

Generation of a neutralization-resistant CCR5 tropic SHIV-MK38
molecular clone, a derivative of SHIV-89.6

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Chapter 1

Empirical research for the development of prophylactic and therapeutic interventions against HIV infection using nonhuman primate models

1-1 Preface

In the thirty years since acquired immunodeficiency syndrome (AIDS) was first reported in 1981, human immunodeficiency virus (HIV) infection has spread rapidly worldwide. According to the World Health Organization (WHO) and United Nations program on AIDS (UNAIDS), there were 36.9 million HIV-positive individuals in 2014, with 2 million newly infected individuals and 1.2 million HIV-related deaths. Thus, AIDS remains a significant global public health issue. Combined antiretroviral therapy (cART) has contributed to the dramatic reduction in mortality; however, several unresolved concerns remain such as drug-resistance, side effects and medical costs. While cART has shown some significant advances, it has failed to completely eradicate latent virus in the host, and fundamental therapeutic interventions for AIDS have not been established. In addition, effective HIV vaccines remain elusive. Animal models are important tools for understanding viral pathogenesis and evaluating preventive interventions. Particularly, non-human primate (NHP) models are used for AIDS research. This chapter states the current status of non-human primate models for AIDS.

1-2 Phylogenetic relationship between HIV and SIV

Soon after HIV was recognized, research efforts focused on where and how the AIDS virus emerged. SIV, which is similar to HIV, was isolated from rhesus macaques that died of AIDS in a U.S. primate center. A number of African-origin monkeys, including the African Green Monkey, (AGM) retained antibodies against SIV, whereas Asian-origin macaques, including rhesus macaques in the wild, showed no SIV infection. Therefore, it was hypothesized that the AIDS virus was derived from African-origin monkeys. However, genetic analysis of SIV from AGMs refuted the simple scenario of transmission from AGM to human. Although it was tempting to lump all the monkey-derived viruses together, the abundance of a variety of viruses complicated the phylogenic relationship.

Fukasawa *et al.* (1988) clarified the full-length base sequence of SIVagm, (SIV isolated from AGM) and showed that HIV did not come directly from SIVagm. In addition, Tsujimoto *et al.* (1989) reported that the full-length sequence of mandrill-derived SIV (SIVmnd), contributing to the full picture of a variety of primate lentivirus. At almost the same time, a virus isolated from Sooty Mangabey in West Africa (SIVsmm) was found to be similar to HIV-2, which was prevalent in West Africa (Hirsch *et al.*, 1989). SIVcpz derived from chimpanzee was found to be similar to

HIV-1 (Huet *et al.*, 1990). Thus, phylogenic analysis revealed relationships between HIV-1 and HIV-2 and the primate lentiviruses including SIV (Fig. 1-1).

1-3 SIV model

After initial reports of HIV-1 infection, research at the molecular/cellular level advanced significantly, with a decline in analytical research regarding the mechanisms of pathogenesis and disease progression to AIDS. Thus, the fundamental mechanisms of HIV-1 progression to AIDS remain unclear. Although important knowledge could be obtained from human clinical samples, such samples have limitations such as unidentified infection times, analysis with only peripheral blood, and multiple effects of antiretroviral drugs. Therefore, an experimental animal model is essential for analyzing the disease progression mechanism and to develop prophylactic and therapeutic interventions against AIDS. However, the narrow host range of HIV-1 is a significant limitation, and *in vivo* HIV-1-infection animal experiments are difficult to perform. To avoid these limitations, SIV, which is similar to HIV-2, infects Asian-origin macaques including rhesus monkeys and cynomolgus monkeys, causes AIDS-like symptoms, and has been used as an animal model.

The importance of HIV research using monkeys has been recognized in Europe and the U.S. The U.S. developed eight primate centers, modified the monkey breeding protocols and physical building systems, and augmented research grants to researchers. SIVmac was isolated from rhesus monkeys that died of AIDS in the U.S. primate

centers in the 1980s. Phylogenetic analysis indicated that SIVsmm was transmitted from sooty mangabey during breeding. The SIVmac/macaque model is currently most widely used in the field of AIDS research. SIV causes AIDS in inoculated rhesus macaques within 1 or 2 years. The pathological condition of the SIV/macaque model is comparable to HIV-1-positive individuals, making the SIV/macaque an excellent model for examining the pathological condition and pathogenesis during HIV infection.

SIV inoculation experiments were central to the development of knowledge in AIDS pathological research. In these studies, tissue biopsies were analyzed continuously and at regular time intervals, a process that would be impossible to perform in humans. SIV research in AIDS led to the following important conclusions: 1) SIV was the causative factor of simian AIDS, and the pathological condition of SIV-infected monkeys was very similar to that of HIV-positive individuals; 2) HIV disease was zoonotic; 3) cellular immunity was important to suppress viral replication; 4) SIV-attenuated vaccine could prevent SIV infection; and 5) the primary anatomical compartment targeted by HIV and SIV was the intestine (Gardner *et al.*, 2003; Veazey *et al.*, 1998).

Recently, an *in vivo* experimental system with combined antiretroviral therapy (cART) was established in many groups. Horiike *et al.* (2012) established the system by

the oral administration. For example, SIV-infected macaques were fed anti-HIV drug-kneaded biscuits over time. The SIV/macaque model treated with cART over 1 year showed no accumulation of SIV mutations in SIV-infected monkeys during cART, indicating no transmission to uninfected cells in SIV-infected monkeys during cART (Oue *et al.*, 2013). This cART-treated primate model is expected to promote the development of new therapeutic agents in the near future.

However, the SIV/macaque model has some limitations. The low homology of Env between HIV and SIV does not show reciprocal neutralizing antibody cross-reactive (Javaherian *et al.*, 1992; Yuste *et al.*, 2006). Therefore, the SIV/macaque model cannot be used for evaluating anti-HIV neutralizing antibody. To evaluate anti-HIV neutralizing antibody in NHP models, it is essential for SIV to carry HIV Env antigenicity and structures. Therefore, the chimeric simian/human immunodeficiency virus (SHIV), which carries HIV *env* gene on the basis of SIVmac, was generated (Shibata *et al.*, 1991).

1-4 SHIV model

The SIV/macaque model is an excellent model for analyzing the pathological conditions and pathogenesis in HIV infection. However, this model is not suited for analyzing the role of neutralizing antibody in protective immunity due to the low homology of envelop proteins (Env) between SIV and HIV, which results in different Env structures and antigenicities. To address this issue, SHIV, which substitutes HIV-1 *env*, *rev*, *tat* and *vpu* genes into the corresponding region of SIV in the SIV genome was generated (Fig. 1-2) (Shibata *et al.*, 1991). A variety of pathogenic and non-pathogenic SHIV strains from the generated SHIV were obtained (Reimann *et al.*, 1996). SHIV-89.6 rapidly reduced CD4⁺ T cells and induced the AIDS-like symptoms after two serial monkey-to-monkey passages. The isolated SHIV-89.6P is the most widely used for vaccine evaluation.

However, the clinical trial of HIV vaccine conducted by Merck in 2007 was suddenly interrupted, as the vaccine candidate having a protective effect in a SHIV/macaque model showed no protection in HIV infection in the human clinical trial (Watkins *et al.*, 2008). Furthermore, the vaccine group probably demonstrated higher viremia than the placebo group. Thus, the SHIV/macaque model overestimated the preventive effect of the vaccine candidate. This fact indicated that infections with HIV

and SIV were more difficult to control than SHIV infection. Such differences could be due to the fact that most circulating highly pathogenic SHIV differed from HIV/SIV with regard to which co-receptors the viruses used and, consequently, the target cell and pathogenesis patterns differed (Watkins *et al.*, 2008). Circulating highly pathogenic SHIV preferentially infects naïve T cells with high affinity to CXCR4, and peripheral CD4⁺ T cells exhibit rapid depletion. On the other hand, SIV and HIV have high affinity to CCR5 and preferentially infect memory T cells residing in the effector site such as the intestinal mucosa (Nishimura *et al.*, 2004). Although many studies used circulating SHIV that was CXCR4-tropic, CCR5-tropic virus was thought to be important in human AIDS pathological conditions, prompting further studies with CCR5-tropic SHIV.

Intravenous inoculation with highly pathogenic CXCR4 tropic SHIV-KS661 caused dramatic AIDS pathological conditions with persistent infection and high viremia due to the rapid deletion of CD4⁺ T cells and the lack of an antibody response against SHIV-KS661. Intrarectal inoculation with SHIV-KS661 resulted in an antibody response that controlled viremia below the detection limit after transient viral growth (Inaba *et al.*, 2010). In contrast, SIV caused persistent infection with stably high viremia despite the antibody response (Fig. 1-3). Therefore, Miura's group built a hypothesis

that the sensitivity of viruses against neutralizing antibody is associated with persistent infection in individuals.

To conduct the simple comparative analysis with the most widely used SHIV-89.6P, Matsuda *et al.* (2010) tried to generate CCR5 tropic and neutralization-resistant SHIV derived from SHIV-KS661, a derivative of SHIV-89.6. Five amino acids of Env from the highly pathogenic SHIV-KS661 were introduced to switch the tropism from CXCR4-tropic to CCR5-tropic. Then, the virus was acclimated to rhesus monkey by animal-to-animal passage (Matsuda *et al.*, 2010). The monkey-adapted virus was a genetically diverse SHIV-MK38, and showed neutralization-resistance against autologous plasma compared with the parental SHIV-KS661. To understand the relationship between neutralization resistance of the virus and persistent infection in individuals, I decided to analyze the detailed neutralization phenotype of SHIV-MK38 and performed the *in vivo* rectal inoculation experiment.

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1-6 Figures

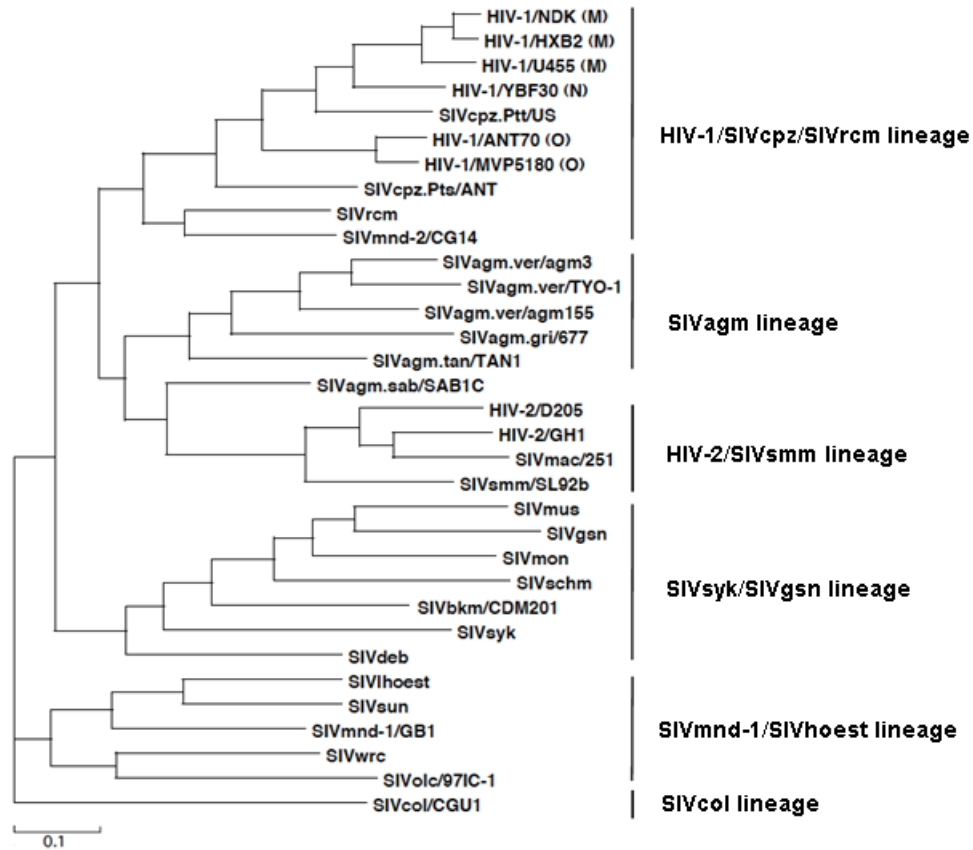


Fig. 1-1 Molecular evolutionary phylogenetic tree of lentivirus in a primate model. The phylogenetic tree was generated using a maximum-likelihood method based on the base sequence of the *pol* gene. HIV-1 is derived from SIVcpz, which is retained in chimpanzees residing in Central Africa. HIV-2 is derived from SIVsmm, which is retained by sooty mangabeys residing in West Africa. SIV, which is used as a macaque AIDS model, is the most common SIVmac isolated from a rhesus macaque with AIDS-like symptoms during breeding in the primate center in the U.S. SIVmac is derived from SIVsmm (similar to HIV-2).

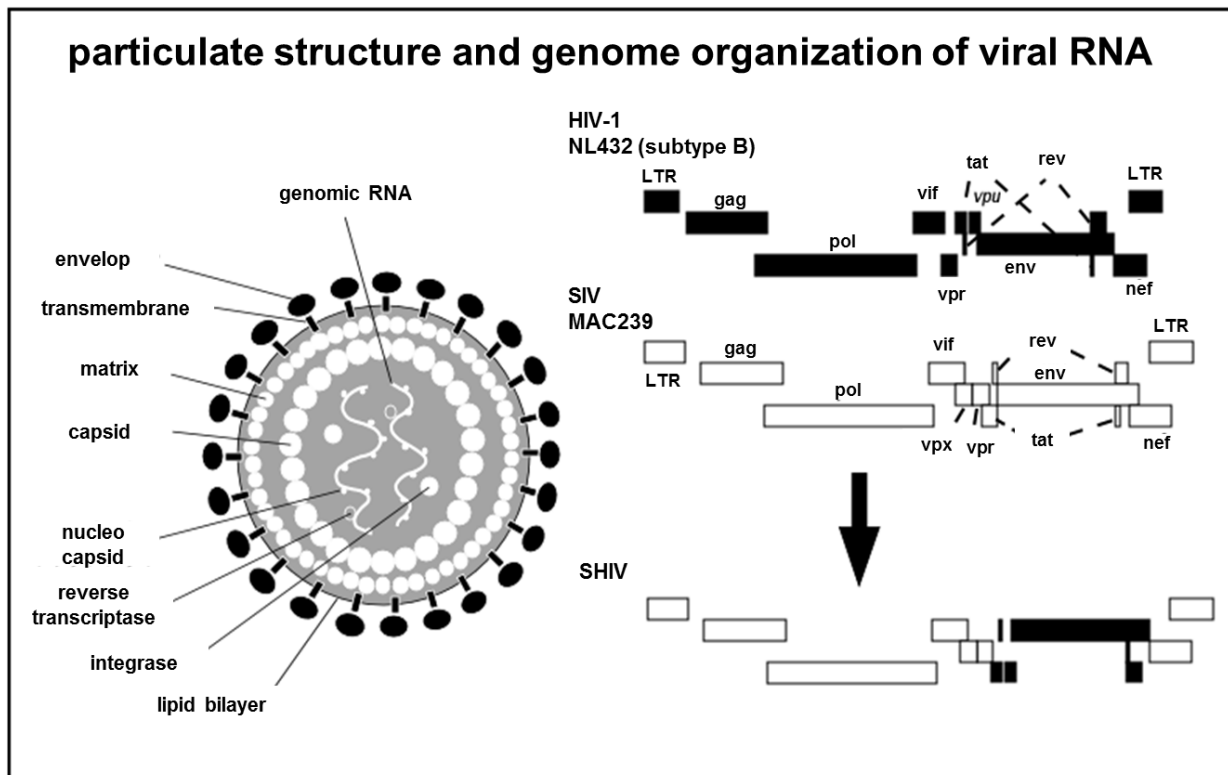


Fig. 1-2 Cartoons of the particulate structure and genomic organization of SHIV, the chimera virus of HIV-1 and SIV that infects the macaca, such as rhesus macaques. Black boxes represent HIV-1-derived genes and white boxes represent SIV-derived genes. SHIV is the chimera virus encoding HIV-1-derived *env* gene and part of the accessory genes based on SIV.

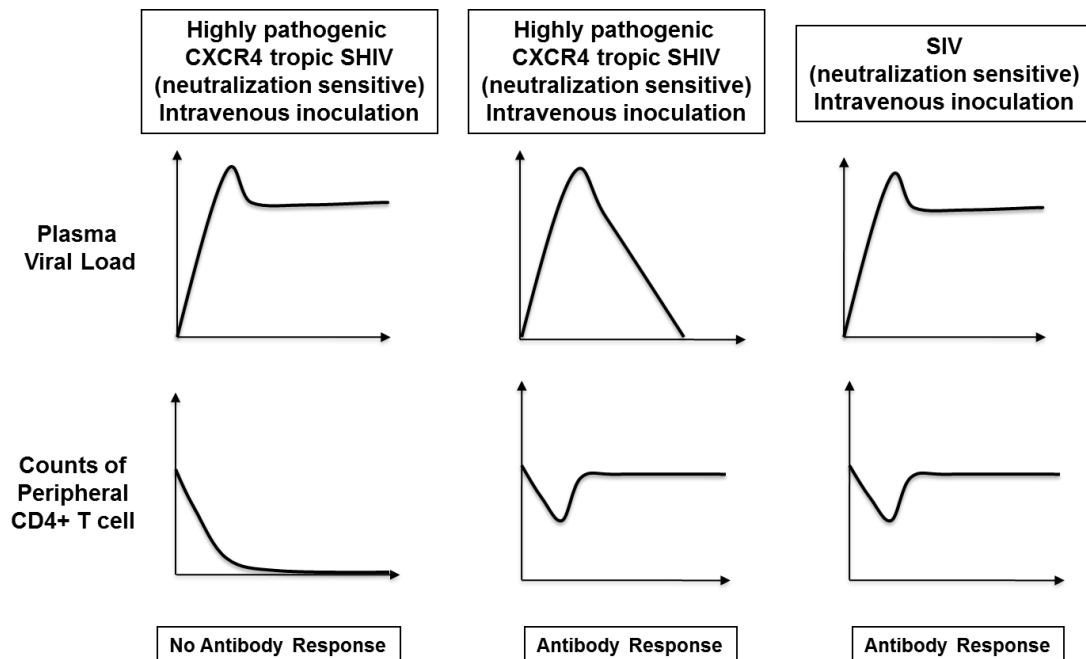


Fig. 1-3 Comparison between the pathogenesis of highly pathogenic CXCR4 tropic SHIV and SIV. When highly pathogenic CXCR4 tropic SHIV is intravenously inoculated to rhesus macaques, it quickly depleted peripheral CD4⁺ T cells and exhibited high viremia and persistent infection without an antibody response in the host. However, when highly pathogenic CXCR4 tropic SHIV was intrarectally inoculated, viremia was suppressed below the detection limit because the recovery of peripheral CD4⁺ T cells after the transient reduction renders the antibody response active. On the other hand, when SIV is intrarectally inoculated, it showed a persistent infection with high viremia despite the antibody response that occurs in the host.

Chapter 2

Generation of a CCR5 tropic and neutralization-resistant SHIV-MK38

molecular clone derived from SHIV-89.6

2-1 Abstract

Previously, a new genetically diverse CCR5 (R5) tropic simian/human immunodeficiency virus (SHIV-MK38) adapted to rhesus monkeys became more neutralization resistant to SHIV-infected plasma than did the parental SHIV-KS661 clone. Here, to clarify the significance of the neutralization-resistant phenotype of SHIV in a macaque model, I initially investigated the precise neutralization phenotype of the SHIVs, including SHIV-MK38 molecular clones, using SHIV-MK38-infected plasma, a pooled plasma of HIV-infected individuals, soluble CD4 (sCD4), and anti-HIV-1 neutralizing monoclonal antibodies, the epitopes of which were known. The results show that SHIV-KS661 had tier 1 neutralization sensitivity, but monkey-adapted R5 tropic SHIV-MK38 acquired neutralization resistance similar to that of tier 2 or 3 as a clone virus. Sequence analysis of the *env* gene suggests that the neutralization-resistant phenotype of SHIV-MK38 was acquired by conformational changes in Env associated with the net charge and potential N-linked glycosylation sites. To examine the relationship between neutralization phenotype and stably persistent infection in monkeys, I performed *in vivo* rectal inoculation experiments using an SHIV-MK38 molecular clone. The results show that one of three rhesus monkeys exhibited durable infection with a plasma viral load of 10^5 copies/ml despite the high antibody responses

that occurred in the host. While further improvements are required in the development of a challenge virus, it will be useful to generate a neutralization-resistant R5 tropic molecular clone of the SHIV-89.6 lineage commonly used for vaccine development, a result that can be used to explore the foundation of AIDS pathogenesis.

2-2 Introduction

Simian immunodeficiency virus (SIV)/macaque models of acquired immunodeficiency syndrome (AIDS) play important roles as surrogates of human immunodeficiency virus type 1 (HIV-1) in understanding AIDS pathogenesis and in vaccine development. Although SIV is an excellent model of HIV-1, homology of the *env* gene between SIV and HIV-1 is low (Yuste *et al.*, 2006), and consequently reciprocal neutralizing antibodies do not show cross-reactivity (Javaherian *et al.*, 1992). Thus, an important limitation of the SIV/macaque model is that the protective effects of neutralizing antibodies against HIV-1 cannot be examined. Therefore, simian/human immunodeficiency viruses (SHIVs) constructed by inserting HIV-1 *env*, *tat*, *vpu*, and *rev* into the SIV backbone have been developed to carry the antigenicity of HIV-1 Env into SIV (Shibata *et al.*, 1991). Highly pathogenic SHIVs, which persistently infected rhesus macaques with a high set point following a high viral load peak, were obtained using *in vivo* adaptation to macaques (Reimann *et al.*, 1996; Shinohara *et al.*, 1999). Because highly pathogenic SHIVs are useful in evaluating anti-HIV-1 neutralizing antibodies, SHIV-infected macaque models have been used in many preclinical investigations, even though SHIVs can also be used to evaluate cytotoxic T lymphocytes against SIV (Shiver *et al.*, 2002; Letvin *et al.*, 2006; O'Connell *et al.*,

2012). However, a cytotoxic T lymphocyte-inducing vaccine candidate that showed protective effects in an SHIV/macaque model failed to prevent viral replication in both a human clinical trial and an SIV/macaque model (Casimiro *et al.*, 2005; Buchbinder *et al.*, 2008). These results show that the vaccine-induced protective effect is not properly evaluated in SHIV/macaque models and indicate that the previously used SHIVs need to be improved.

The Env trimers are exposed on the surface of retroviruses. Recently, several broadly neutralizing antibodies against specific parts of Env have been found, including VRC01 (targeting the CD4 binding site (CD4bs)), PG9 (targeting the V1/V2 region), PGT121 (targeting the V3 region), and 2F5 (targeting the membrane proximal exterior region (MPER)) (Kwong *et al.*, 2013). These antibodies are important for vaccine development. Env is not only a target of neutralizing antibodies but also a determinant of co-receptor tropism. According to the current model of HIV entry into host cells, the V3 region of Env interacts with co-receptors and is important for the change of co-receptor tropism. Co-receptor switching is generally associated with the net charge of V3 (Fouchier *et al.*, 1992; Shioda *et al.*, 1994) and there are specific amino acids within the V3 region responsible for co-receptor tropism (Cordoza *et al.*, 2007).

Based on the Env features described above, to improve previously used SHIVs, two differences between HIV-1 initial clinical isolates and previously used SHIVs were focused on. The first difference is the co-receptor tropism of the viruses. Most highly pathogenic SHIVs predominantly use CXCR4 (X4) as a co-receptor and preferentially infect naïve CD4⁺ T lymphocytes, which highly express CXCR4 (Mehandru, 2007). Highly pathogenic SHIVs deplete CD4⁺ T lymphocytes in peripheral blood with rapid reduction in a few weeks, which quickly results in AIDS (Sadjapour *et al.*, 2004; Reimann *et al.*, 2005; Fukazawa *et al.*, 2008). On the other hand, HIV-1 initial clinical isolates and SIVs use CCR5 (R5) as a co-receptor. They preferentially infect memory CD4⁺ T lymphocytes, which highly express CCR5 (Mehandru, 2007) and rapidly decrease CD4⁺ T lymphocytes in effector sites such as the intestine, while they cause gradual loss of naïve CD4⁺ T lymphocytes in peripheral blood (Veazey *et al.*, 1998; Mehandru, 2007; Wang *et al.*, 2007).

The second difference is the level of neutralization sensitivity. As previously reported, HIV-1 primary isolates are relatively resistant against anti-HIV-1 neutralizing antibodies compared to T-cell line-adapted laboratory strains (Moore *et al.*, 1995). Based on these results, most previously used SHIVs were thought to be neutralization sensitive because they contained the *env* gene of laboratory strains obtained by

passaging through T-cell lines. Another report showed that the neutralization phenotype against neutralizing antibodies can be categorized as follows: tier 1 (neutralization sensitive), tier 2 (neutralization resistant), and tier 3 (extremely neutralization resistant) (Seaman *et al.*, 2010). The majority of HIV-1 clinical isolates are categorized as tier 2, while HIV-1 laboratory strains are categorized as tier 1. Therefore, it is important to generate a challenge virus with greater than tier 2 neutralization resistance to evaluate its protective effect on vaccine candidates.

As described above, co-receptor use and the neutralization phenotype of the virus were focused on and a previously used SHIV started to be improved. Miura's group selected the SHIV-89.6 lineage as a parental strain because SHIV-89.6P, a derivative of SHIV-89.6, has been used most widely for the development of vaccines and therapeutic interventions. Miura's group reasoned that generation of neutralization resistant R5 tropic SHIV derived from SHIV-89.6 would be valuable with regard to comparing data between the virus and SHIV-89.6P. Hence, Matsuda *et al.* (2010) generated SHIV-MK38 from SHIV-KS661, a derivative of SHIV-89.6, by introducing five amino acid substitutions in the V3 region to alter co-receptor use and two *in vivo* passages to adapt the virus to rhesus monkeys (Shinohara *et al.*, 1999; Matsuda *et al.*, 2010). SHIV-MK38 is R5 tropic and highly competent in replicating in rhesus

macaques, and becomes neutralization resistant to autologous plasma in these SHIV-infected monkeys compared with original SHIV-KS661 and SHIV-MK1, which are the R5 virus prior to acclimation to monkeys (Matsuda *et al.*, 2010). However, SHIV-MK38 acquires genetic diversity by accumulating mutations during *in vivo* passages and the precise nature of its neutralization resistance remains unclear. In this study, I generated a molecular clone of SHIV-MK38 and examined its neutralization phenotype and infectivity in rhesus monkeys.

2-3 Results

Generation of SHIV-MK38 infectious molecular clones

To examine the precise nature of neutralization resistance in the SHIV-MK38 strain, I generated molecular clones of SHIV-MK38. A virus neutralization phenotype is determined based on membrane-external regions of the Env protein. Therefore, viral RNA of SHIV-MK38 was extracted and its *env* gene was amplified by PCR following cDNA synthesis. The amplified *env* fragments were replaced with the equivalent region of the original SHIV-KS661 using restriction enzyme sites *Sph*I and *Xho*I (Fig. 2-1). By cloning, six infectious molecular clones of SHIV-MK38 were obtained and named MK38#811, #812, #813, #814, #816, #818.

Neutralization analysis of SHIV-MK38 and SHIV-MK38 molecular clones

To explore whether the obtained six molecular clones reflect the neutralization phenotype of SHIV-MK38, I performed a neutralization assay using plasma from a SHIV-MK38-infected monkey at 16 weeks post-inoculation (Fig. 2-2a). Matsuda *et al.* previously reported that the plasma neutralized SHIV-KS661 and SHIV-MK1, but did not neutralize SHIV-MK38 (Matsuda *et al.*, 2010). While SHIV-KS661 and SHIV-MK1 were neutralized by the plasma and their infectivity decreased to less than

22%, SHIV-MK38 was not neutralized at all in the presence of the plasma, which was consistent with previous data indicating that SHIV-MK38 was neutralization resistant. In six molecular clones, MK38#814 and MK38#818 infection was not inhibited by the plasma, and infectivity reached 114% and 175%, respectively, suggesting that MK38#814 and MK38#818 clones, like parental SHIV-MK38 strain, were neutralization resistant. The infectivity values of the other molecular clones (MK38#811, MK38#812, MK38#813, and MK38#816) were 6.4%, 4.2%, 0.41%, and 6.2%, respectively. Therefore, of the six molecular clones, MK38#814 and MK38#818 were neutralization resistant against autologous plasma from SHIV-MK38-infected monkeys, similar to parental SHIV-MK38 strain.

To investigate whether the neutralization resistant property of SHIV-MK38, MK38#814, and MK38#818 represented strain-specific resistance or strain-independent general resistance, a neutralization assay was performed using pooled plasma from HIV-1-infected individuals (Fig. 2-2b). I found that the ID₅₀ of HIV-1 reference clone 6535.3 (categorized as tier 1) was a 1:433 dilution, while that of PVO.4 (categorized as tier 3) was a 1:112 dilution. The ID₅₀ values of SHIV-KS661 and SHIV-MK1 were 1:437 and 1:353 dilution, respectively, and closer to 6535.3. On the other hand, the ID₅₀ values of SHIV-MK38 and MK38#818 were 1:159 and 1:98 dilution, respectively, and

closer to PVO.4. The ID_{50} of SHIV-MK#814 was 1:267 dilution, representing an intermediate value.

Next, I performed a neutralization assay against anti-HIV-1 neutralizing monoclonal antibodies, the epitopes of which are known and also soluble CD4 (sCD4) (Fig. 2-2c). I used three types of anti-HIV-1 neutralizing monoclonal antibody: KD-247 (which recognizes GPGR in the V3 region), and 4E10 and 2F5 (which recognize NWFDIT and ELDKWA, respectively, in the conserved membrane-proximal external region (MPER) of gp41) (Purtscher *et al.*, 1994; Stiegler *et al.*, 2001; Eda *et al.*, 2006). While the IC_{50} of SHIV-KS661 against KD-247 was 0.59 $\mu\text{g/ml}$, the IC_{50} values of SHIV-MK38, MK38#814, and MK38#818 were greater than 50 $\mu\text{g/ml}$, suggesting that SHIV-MK38, MK38#814, and MK38#818 showed approximately 100 times greater neutralization resistance to KD-247 than did SHIV-KS661. The IC_{50} of PVO.4 against KD-247 was greater than 50 $\mu\text{g/ml}$. The data show that SHIV-MK38, MK38#814, and MK38#818 had levels of neutralization resistance against KD-247 that were comparable to that of PVO.4. Next, the IC_{50} values of SHIV-MK38, MK38#814, MK38#818, and PVO.4 against 4E10 were found to be 37.2 $\mu\text{g/ml}$, 2.19 $\mu\text{g/ml}$, 9.27 $\mu\text{g/ml}$, and 9.51 $\mu\text{g/ml}$, respectively, while that of SHIV-KS661 was 0.51 $\mu\text{g/ml}$, which showed that SHIV-MK38 and MK38#818 had more than 18 times greater neutralization resistance

to 4E10 than did SHIV-KS661. On the other hand, compared to PVO.4, SHIV-MK38 showed about four times greater neutralization resistance to 4E10, but the neutralization resistance of MK38#818 was equivalent to that of PVO.4. The IC₅₀ values of SHIV-MK38, MK38#814, MK38#818, and PVO.4 against 2F5 were 36.6 µg/ml, 2.03 µg/ml, 7.64 µg/ml, and greater than 50 µg/ml, respectively, while that of SHIV-KS661 was 1.00 µg/ml. The data indicate that SHIV-MK38, MK38#818, and PVO.4 showed more than seven times greater neutralization resistance to 2F5 than did SHIV-KS661. sCD4 reacts with HIV-1 gp120 and inhibit viral infection. The IC₅₀ of PVO.4 against sCD4 exceeded 5.0 µg/ml, indicating that it is resistant to inhibition. The IC₅₀ of SHIV-MK38 against sCD4 was 1.4 µg/ml, while that of SHIV-KS661 was 0.1 µg/ml, indicating that the inhibition resistance of SHIV-MK38 was 14-fold greater than that of KS661. The IC₅₀ values of MK38#814 and MK38#818 were 0.2 and 0.6 µg/ml, respectively. MK38#818 showed mild inhibition resistance compared with SHIV-MK38. Taken together, these results suggest that parental highly pathogenic X4 tropic SHIV-KS661 is neutralization sensitive, similar to the tier 1 virus, but that monkey-adapted R5 tropic SHIV-MK38, which acquires the general neutralization resistant phenotype as a clone virus, is similar to tier 2 or 3 viruses.

Sequence analysis of MK38#814 and MK38#818

Because Env is the primary target for neutralizing antibodies, mutations in Env may be involved in acquiring neutralization resistance. Therefore, nucleotide sequences of the *env* gene of MK38#814 and MK38#818 were determined and amino acid mutations were deduced. No mutation was detected in the epitopes of any of the three types of anti-HIV-1 neutralizing monoclonal antibodies examined in this study and also in CD4bs, indicating that those neutralization epitopes and CD4bs were kept in SHIV-MK38#814 and SHIV-MK38#818. When the amino acid sequences of MK38#814 and MK38#818 were compared with the original SHIV-KS661, there were 17 amino acid mutations, except for the five mutations for changing co-receptor usage (Table 2-1). In total, 11 of the 17 mutations were related to changes in potential N-linked glycosylation sites (PNGS) (5/11) and/or net charges (8/11). Regarding the structural model of Env, most of the amino acid substitutions were located outside of Env trimeric structure including the V1/V2 loop region. There are some mutations (282, 389 and 422) close to CD4bs on the three-dimensional structure (Fig. 2-3). This suggests that the neutralization-resistant phenotype of SHIV-MK38 was acquired by conformational changes in the outside of specific Env structure associated with the net charge and PNGS keeping the each neutralizing epitope.

Co-receptor use of SHIV-MK38 molecular clones

To confirm that MK38#814 and MK38#818 are R5 tropic, an assay to determine virus co-receptor tropism was performed with small molecule inhibitors (Fig. 2-4). For SIVmac239, used as a positive control for the R5 tropic virus, dose-dependent inhibition was observed in the presence of AD101 (R5 inhibitor) alone or with both AD101 and AMD3100 (X4 inhibitor). For HIV-1 NL4-3, used as a positive control for the X4-tropic virus, dose-dependent inhibition was observed in the presence of AMD3100 alone or with both AD101 and AMD3100. As the infectivity of MK38#814 and MK38#818 showed a reduction in dose-dependent inhibition in the presence of AD101 alone or with both AD101 and AMD3100, the reaction curve of the molecular clones was similar to that of SIVmac239. These data indicate that MK38#814 and MK38#818 were R5 tropic.

***In vitro* replication in rhesus macaque peripheral blood mononuclear cells**

In vitro replication competency in rhesus macaque peripheral blood mononuclear cells (PBMCs) was assayed using MK38#814 and MK38#818 (Fig. 2-5). The cultured supernatants were collected every day up to 12 days post-inoculation, after

which reverse transcriptase (RT) activity was measured. The peak RT activity of SHIV-MK38 was attained at 6 days post-inoculation at a value of 4287 CPM. The RT activities of MK38#814 and MK38#818 peaked at 7 days post-inoculation at 2768 CPM and 5931 CPM, respectively. SIVmac239 had the highest RT activity at 4 days post-inoculation. SHIV-MK1 RT activity was 5066 CPM following the peak at 9 days post-inoculation. Thus, the replication of SHIV- MK38#818 in rhesus PBMCs was comparable to that of parental SHIV-MK38, although the replication was slower and the peak value was lower relative to SIVmac239.

***In vivo* inoculation experiment involving MK38#818**

Based on the above results, I determined that MK38#818 reflected the improved behavior of the parental SHIV-MK38 strain. Therefore, to examine whether MK38#818 established a durable infection in rhesus macaques, three macaques (MM596, MM597, and MM599) were inoculated intrarectally with 10,000 TCID₅₀ of MK38#818. The results show that all monkeys generated peak plasma viral loads ranging from 1.6×10^7 to 2.2×10^7 copies/ml at 2 weeks post-inoculation (Fig. 2-6a). MM597 maintained set point 10^5 copies/ml until 12 weeks post-inoculation, which established a durable infection. However, the plasma viral load in MM596 was 3.9×10^2 copies/ml at 12

weeks post-inoculation, decreasing to nearly below the detection limit. MM599 also had no set point and at 9 weeks post-inoculation the plasma viral load had decreased to below the detection limit. Therefore, these data show that MK38#818 exhibited robust replication in MM597, but was suppressed in MM596 and MM599.

I also analyzed counts of circulating CD4⁺ T lymphocytes (Fig. 2-6b). All monkeys showed no reduction in either total or memory CD4⁺ T lymphocytes in peripheral blood.

To examine the influence of viral replication in the effector site where most memory CD4⁺ T lymphocytes reside, rectal tissues were collected from all three monkeys and the percentage of CD4⁺ T lymphocytes in rectal mononuclear cells was assessed (Fig. 2-6c). All three monkeys showed a transient reduction in the percentage of CD4⁺ T lymphocytes in rectal mononuclear cells. MM597, the monkey that was persistently infected with a high viral load, showed a decrease from 68% to 12% over a few weeks, indicating that MK38#818 preferentially infected memory CD4⁺ T lymphocytes in effector sites and caused a rapid reduction in MM597.

To examine whether antibody responses occurred in rhesus monkeys, I performed a particle agglutination assay with the plasmas at 0, 2, 4, 6, 8, 10, and 12 weeks post-inoculation (Table 2-2). Although no antibody response was observed up to

2 weeks post-inoculation in MM596 and up to 4 weeks post-inoculation in MM597 and MM599, antibody responses were detected from 4 weeks post-inoculation onward in MM596 and 6 weeks post-inoculation onward in MM597 and MM599. Furthermore, to assess the neutralizing activity of MK38#818-infected monkey plasma, I performed a neutralization assay using plasma from each monkey obtained 0, 6, and 12 weeks post-inoculation. The 12-week post-inoculation plasma from MM597 neutralized SHIV-KS661, MK38#818, and NF462, which possess the extremely neutralization-sensitive Tier 1A reference strain HIV-1 SF162 *env* gene (Kawamura *et al.*, 1994), with an ID₅₀ of 1:33.2, 1:49, and 1:208.4, respectively (Table. 2-2). The 6-week post-inoculation plasma from MM597 neutralized only the extremely neutralization-sensitive NF462, and the ID₅₀ was 1:34.1. The other plasma samples did not show neutralizing activity. These results show that MK38#818 replicated in MM597, even in the presence of the neutralizing antibody response.

2-4 Discussion

Previously, a new R5 tropic SHIV generated from the highly pathogenic SHIV-KS661, a derivative of SHIV-89.6, was adapted to rhesus monkeys and became neutralization resistant against autologous plasma (Matsuda *et al.*, 2010). Therefore, in this study I generated SHIV-MK38 molecular clones and examined the precise neutralization phenotype of SHIV-MK38 and its molecular clones. I found that SHIV-MK38 did not merely escape from strain-specific antibodies by accumulating variants, but acquired neutralization resistance against various antibodies as a molecular clone. Since SHIV-MK38 is a derivative of SHIV-89.6, data on genetic mutations associated with the pathogenesis can be directly compared with those of SHIV-89.6P, which is the most widely used SHIV for understanding HIV-1 pathogenesis and in vaccine development.

To examine how SHIV-MK38 acquired the neutralization-resistant phenotype, I analyzed the sequences of the *env* genes of neutralization-resistant molecular clones. In addition, the mutational positions that might be associated with the acquisition of neutralization resistance were analyzed based on the predicted structure. There was no direct mutation of the neutralizing epitopes, while there were several mutations in the V1/V2 region and outside of Env trimeric structure, which might lead to conformation

changes and block access of neutralizing antibodies. To date, there have been several hypotheses regarding the mechanism for the neutralization resistance of HIV-1, which are glycan shield (prevent access of neutralizing antibodies to conserved epitopes by adding, losing, and shifting PNGS) and the number of positive net charge (Wei *et al.*, 2003). Because most of the mutations found in MK38#814 and MK38#818 were associated with PNGS and net charge, these mechanisms might lead to the acquisition of the neutralization resistant phenotype.

Why did the parental X4 tropic SHIV-KS661 maintain the neutralization sensitive phenotype after acclimation to monkeys, although its derivative R5 tropic SHIV-MK1 became the neutralization resistant SHIV-MK38 upon post-monkey adaptation? I suggest that the co-receptor tropism of the viruses may be associated with these observations. The remarkable difference between R5 tropic and X4 tropic viruses is in the values of V3 net positive charge. Previous studies found that T-cell-adapted viruses had high values for V3 net positive charge (O'Brien *et al.*, 1996; Cardozo *et al.*, 2007). Now, the co-receptor use of the viruses by controlling V3's net positive charge was able to be altered (Cardozo *et al.*, 2007). The values for the net positive charge of V3 are generally higher in X4 tropic viruses than in R5 tropic viruses. Naganawa *et al.* (2008) predicted that a decrease in V3 net positive charge leads to less exposed V3 from

a structural standpoint, resulting in immunological escape from neutralizing antibodies. This prediction may support the hypothesis of “open” and “closed” conformations of Env, which is associated with the accessibility of antibodies. Repits *et al.* (2008) investigated the amino acid mutations related to the change in net charge of the virus Env isolated before and after AIDS; most of the mutations related to increased net positive charge were found in variable regions, excluding V3. This suggests that X4 tropic viruses emerge only later during the course of infection and they may not reflect the “closed” form of Env due to some conformational limitations. Thus, the difference in co-receptor tropism determined by V3 linking its net charge may determine the structural constraints of the entire Env protein structure, affecting the neutralization resistant phenotype of the virus.

It is important to consider the extensive mutation of the HIV-1 Env during monkey-to-monkey passages since frequently used SHIVs are adapted to the monkey in order to become pathogenic (Etemad-Moghadam *et al.*, 2000; Hsu *et al.*, 2003; Gautam *et al.*, 2012). Boyd *et al.* (2015) reported that the SHIV adapted to utilize the monkey CD4 molecule, resulting in conformational changes that exposed epitopes in the variable regions. Although the corresponding mutations associated with increased monkey CD4-mediated entry in their report were not observed in Miura’s study, there

were some mutations around CD4bs and several direct mutations in the V1/V2 region, which might be involved in increased monkey CD4-mediated entry.

Although SHIV-KS661 is highly pathogenic by intravenous inoculation, it is easily controlled by host immune responses when intrarectally inoculated into monkeys (Inaba *et al.*, 2010). In this study, I showed that the neutralization-resistant R5 tropic SHIV-MK38#818 molecular clone derived from SHIV-KS661 can cause stably persistent infection with high viral load via intrarectal inoculation despite the immune responses that occurred in the host. Currently, SHIV_{AD8}, categorized as a tier 2 neutralization-resistant phenotype, is widely used as an SHIV challenge virus for a variety of vaccine developments (Nishimura *et al.*, 2010; Shingai *et al.*, 2012, Gautam *et al.*, 2012; Shingai *et al.*, 2013; Gardner *et al.*, 2015; Francica *et al.*, 2015). However, use of SHIV_{AD8} as the only challenge virus is very risky for precise evaluation in vaccine candidates and anti-HIV-1 neutralizing antibodies. Hence, it is important to generate some challenge viruses. MK38#818 is inferior to SHIV_{AD8} in terms of pathogenesis. However, there is still room to improve MK38#818 because the genetically diverse SHIV-MK38 exhibited stably persistent infection with high viral load via intravenous inoculation in all three monkeys (Matsuda *et al.*, 2010). MK38#818 was the best molecular clone of the six clones obtained in this study, but

may not be the best among all SHIV-MK38 variations because the neutralization resistance of MK38#818 was not as strong as that of SHIV-MK38 in the assay using neutralizing monoclonal antibodies. Therefore, I could select a better clone than MK38#818. It is also possible that there are unknown mechanisms, aside from neutralization phenotype, that are related to stably persistent infection in rhesus monkeys. Mutation sites exterior to the Env, for example, may be important in acquiring stable infection with high viral load in monkeys. Obtaining better clones will provide valuable information regarding the molecular basis of pathogenesis for AIDS through comparative analysis of the SHIV-89.6 lineage.

2-5 Materials and Methods

Viruses

The stock viruses were prepared with 293T cells by transfection or with C8166-CCR5 cells by infection (Matsuda *et al.*, 2010; Seaman *et al.*, 2010). The titration was performed with TZM-bl cells for *in vitro* assays and with C8166-CCR5 cells for the *in vivo* inoculation experiment. The titration procedure with TZM-bl cells was performed as previously described (Otsuki *et al.*, 2014). The procedure involving C8166-CCR5 cells was performed by limiting dilution.

Cells

C8166-CCR5 cells (Shimizu *et al.*, 2006) were maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT). 293T cells (DuBridge *et al.*, 1987) were maintained in Dulbecco's Modified Eagle Medium (D-MEM) (Wako Pure Chemicals, Osaka, Japan) supplemented with 10% FBS and 1 mM L-glutamine. TZM-bl cells (Platt *et al.*, 1998) from the NIH AIDS research and reference reagent program were maintained in D-MEM with 10% FBS, 1 mM L-glutamine, and 1 mM sodium pyruvate. PBMCs were prepared with the Ficoll density

gradient separation method. For this procedure, lymphocyte separation medium 1077 for humans (Wako Pure Chemicals) was used as a separation solution. Residual red blood cells were removed using ACK lysing buffer (0.15 M NH_4Cl , 10 mM KHCO_3 , 1.0 mM EDTA-2Na). PBMCs were stored in a deep freezer (-80°C) or maintained in RPMI-1640 (Invitrogen) supplemented 10% FBS, 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), 1 mM sodium pyruvate (MP Biomedicals, Milwaukee, WI), 50 nM 2-mercaptoethanol (Nacalai Tesque), and 40 $\mu\text{g/ml}$ gentamicin (Nacalai Tesque).

Generation of infectious molecular clones

To construct SHIV-MK38 molecular clones, viral RNA was extracted using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. cDNA including the *env* gene was synthesized by extracted RNA through the reverse transcriptase reaction using primer SHenv9R (GAGGCATATGTTAGATACCC) and Super Script III First-Strand Synthesis SuperMix (Invitrogen, Carlsbad, CA). Approximately 3.0 kbp of the region including the *env* gene from the cDNA as a template was amplified by the polymerase chain reaction (PCR) using primer SIV20F (CTCCAGGACTAGCATAAATGG) and SHenv6R (GCTGAAGAGGCACAGGCTCCG). PCR was performed as follows: one

cycle of denaturation (98°C, 2 min), 45 cycles of amplification (98°C, 10 s/ 61°C, 30 s/ 68°C, 4 min), and an additional cycle for final extension (68°C, 7 min) using KOD FX buffer, 0.4 mM deoxynucleoside triphosphate, 0.3 μ M primer, 0.02 units KOD FX (TOYOBO, Osaka, Japan), and a template. After the PCR product had been purified using a NucleoSpin Gel and PCR Clean-up (TaKaRa, Shiga, Japan), 8 μ g purified PCR product was reacted to digest with 10 units of restriction enzymes, *Sph*I and *Xho*I, at 37°C for 8 h. After electrophoresis, the 2.7 kbp band was collected from an agarose gel [0.8% agarose S powder (Wako Pure Chemicals, Osaka, Japan) and 0.5 mg/ml ethidium bromide in 1 $\times$ TBE buffer (89 mM Tris/Borate, 2 mM EDTA)] and purified. To insert purified DNA into pSHIV-KS661, pSHIV-KS661 was processed as described above and the 10 kbp band was collected by agarose gel electrophoresis and purified. Using Ligation Mix (TaKaRa), *env* depleted pSHIV-KS661 was reacted with the insert DNA at a ratio of 1:3.5. Cloning was then conducted by transforming the obtained plasmid DNA into stbl-2. Six molecular clones were obtained and labeled as MK38#811, #812, #813, #814, #816, and #818.

Neutralization assays

To evaluate virus neutralization resistance, I performed neutralization assays using plasma at 16 weeks post-inoculation from SHIV-MK38-infected monkeys, a pooled plasma of HIV-1 infected individuals (ZeptoMetrix, Buffalo, NY), and anti-HIV-1 monoclonal neutralization antibodies. Luciferase activity was measured using TZM-bl cells in the assay (Wei *et al.*, 2002). For plasmas collected from the infected monkeys, the infectivity was defined as 100% in the presence of week 0 post-inoculation. In the case of the pooled plasma of HIV-1-infected individuals, MK38#818-infected monkey plasma, anti-HIV-1 neutralizing monoclonal antibodies and sCD4, the infectivity was defined as 100% in the absence of the pooled plasma, monoclonal antibodies or sCD4. The pooled plasma, MK38#818-infected monkey plasma, neutralizing monoclonal antibodies and sCD4 were serially diluted and 50% inhibition dilution (ID₅₀) or 50% inhibition concentration (IC₅₀) was determined.

At 16 weeks post-inoculation, plasma from SHIV-MK38-infected monkey was inactivated by digesting at 56°C for 30 min. Plasma was then diluted by 1:40 and was pre-incubated with 100 TCID₅₀ of viruses at 37°C for 60 min. After the reaction, 5000 TZM-bl cells were cultured with a pre-incubated mixture under 5 µg/ml of DEAE dextran at 37°C for 48 h. To measure luciferase activity, 50 µl of culture cell lysate

solution (Toyo Ink Group, Tokyo, Japan) were added to each well and the plate was agitated for 15 min. In total, 30 μ l of lysates were transferred to a Nunc F96 MicroWell white plate (Thermo Scientific, Waltham, MA) and the luminescent substrate was added to each well. The luciferase activity was then calculated with Mikrowin (Berthold Technologies, Bad Wildbad, Germany) using Tri Star LB941 (Berthold Technologies). For pooled plasma of HIV-1 infected individuals and MK38#818-infected monkey plasma, the plasma was diluted two-fold from 1:50 to 1:3200. Subsequently, ID₅₀ was calculated as described above.

For anti-HIV-1 monoclonal neutralization antibodies, three types of anti-HIV-1 monoclonal neutralizing antibodies were used. These were KD-247, which recognizes the epitope GPGR in the V3 region of gp120 and was kindly provided by the Chemo-Sero-Therapeutic Research Institute; 4E10, which recognizes the epitope NWFDIT, and 2F5, which recognizes the epitope ELDKWA, both distributed by Dr. Hermann Katinger through the NIH AIDS reagent program. These monoclonal antibodies were diluted four-fold: KD-247 and 2F5 from 50 μ g/ml to 0.012 μ g/ml, and 4E10 from 40 μ g/ml to 0.010 μ g. IC₅₀ values were then calculated as described above.

For sCD4, sCD4 was diluted four-fold from 5.0 to 0.0003 μ g/ml. Subsequently, the IC₅₀ was calculated as described above.

Inhibition of viral replication by small molecule inhibitors

The inhibitor, AD101, was kindly provided by Dr. Julie Strizki at the Schering Plough Research Institute. AMD3100 was purchased from Sigma-Aldrich (St. Louis, MO). The procedure for inhibition assays was performed as previously described (Otsuki *et al.*, 2014). Inhibitors were diluted three-fold from 4 μ M to 0.005 μ M in this study.

***In vitro* replication of the viruses in PBMCs**

PBMCs were isolated from a non-infected monkey and cultured with medium, adding 10% (vol/vol) FBS, 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), and 1 mM sodium pyruvate (MP Biomedicals, Milwaukee, WI). PBMCs were stimulated with 25 μ g/ml concanavalin A (Sigma-Aldrich) on day 1, after which the cells were cultured with 160 units/ml of human recombinant IL-2 (Wako Pure Chemicals). After 3 days, stimulated PBMCs were spinoculated with each virus at MOI = 0.1 at $1,200 \times g$ for 60 min to achieve efficient infection (O'Doherty *et al.*, 2000). The supernatants in cultured cells were collected for up to 12 days and the RT activity in each supernatant was measured.

Sequencing analysis

Nucleic acid sequences of MK38#814 and MK38#818 in plasmid DNA were determined with the Big Dye Terminator v3.1 Cycle Sequencing Kit 3.1 (Applied Biosystems, Foster City, CA). The reaction conditions were as follows: one cycle of 96°C for 2 min, followed by 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The PCR product was purified with CleanSEQ (Beckman Coulter, Brea, CA) and analyzed using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Virus inoculation

Indian-origin rhesus macaques were used in accordance with the institutional regulations approved by the Committee for Experimental Use of Nonhuman Primates of the Institute for Virus Research, Kyoto University, Kyoto, Japan. Monkeys were housed in a biosafety level-3 facility and all procedures were performed in this facility. Blood collection, rectal biopsy, and virus intrarectal inoculation (10,000 TCID₅₀ of MK38#818) were performed under ketamine hydrochloride (Daiichi Sankyo, Tokyo, Japan). The virus was inoculated into the anus as described previously and then stood for 30 min (Miyake *et al*, 2006). The plasma viral load was measured by quantitative

RT-PCR as previously reported (Miyake *et al.*, 2006). The detection limit is 200 copies/ml.

Rectal biopsy

In total, 10 pieces of rectal tissue were collected using biopsy forceps (Olympus, Tokyo, Japan). Rectal tissue was washed with MACS buffer (Miltenyi Biotec, Bergisch Gladbach, Germany) and then incubated in pre-incubation buffer [1% BSA, 10 mM HEPES (Nacalai Tesque), 1 mM DTT (Sigma-Aldrich) in HBSS (Nacalai Tesque)] at 37°C for 30 min with mild shaking. Rectal tissue was washed with MACS buffer and then with 10 mM HEPES in HBSS. Rectal tissue was then digested using a Lamina Propria Dissociation Kit (Miltenyi Biotec GmbH) in a C tube (Miltenyi Biotec GmbH) at 37°C for 30 min using the m_brain_01_01 program of gentleMACS Octo Dissociator (Miltenyi Biotec). Rectal tissue was then processed using the m_intestine_01 program of gentleMACS Octo Dissociator (Miltenyi Biotec). Rectal mononuclear cells were collected through a 70-µm strainer (BD PharMingen, Tokyo, Japan).

Flow cytometry

To analyze total CD4⁺ T lymphocytes, naïve CD4⁺ T lymphocytes (CD4⁺CD28⁺CD95⁻), and memory CD4⁺ T lymphocytes (CD4⁺CD28⁻CD95⁺) (Pitcher *et al.*, 2002), whole blood and rectal samples were stained with seven fluorescent antibodies: Pacific Blue-conjugated anti-human CD3 antibody (Clone SP34-2, BD PharMingen), PerCP-Cy5.5-conjugated anti-human CD4 antibody1 (Clone L200, BD PharMingen), allophycocyanin-Cy7 (APC-Cy7)-conjugated anti-human CD8 antibody (Clone SK1, BD PharMingen), fluorescein isothiocyanate (FITC)-conjugated anti-human CD20 antibody (Clone L27, BD PharMingen), phycoerythrin (PE)-conjugated anti-human CD28 antibody (Clone CD28.2 BD PharMingen), and APC-conjugated anti-human CD95 (Clone DX2, BD PharMingen). Whole blood and rectal samples were reacted with lysing solution (BD BioScience, San Diego, CA) for 10 min to lyse red blood cells. Each sample was then analyzed with a FACS Canto II analyzer (BD BioScience). The absolute numbers of total CD4⁺ T lymphocytes, naïve CD4⁺ T lymphocytes, and memory CD4⁺ T lymphocytes in peripheral blood were calculated using KX-21 (Sysmex, Kobe, Japan).

Determination of antibody titers

Anti-HIV antibody titers were determined using a commercial particle agglutination kit (Serodia-HIV1/2; Fujirebio). Isolated plasma samples were serially diluted and assayed as described previously (Inaba *et al.*, 2010). The end point of the highest dilution giving a positive result was determined to be the titer.

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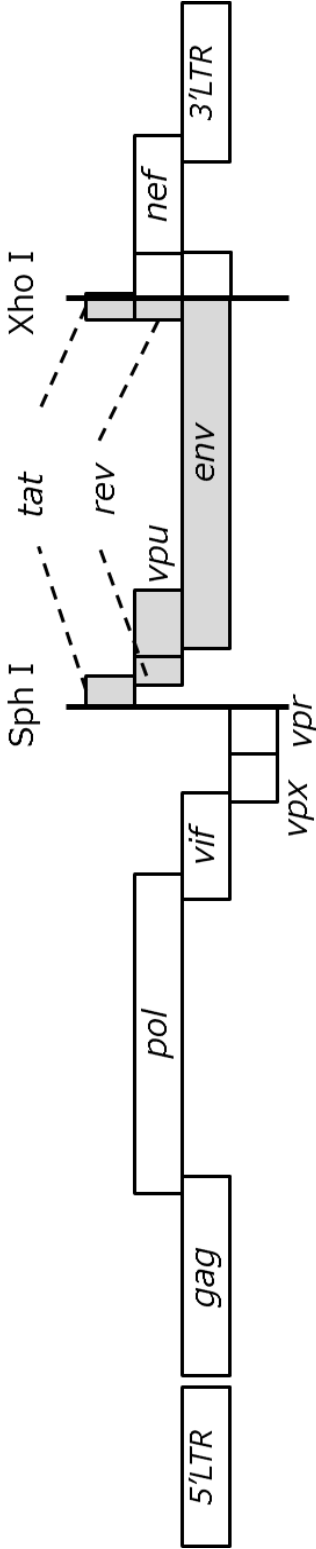


Fig. 2-1 Genomic organization of SHIV-MK38 molecular clones. SHIV-MK38 molecular clones were generated by replacing the *Sph*I-*Xho*I fragment of SHIV-MK38 with the equivalent region of SHIV-KS661. Gray and white indicate the regions derived from SHIV-MK38 and SHIV-KS661, respectively.

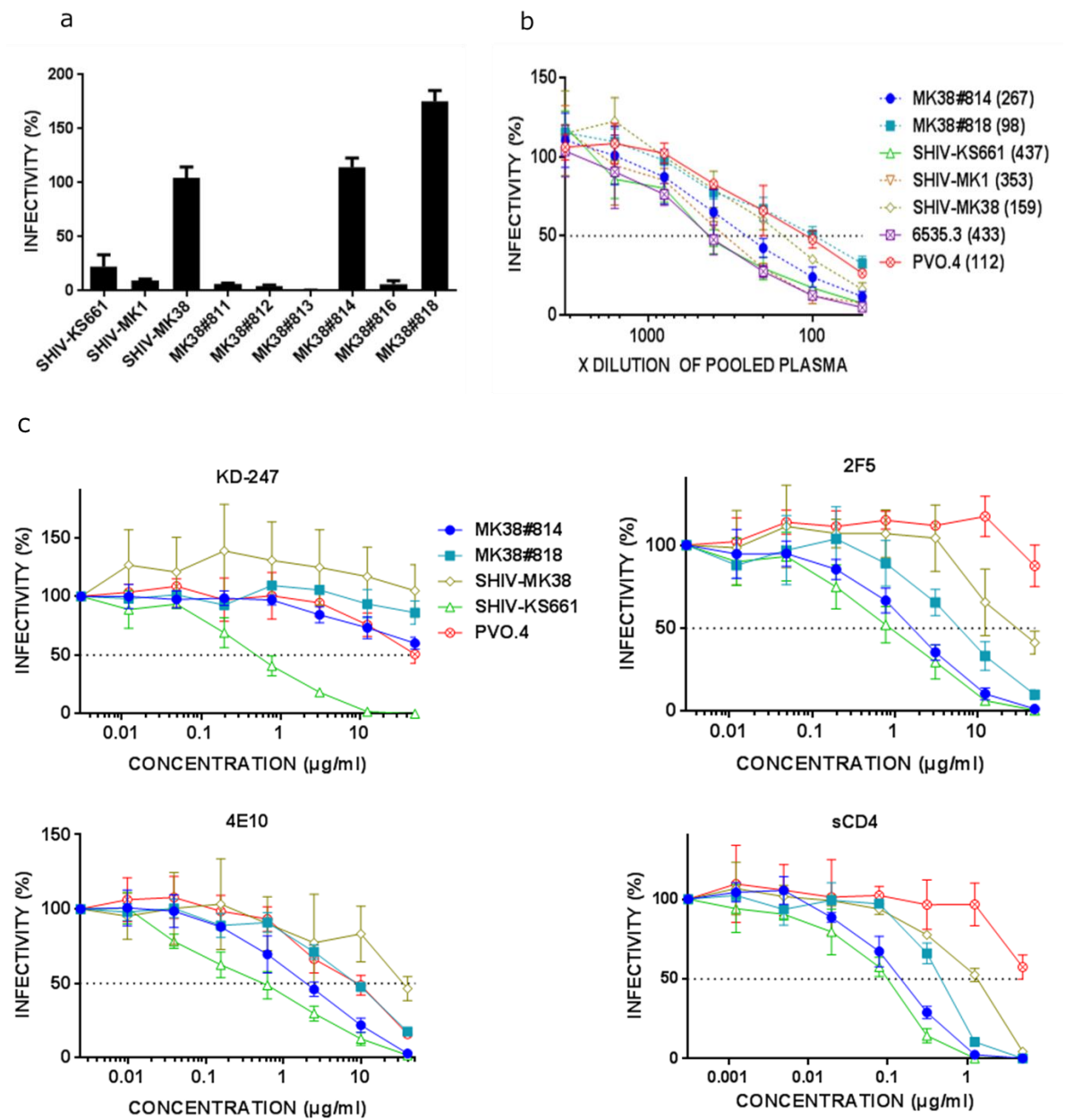


Fig. 2-2 Analysis of the neutralization resistance of each virus. (a) Screening of SHIV-MK38 clones that reflected the neutralization resistance of SHIV-MK38. After pre-incubating 100 TCID₅₀ of each virus and 16-week post-inoculation plasma of SHIV-MK38-infected monkeys, TZM-bl cells were cultured with the mixture for 48 h and luciferase activity was measured. Plasma was diluted 1:40. Infectivity in the presence of 0-week post-inoculation plasma was defined as 100%. (b) Neutralization resistance of each virus to pooled plasma of HIV-1-infected individuals. After pre-incubating 100 TCID₅₀ of each virus and pooled plasma, TZM-bl cells were cultured with the mixture at 37°C for 48 h and their luciferase activities were measured. Pooled plasma was diluted two-fold from 1:50 to 1:3200. The value in brackets represents the 50% inhibition dilution (ID₅₀). (c) Analysis of the neutralization resistance of each virus to three kinds of anti-HIV-1 neutralizing monoclonal antibody and sCD4. After pre-incubating 100 TCID₅₀ of each virus and each HIV-1 neutralizing monoclonal antibody or sCD4, TZM-bl cells were cultured with the mixture for 48 h, after which luciferase activity was measured. KD-247 and 2F5 were diluted four-fold from 50 to 0.012 µg/ml, 4E10 was diluted four-fold from 40 to 0.010 µg/ml and sCD4 was diluted four-fold from 5 to 0.0003 µg/ml.

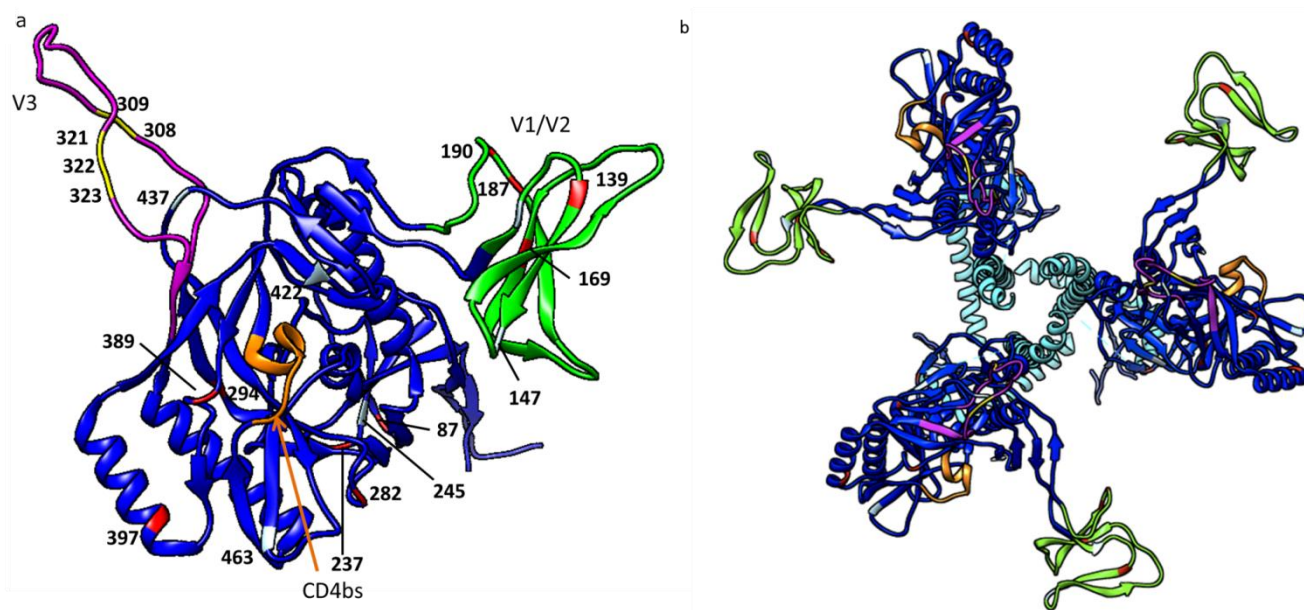


Fig. 2-3 Model of gp120 (a) and trimeric structure of Env (b).

The model is cited from PDB:3J70 (Rasheed *et al.*, 2015) and visualized by UCSF Chimera software. The mutations associated with MK38#814 and MK38#818 were fitted into the model. The part of gp120 and gp41 are colored with blue and cyan, respectively. The domains of V1/V2, V3 and CD4bs are highlighted with green, magenta and orange colors, respectively. The mutation sites associated with co-receptor switching in V3 are indicated with yellow color. The mutation sites associated with PNGS and/or net charge are indicated with red color. Other mutation sites are indicated with light blue.

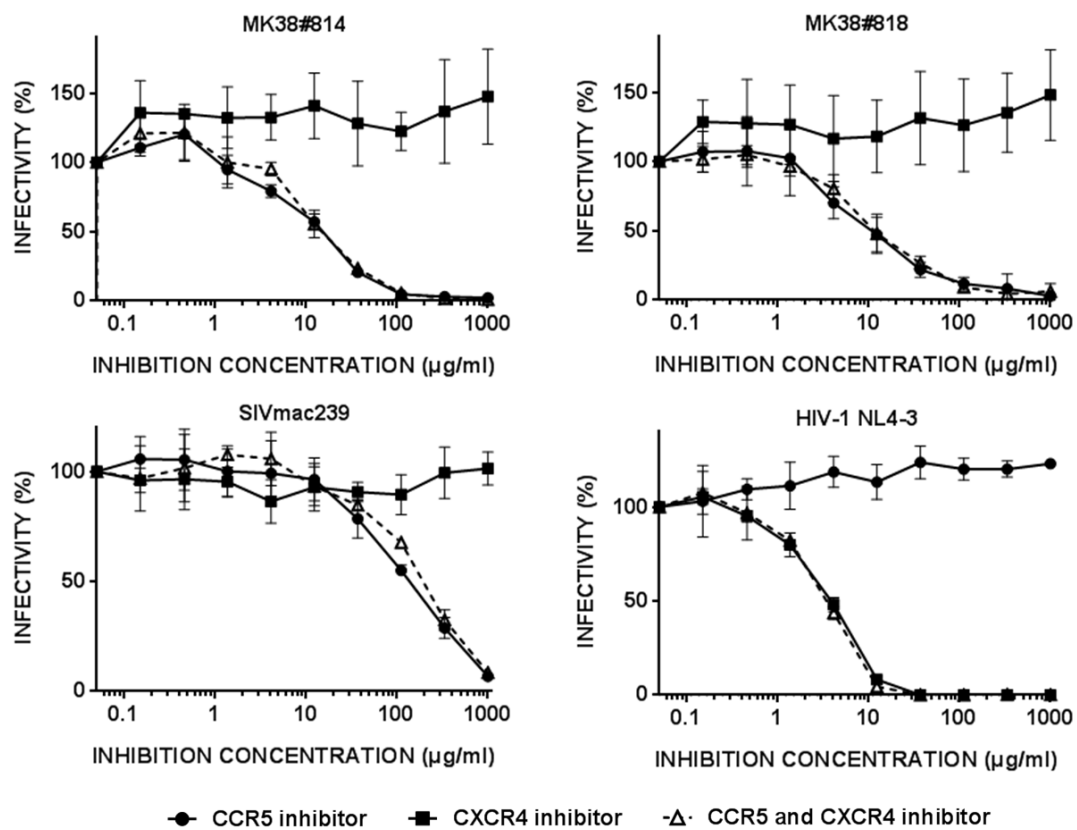


Fig. 2-4 Co-receptor tropism of SHIV-MK38 clones.

After pre-incubating small-molecule inhibitors and TZM-bl cells, 100 TCID₅₀ of each virus was infected with the TZM-bl cells, and luciferase activity was measured. Small-molecule inhibitors were diluted three-fold from 1000 to 0.005 μM; 100% infectivity was defined as the absence of small-molecule inhibitors.

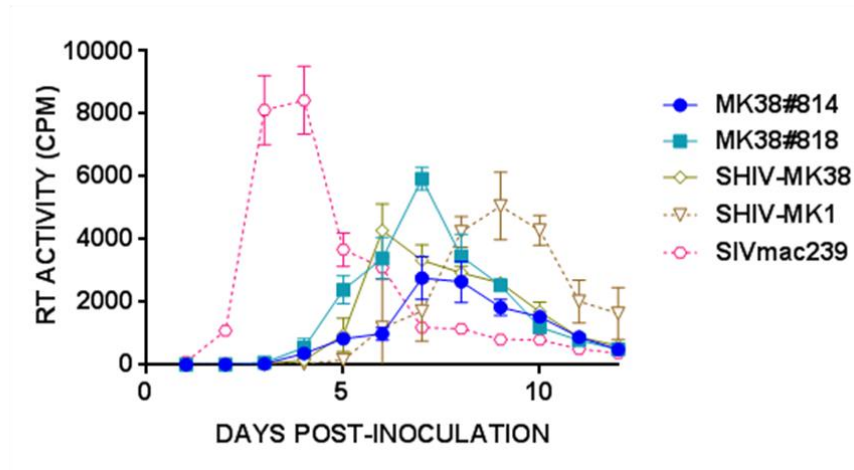


Fig. 2-5 *In vitro* viral replication in rhesus macaque PBMCs (rhPBMCs).

Each virus was spinoculated with rhPBMCs at multiplicity of infection (MOI) = 0.1.

Reverse Transcriptase (RT) activity in culture supernatants was measured up to 12 days post-inoculation.

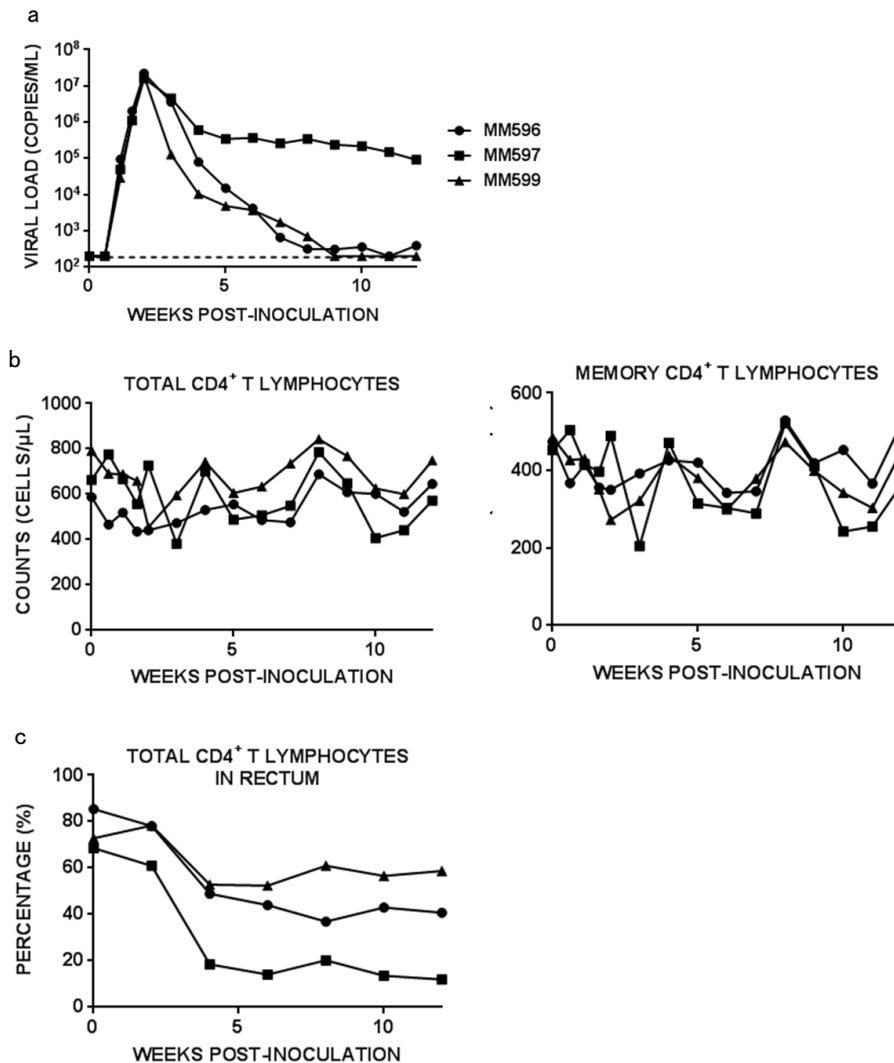


Fig. 2-6 Experimental infection of MK38#818 in rhesus monkeys.

(a) Plasma viral loads were measured at various time points. The detection limit is 200 copies/ml. (b) Kinetics of circulating CD4⁺ T lymphocytes. The absolute number of circulating CD4⁺ T lymphocytes was measured. (c) Kinetics of the percentage of CD4⁺ T lymphocytes in rectal mononuclear cells. Rectal tissue was collected by biopsy forceps every 2 weeks. Isolated rectal mononuclear cells were analyzed to measure the percentage of CD4⁺ T lymphocytes.

Table 2-1. List of mutations in *env* gene

Site	SHIV-KS661	SHIV-MK1	MK38#814	MK38#818	Region	Remarks
87	G	G	G	E	C1	negative
133	N	N	S	S	V1	
139	N	N	D	N	V1	negative loss
147	S	S	G	G	V1	
169	N	N	D	D	V2	negative
187	K	K	E	E	V2	negative
190	S	S	N	N	V2	shift
237	N	N	D	T	C2	negative loss
245	V	V	I	V	C2	
282	D	D	N	N	C2	positive
294	S	S	S	F	C2	loss
308	E	K	K	K	V3	co-receptor
309	R	S	S	S	V3	co-receptor
321	R	T	T	T	V3	co-receptor
322	R	G	G	G	V3	co-receptor
323	N	D	D	D	V3	co-receptor
389	A	A	T	T	V4	addition
397	N	N	D	D	V4	negative
422	N	N	T	N	C4	
437	T	T	A	A	C4	
463	T	T	T	P	V5	
555	R	R	K	R	gp41	positive

Gray boxes represent the change of net charges.

Dark gray boxes represent the change of N-glycosylation sites.

White boxes represent the mutations involved in changing co-receptor usage.

GenBank accession number of MK38#814 and MK38#818 are LC108991 and LC108992, respectively.

Neutralization sensitive SHIV-MK38 clone, MK38#812, was also registered to GenBank (GenBank accession number LC108990)

Table 2-2. Anti-HIV antibody titers in infected monkeys

Time (weeks)	MM596	MM597	MM599
0	<32 (-)	<32 (-)	<32 (-)
2	<32	<32	<32
4	512	<32	<32
6	512 (-)	2048 (34.1)	256 (-)
8	1024	4096	512
10	2048	4096	1024
12	4096 (-)	16384 (208.4)*	1024 (-)

The values of the brackets show ID₅₀ of HIV-1 reference strain, NF462.

- in the brackets represents not detected.

Asterisk represents the weak neutralization activity against SHIV-KS661 and MK38#818.

The ID₅₀ values were 1:33.2 and 1:49, respectively.

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