas.1114981109

190. Asmuth DM, Murphy RL, Rosenkranz SL, Lertora JJL, Kottilil S, Cramer Y, et al. Safety, tolerability, and mechanisms of antiretroviral activity of pegylated interferon Alfa-2a in HIV-1-monoinfected participants: a phase II clinical trial. Journal of Infectious Diseases. 2010;201: 1686–1696. doi:10.1086/652420

191. Hubbard JJ, Greenwell-Wild T, Barrett L, Yang J, Lempicki RA, Wahl SM, et al. Host

Gene Expression Changes Correlating With Anti-HIV-1 Effects in Human Subjects After Treatment With Peginterferon Alfa-2a. Journal of Infectious Diseases. 2012;205: 1443–1447. doi:10.1093/infdis/jis211

192. Asmal M, Hellmann I, Liu W, Keele BF, Perelson AS, Bhattacharya T, et al. A signature in HIV-1 envelope leader peptide associated with transition from acute to chronic infection impacts envelope processing and infectivity. PLoS ONE. 2011;6: e23673. doi:10.1371/journal.pone.0023673

193. Postler TS, Desrosiers RC. The tale of the long tail: the cytoplasmic domain of HIV-1 gp41. Journal of Virology. 2013;87: 2–15. doi:10.1128/JVI.02053-12

194. Thippeshappa R, Ruan H, Wang W, Zhou P, Kimata JT. A Variant Macaque-Tropic Human Immunodeficiency Virus Type 1 Is Resistant to Alpha Interferon-Induced Restriction in Pig-Tailed Macaque CD4+ T Cells. Journal of Virology. 2013;87: 6678–6692. doi:10.1128/JVI.00338-13

195. Besnier C, Takeuchi Y, Towers G. Restriction of lentivirus in monkeys. Proc Natl Acad Sci USA. 2002;99: 11920–11925. doi:10.1073/pnas.172384599

196. Mashiba M, Collins DR, Terry VH, Collins KL. Vpr Overcomes Macrophage-Specific Restriction of HIV-1 Env Expression and Virion Production. Cell Host and Microbe. 2014;16: 722–735. doi:10.1016/j.chom.2014.10.014

197. Song B, Javanbakht H, Perron M, Park DH, Stremlau M, Sodroski J. Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. Journal of Virology. 2005;79: 3930–3937. doi:10.1128/JVI.79.7.3930-3937.2005

198. Brandenberg OF, Magnus C, Rusert P, Regoes RR, Trkola A. Different Infectivity of HIV-1 Strains Is Linked to Number of Envelope Trimers Required for Entry. PLoS Pathog. 2015;11: e1004595. doi:10.1371/journal.ppat.1004595

199. Fowler DM, Fields S. Deep mutational scanning: a new style of protein science. Nat Methods. 2014;11: 801–807. doi:10.1038/nmeth.3027

200. Thyagarajan B, Bloom JD. The inherent mutational tolerance and antigenic evolvability of influenza hemagglutinin. Elife. 2014;3. doi:10.7554/eLife.03300

201. Meyerson NR, Sharma A, Wilkerson GK, Overbaugh J, Sawyer SL. Identification of Owl Monkey CD4 Receptors Broadly Compatible with Early-Stage HIV-1 Isolates. Journal of Virology. 2015;89: 8611–8622. doi:10.1128/JVI.00890-15

202. Haas J, Park EC, Seed B. Codon usage limitation in the expression of HIV-1 envelope glycoprotein. Curr Biol. 1996;6: 315–324.

203. Lepelley A, Louis S, Sourisseau M, Law HKW, Pothlichet J, Schilte C, et al. Innate sensing of HIV-infected cells. PLoS Pathog. 2011;7: e1001284. doi:10.1371/journal.ppat.1001284

204. Gao D, Wu J, Wu Y-T, Du F, Aroh C, Yan N, et al. Cyclic GMP-AMP Synthase Is an Innate Immune Sensor of HIV and Other Retroviruses. Science. 2013. doi:10.1126/science.1240933

205. Monroe KM, Yang Z, Johnson JR, Geng X, Doitsh G, Krogan NJ, et al. IFI16 DNA Sensor Is Required for Death of Lymphoid CD4 T Cells Abortively Infected with HIV. Science. 2013. doi:10.1126/science.1243640

206. Brass AL, Dykxhoorn DM, Benita Y, Yan N, Engelman A, Xavier RJ, et al. Identification of host proteins required for HIV infection through a functional genomic screen. Science. 2008;319: 921–926. doi:10.1126/science.1152725

207. Shalem O, Sanjana NE, Zhang F. High-throughput functional genomics using CRISPR–Cas9. Nat Rev Genet. Nature Publishing Group; 2015;16: 299–311. doi:10.1038/nrg3899

208. Hansen SG, Ford JC, Lewis MS, Ventura AB, Hughes CM, Coyne-Johnson L, et al. Profound early control of highly pathogenic SIV by an effector memory T-cell vaccine. Nature. Nature Publishing Group; 2011;473: 523–527. doi:10.1038/nature10003

209. Hansen SG, Piatak M Jr, Ventura AB, Hughes CM, Gilbride RM, Ford JC, et al. Immune clearance of highly pathogenic SIV infection. Nature. Nature Publishing Group; 2013;: 1–7. doi:10.1038/nature12519

210. Burton DR, Ahmed R, Barouch DH, Butera ST, Crotty S, Godzik A, et al. A Blueprint for HIV Vaccine Discovery. Cell Host and Microbe. Elsevier Inc; 2012;12: 396–407. doi:10.1016/j.chom.2012.09.008

211. Sanders RW, Derking R, Cupo A, Julien J-P, Yasmeen A, de Val N, et al. A Next-Generation Cleaved, Soluble HIV-1 Env Trimer, BG505 SOSIP.664 gp140, Expresses Multiple Epitopes for Broadly Neutralizing but Not Non-Neutralizing Antibodies. PLoS Pathog. 2013;9: e1003618. doi:10.1371/journal.ppat.1003618

212. Sanders RW, van Gils MJ, Derking R, Sok D, Ketas TJ, Burger JA, et al. HIV-1 neutralizing antibodies induced by native-like envelope trimers. Science. 2015. doi:10.1126/science.aac4223

213. McGuire AT, Hoot S, Dreyer AM, Lippy A, Stuart A, Cohen KW, et al. Engineering HIV envelope protein to activate germline B cell receptors of broadly neutralizing anti-CD4 binding site antibodies. J Exp Med. 2013;210: 655–663. doi:10.1084/jem.20122824

214. Doria-Rose NA, Schramm CA, Gorman J, Moore PL, Bhiman JN, Dekosky BJ, et al. Developmental pathway for potent V1V2-directed HIV-neutralizing antibodies. Nature. 2014. doi:10.1038/nature13036