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Infection of Monkeys by Simian-human Immunodeficiency Viruses with Transmitted/ founder Clade C HIV-1 Envelopes

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Abstract

Simian-human immunodeficiency viruses (SHIVs) that mirror natural transmitted/founder (T/F) viruses in man are needed for evaluation of HIV-1 vaccine candidates in nonhuman primates. Currently available SHIVs contain HIV-1 *env* genes from chronically-infected individuals and do not reflect the characteristics of biologically relevant HIV-1 strains that mediate human transmission. We chose to develop clade C SHIVs, as clade C is the major infecting subtype of HIV-1 in the world. We constructed ten clade C SHIVs expressing Env proteins from T/F viruses. Three of these ten clade C SHIVs (SHIV KB9 C3, SHIV KB9 C4 and SHIV KB9 C5) replicated in naïve rhesus monkeys. These three SHIVs are mucosally transmissible and are neutralized by sCD4 and several HIV-1 broadly neutralizing antibodies. However, like natural T/F viruses, they

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exhibit low Env reactivity and a Tier 2 neutralization sensitivity. Of note, none of the clade C T/F SHIVs elicited detectable autologous neutralizing antibodies in the infected monkeys, even though antibodies that neutralized a heterologous Tier 1 HIV-1 were generated. Challenge with these three new clade C SHIVs will provide biologically relevant tests for vaccine protection in rhesus macaques.

Keywords

SHIV; HIV-1 Clade C; Transmitted/founder Env; Mucosal transmission

INTRODUCTION

Chimeric simian-human immunodeficiency viruses (SHIVs) were developed for studies of pathogenicity and preclinical assessment of candidate HIV-1 vaccines in nonhuman primate (NHP) models (Sato and Johnson, 2007). Chimeric viruses that contain HIV-1 *tat, rev, vpu* and *env genes,* with the remainder of the virus originating from the simian immunodeficiency virus (SIV), have been used as challenge viruses to assess the ability of HIV-1 envelope glycoprotein (Env)-based vaccines to elicit antibodies that prevent infection. However, currently available SHIV challenge stocks have limitations. Many HIV-1 Envs do not produce viable viruses when introduced into the SIV backbone. One of the first SHIVs to be generated that replicated robustly in rhesus monkeys and caused an AIDS-like illness was SHIV-89.6P (Reimann et al., 1996a; Reimann et al., 1996b). However, this dual-tropic (CXCR4/CCR5-tropic) virus exhibited *in vivo* preference for CXCR4, unlike the CCR5-tropism of transmitted HIV-1 variants; thus, in infected monkeys, SHIV 89.6P preferentially targeted naïve CD4+ T cells, a situation very different from acute HIV-1 infection in humans (Igarashi et al., 2003; Nishimura et al., 2004). SHIVs with exclusively CCR5-tropic envelopes have been generated; however viral loads and CD4+ T cell loss in animals infected with these SHIVs have been variable (Pahar et al., 2007; Pal et al., 2003; Parren et al., 2001; Tan et al., 1999).

The Envs of currently utilized SHIVs for which challenge stocks are available, such as SHIV SF162P3 (Harouse et al., 1999) and SHIV BaLP4 (Pal et al., 2003), were isolated from individuals chronically infected with HIV-1. As a result, these Envs were exposed to extensive humoral and cellular immune pressure within the infected individuals from whom they were isolated. Moreover, to achieve consistency and higher replicative efficiency *in vivo*, these SHIVs have been extensively passaged in monkeys. There is evidence from HIV-infected humans and SIV-infected rhesus monkeys that viral *env* genes accrue mutations during the course of infection that allow them to escape from autologous neutralizing antibodies (Mikell et al.; Moore et al., 2009; van Gils et al.; Yeh et al.). It is thus likely that the neutralization sensitivities of the chronic Envs used in current SHIVs are different from those of the transmitted/ founder (T/F) viruses that establish infections in humans, and use of SHIVs that contain such chronic Envs may bias the results of antibody protection studies in NHP.

A recent manuscript describes development of a new CCR5-tropic SHIV expressing T/F Env from HIV-1 Clade B (Del Prete et al., 2014). Both dual-tropic and CCR5-tropic SHIVs containing Envs from clade C HIV-1 have previously been reported (Cayabyab et al., 2004; Chen et al., 2000; Humbert et al., 2008; Ren et al., 2013; Siddappa et al., 2009; Song et al., 2006). Some of these CCR5-tropic, clade C SHIVs encoded *env* genes that were isolated from recently infected subjects (Humbert et al., 2008; Ren et al., 2013). However, these SHIVs have been passaged extensively in monkeys. Therefore, the envelopes encoded in these SHIVs may have undergone sequence alterations compared with the parental envelopes in the T/F viruses.

We hypothesized that SHIVs containing T/F HIV-1 *env* genes would be able to better recapitulate the mucosal transmission physiology of acute HIV infection, and thus more accurately reflect the sensitivity of transmitted HIV-1 Envs to antibody-mediated neutralization. Approximately 80% of individuals who are infected via heterosexual contact are infected by one founder virus (Keele et al., 2008). One of the early pathogenic SHIVs, KB9, began with the introduction of *tat, rev, vpu* and *env* genes from a chronic clade B HIV-$1_{89.6}$ into the SIVmac239 backbone. The resulting virus was passaged in monkeys to produce the pathogenic SHIV 89.6P; SHIV KB9 is an infectious, pathogenic molecular proviral clone derived from SHIV 89.6P-infected cells (Karlsson et al., 1997). Because KB9 was a viable SHIV, we utilized the KB9 architecture to generate novel SHIVs with the *env* genes of CCR5-tropic, Clade C T/F HIV-1 from acutely infected individuals from South Africa. Three clade C SHIVs with T/F Envs that are phylogenetically diverse infected rhesus macaques after atraumatic mucosal exposure. These three clade C SHIVs are the first clade C SHIVs that encode *env* genes isolated from T/F viruses at the earliest stages of infection (Parrish et al., 2012). Clade C is the major infecting subtype of HIV-1 infections globally. Our results provide insights into the ability of Clade C T/F Envs to mediate mucosal transmission, to generate neutralizing antibodies, and to establish persistent infections in primates. Development of these three clade C SHIVs containing phylogenetically diverse T/F *env* genes will greatly expedite pre-clinical vaccine efficacy studies as well as monoclonal antibody passive infusion studies in the rhesus monkey model.

MATERIALS AND METHODS

Construction of Clade C T/F SHIVs

We utilized *env* genes from ten clade C T/F viruses from South Africa (C1-19912872, C2-21197826, C3-21283649, C4-20927783, C5-1245045, C6-20258279, C7-19157834, C8-20915593, C9-20965238, and C10-21197826) to construct SHIVs. Some of these T/F viruses (C3-C7) have already been reported (Parrish et al., 2012). Clones C1, C2, C8, C9 and C10 were also isolated from clade C-infected subjects at Fiebig stage I/II of infection (personal communication). GenBank accession numbers for all ten viruses are provided in Table S1 of the Supplemental Material. These ten clade C T/F *env* genes were cloned into the SHIV KB9 backbone. A *Cla1* restriction site was introduced immediately upstream of the *env* ATG and an Age1restriction site upstream of the 3' gp41 HIV-SIV recombination junction of SHIV KB9. *Cla1* and *Age 1* restriction sites were introduced into the T/F *env* sequences as well to facilitate cloning of the T/F *env* in SHIV KB9. As a result of this

cloning strategy, exon 2 of *tat, rev* and 3' half of *vpu* of SHIV KB9 were replaced by those of the new T/F virus. However, exon 1 of *tat*, *rev* as well as the 5'end of *vpu*, which lie outside *env*, remained from SHIV KB9 (Fig 1). However, only seven of the viruses (C1 through C7) were replication competent, whereas, viruses constructed with *env* genes from C8, C9 and C10 were not.

Study animals

A PCR-based assay was used to select adult Indian-origin rhesus monkeys that were *Mamu-A*01-ve*, *B*08-ve*, *B*17-ve*, and Trim5α heterozygous permissive (Barouch et al., 2000; Letvin et al., 2011). Monkeys were housed at the New England Primate Research Center, Southborough, MA and at Bioqual, Inc, Rockville, MD. All monkeys were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals*. All animal protocols were approved by the Institutional Care and Use Committee.

Viral Isolation and Sequencing

Viral RNA was extracted from monkey plasma using the QIAamp Viral RNA Isolation Kit (Qiagen) and reverse transcribed using SuperScript III (Invitrogen) with the primer *rt1* (GATTGTATTTCTGTCCCTCAC), which recognizes a sequence immediately downstream of the stop codon of the HIV *env* gene of SHIV KB9 (Genbank accession U89134). The viral genomic fragment from the *tat* start codon to the *env* stop codon was amplified using Easy-A High Fidelity (Agilent Technologies) with the primers *p1* (CTAGAAGCATGCTGTAGAGCAAG) and *p2* (GATTGTATTTCTGTCCCTCAC). The RT-PCR product was sequenced by Genewiz, Inc. (South Plainfield, NJ).

Generation of Infectious Virus and Animal Inoculation

Plasmids containing the infectious molecular clones were transfected into 293T cells using Lipofectamine 2000 (Invitrogen). Cell culture supernatants were collected after 48 hours and clarified through a 0.4 micron filter. These supernatants were then used to infect CD8+ T cell-depleted human peripheral blood mononuclear cells (PBMCs). Briefly, PBMCs were isolated from the whole blood of healthy human donors by Ficoll-Hypaque centrifugation and stimulated with 6.25 μg/ mL concanavalin-A (Con-A) and 20 U/ml interleukin-2 (IL-2). The following day, PBMCs were depleted of CD8+ T cells using the CD8+ T cell depletion kit (Stem Cell Technologies). 100 μL of transfected 293T cell supernatant was applied to every 10 million cells, and cells were incubated for between 7 and 10 days. Infected PBMC supernatants were then harvested, clarified of cellular debris by 0.4 micron filtration, and stored at −80 C. Virus was quantified by SIV p27 ELISA (Zeptometrix), and the 50% tissue culture infectious dose ($TCID_{50}$) was determined on TZM-bl cells. *Mamu-A*01-*, *B*08-*, *B*17-*, Trim5α heterozygous permissive animals were inoculated intravenously with 1000 $TCID_{50}$ virus.

Generation of challenge stocks of SHIV KB9 C3, SHIV KB9 C4, and SHIV KB9 C5

Virus challenge stocks of the three clade C SHIVs with T/F Envs were prepared by co-culturing naïve rhesus monkey peripheral blood mononuclear cells (PBMC) with PBMC and lymph node cells from the infected monkeys. At the peak of viremia, 20–30 ml of blood and

lymph node biopsies were taken from the monkeys infected intravenously with SHIV KB9 C3, SHIV KB9 C4 and SHIV KB9 C5. PBMC and lymph node cells from each monkey were enriched for CD4+ T lymphocytes by depleting CD8+ T lymphocytes and were cultured separately in RPMI 1640 supplemented with 10% fetal bovine serum, 6.25 μg/ml Con-A and 20 U/ml IL-2. Forty-eight hours later, the cells were washed and resuspended in RPMI 1640 supplemented with 10% fetal bovine serum, and 20 U/ml IL-2. PBMC from naïve rhesus monkeys that had been stimulated with Con-A were mixed with the PBMC from the infected monkeys at a 1:1 ratio. Virus replication was monitored every other day by measuring the p27 content of the culture supernatants. Supernatants were harvested from days 14–21 of the cultures. The cell-free culture supernatants were frozen in small aliquots to be used as virus challenge stocks for nonhuman primate studies. The viral RNA content and the infectivity of the stocks were determined for all three challenge stocks (Table 1).

CD4+ T lymphocyte subset analyses

CD4+ T lymphocyte subsets were determined by multi-channel flow cytometry for CD3, CD4, CD8, CD28, CD95, CCR5 and CCR7. $CD4^+$ T lymphocyte counts were calculated by multiplying the total lymphocyte count by the percentage of $CD3^+$ $CD4^+$ T cells. Briefly, 100 μl of EDTA-anticoagulated whole blood was stained with anti-CD3-A700 (clone SP34.2), anti-CD4-PerCP Cy5.5 (clone L200), anti-CD8-APC H7 (clone SK1), anti-CCR5-PE (clone 3A9), anti-CD95 APC (clone DX2) all from BD Biosciences, anti-CD28-PE CY7 (clone CD28.2; eBiosciences), and anti-CCR7-FITC (clone 150503; R&D Systems). Fixed cells were collected (30,000 events) on a LSRII instrument using FACSDiva software version 6.1.1 (BD Biosciences) and data were analyzed using FlowJo Software (TreeStar, Ashland, OR).

Plasma Viral RNA Measurement

Plasma viral RNA measurements were performed at CHAVI Viral Core Laboratory, IVQAC, Duke Human Vaccine Institute, Durham, NC. Plasma viral loads were assessed using a Qiagen QIAsymphony DSP Virus/Pathogen Midi Kit using the QIAsymphony SP platform and real-time PCR reaction carried out on the StepOnePlus (Applied Biosystems) instrument. Data from the real-time PCR reaction was analyzed using the StepOnePlus software. The sensitivity of this SIV viral load assay has been shown to be 250 copies per ml.

Neutralization Assays

Neutralizing antibody titers against primary SHIV isolates were measured using a luciferase-based assay in TZM.bl cells as previously described (Montefiori, 2005; Sarzotti-Kelsoe et al., 2013). This assay measures the reduction in luciferase reporter gene expression in TZM.bl cells following infection. Briefly, 3-fold serial dilutions of monoclonal antibody (mAb) reagents or plasma samples were performed in duplicate, and the 50% inhibitory concentration (IC50) titer was calculated as the dilution that caused 50% reduction in relative luminescence units (RLU) compared to virus control wells after subtraction of cell control RLU. All data were analyzed using 5-parameter curve fitting. mAbs 4E10, 2G12, 2F5, b12, PG9, PG16, and VRC01 were obtained commercially (Polymun Scientific), as was soluble human CD4 protein (Progenics). 3BNC117 was kindly provided by Michel

Nussenzweig (Rockefeller University, New York, NY). PGT121, PGT128, PGT145, and PGT151 were generously provided by Dennis Burton (The Scripps Research Institute, La Jolla, CA). Purified Ig were obtained from clade C HIV+ plasma samples purchased from the South African National Blood Services (Johannesburg) and provided by Dr. Lynn Morris.

RESULTS

In vivo selection of clade C T/F env expressing SHIVs

Ten different T/F HIV clade C *env* clones C1 through C10 were isolated from acutely infected subjects sampled during Fiebig stages I/II of infection (Fiebig et al., 2003) as part of a large cohort. These envelopes were shown to be CCR5-tropic (Parrish et al., 2012). The phylogenetic relationships of these ten *env* genes are shown in Fig. 2. As described in the Materials and Methods section, seven of these ten clade C T/F *env* genes (C1 through C7) when cloned into the SHIV KB9 backbone generated replication-competent SHIVs. Infectious virus was generated *in vitro* through transfection of 293T cells, followed by expansion in human PBMCs. Two *Mamu-A*01-*, *B*08-*, *B*17-*, naïve Indian-origin rhesus monkeys were selected for this study. All seven clade C T/F SHIVs (SHIV KB9 C1 through SHIV KB9 C7) were pooled in equal ratios at 100 $TCID_{50}$ for each virus and the virus mixture was intravenously inoculated into two animals, which were followed for the development of plasma viremia. Both the animals developed viremia of 1×10^6 RNA copies/ml by 2 weeks post-inoculation (Fig 3). One of the monkeys (A8V032) controlled SHIV replication, with viremia dropping to undetectable levels by 49 days following inoculation. The other monkey (A8V056) maintained a persistent viremia of 1×10^5 RNA copies/ml up to 130 days post-infection. From the pool of seven T/F SHIVs that were inoculated in these two monkeys, only SHIV KB9 C3, SHIV KB9 C4 and SHIV KB9 C5 were identified in the plasma of monkey A8V056 at day 14. A total of 19 clones were isolated from the plasma of monkey A8V056 on day 14 post-infection; 4 of the 19 clones matched the sequence of SHIV KB9 C3, 14 clones matched the sequence of SHIV KB9 C4 and 1 clone matched the sequence of SHIV KB9 C5. All seven clones isolated from the plasma of monkey A8V056 on day 49 post-infection corresponded to SHIV KB9 C3. SHIV KB9 C3 (2 of 10 clones) and SHIV KB9 C5 (8 of 10 clones) were also detected in that same animal at day 98 post-infection. The sequences of all five clones isolated from monkey A8V032 at day 14 post-infection matched those of SHIV KB9 C4. Sequencing of the isolated clones of SHIV KB9 C3, SHIV KB9 C4 and SHIV KB9 C5 viruses confirmed that no nucleotide changes had occurred by day 98 of infection, compared with the parental clones.

SHIV KB9 C3, SHIV KB9 C4, and SHIV KB9 C5 infection of rhesus monkeys

To confirm the infectivity and persistence of viremia of the three clade C viruses isolated from the pool of seven SHIVs, infectious molecular clones of these three SHIVs were transfected into 293T cells to generate viruses. Previous observations in our laboratory have shown that both SIV as well as SHIVs replicate to higher titers in human PBMC compared to rhesus monkey PBMCs. Therefore, we generated infectious stocks of all three clade C SHIVs by culturing them in human PBMCs for 7 to 14 days, and then harvesting

supernatants for infection of rhesus monkeys. All three challenge stocks were sequenced again. There was no change in nucleotide sequences for SHIV KB9 C4 and SHIV KB9 C5. In SHIV KB9 C3 there was an A→C change in position 2524.

A total of six *Mamu-A*01-, B*08-, B*17-*, Trim5α-heterozygous permissive Indian-origin rhesus macaques were inoculated intravenously with 1000 $TCID_{50}$ of either SHIV KB9 C3, SHIV KB9 C4 or SHIV KB9 C5 (n=2 per virus). All six animals became infected with a peak viremia of at least 10^6 RNA copies/ml by 17 to 21 days after inoculation (Fig 4A). Both monkeys inoculated with SHIV KB9 C3 maintained a persistent viremia of 10^3 RNA copies/ml up to 497 days post-infection. Monkeys inoculated with SHIV KB9 C4 maintained a persistent viremia of 10^3 RNA copies/ml up to 190 days post-infection. One of the monkeys, 42–12, controlled infection around day 300 with undetectable viremia. Both monkeys inoculated with SHIV KB9 C5 maintained a persistent viremia of 10^3 RNA copies/ml up to 190 days post-infection. Monkey 50–12 still has persistent viremia of almost 10^4 RNA copies/ml at 497 days post-infection. No significant decline in total peripheral CD4+ T lymphocyte numbers was detectable in these animals (Figure 4B). Also no decline in memory CD4+ T lymphocytes was observed in these animals (Fig S1).

Mucosal transmissibility of SHIV KB9 C3, SHIV KB9 C4, and SHIV KB9 C5 challenge stocks

Challenge stocks of the three clade C SHIVs were generated as described in the Materials and Methods section. Viral RNA content of the all three stocks and the $TCID_{50}$ as measured on TZM-bl cells are described in Table 1. Sequence analyses of the viruses in the challenge stocks showed no change in nucleotide sequences from the parental viruses. We also confirmed that these three viruses are CCR5-tropic and not dual- or CXCR4-tropic (data not shown). Next we sought to determine the mucosal transmissibility of the challenge stocks of the three clade C SHIVs (Table 1). Undiluted stocks of SHIV KB9 C3 (267,185 $TCID_{50}$) and SHIV KB9 C5 (18,275 $TCID_{50}$) were inoculated into one naïve rhesus monkey each by the intrarectal route. An undiluted stock of SHIV KB9 C4 (53,437 $TCID_{50}$) was inoculated intrarectally into two naïve rhesus monkeys. As shown in Fig 5A, challenge stocks of both SHIV KB9 C3 and SHIV KB9 C5 were able to establish infection in the monkeys when infected by the mucosal route. Monkey 264-12, infected with SHIV KB9 C3, had a peak viremia of 10^8 RNA copies/ml at 14 days post-infection and persistent viremia of 10^3 RNA copies/ml up to 130 days. Monkey 266-12, infected with SHIV KB9 C5, had a peak viremia of 10^7 RNA copies/ml. However, the peak was delayed until day 35 post-infection, but the virus persisted longer than SHIV KB9 C3. At the last measurement at day 329 post-infection, 10^3 RNA copies/ml of virus were detected (Fig. 5A). The SHIV KB9 C4 challenge stock also infected two naïve rhesus monkeys when administered by the intrarectal route. Both monkeys had a peak viremia of $10^{6–7}$ RNA copies/ml between 14–22 days post-infection and persistent viremia of 10^3 RNA copies/ml up to 182 days post-infection (Fig. 5B). No significant change in the absolute number of peripheral total or memory CD4+ T lymphocytes was seen in these monkeys (Fig. S2).

Neutralization Phenotype of SHIV KB9 C3, SHIV KB9 C4, and SHIV KB9 C5

Primate immunodeficiency viruses vary in sensitivity to neutralization by antibodies, and have been classified into tiers accordingly (Seaman et al., 2010). The neutralization tiers of

the three clade C T/F SHIVs were evaluated using TZM-bl cells with a panel of monoclonal antibodies (Table 2). We assessed the neutralization sensitivity of SHIV KB9 C3, SHIV KB9 C4, and SHIV KB9 C5 challenge stock viruses in TZM.bl assays using a panel of broadly neutralizing mAbs (BNAbs) targeting multiple known epitopes on HIV-1 Env. While all three SHIV viruses could be neutralized using soluble human CD4 protein, variable sensitivity to a panel of four BNAbs targeting the CD4 binding site (CD4bs) was observed (Table 2). SHIV KB9-C3 was moderately sensitive to three of the CD4bs mAbs (b12, VRC01, 3BNC117), SHIV KB9-C5 was sensitive to one (VRC01), and SHIV KB9 C4 was resistant to all CD4bs BNAbs tested. All SHIV viruses were resistant to neutralization by BNAbs 4E10 and 2F5 that target the MPER-region of gp41, but demonstrated sensitivity to the more potent mAb 10E8. We used a series of 7 antibodies targeting either V3-glycan (2G12, PGT121, PGT128) or V1/V2 (CH01, PG9, PG16, PGT145) epitopes and observed moderately positive titers against SHIV KB9 C4 and SHIV KB9 C5, whereas SHIV KB9 C3 was resistant to all seven of these BNAbs.

In addition to using epitope-specific mAbs, we further assessed the overall relative neutralization sensitivity of the three SHIVs using a diverse set of purified Ig samples from HIV-1-infected individuals. All viruses were resistant to neutralization by a pooled polyclonal clade B HIVIG, but SHIV KB9 C3 and SHIV KB9 C5 were sensitive to a pooled polyclonal clade C HIVIG (Table 2). Six purified Ig samples from clade C HIV-1-infected individuals from South Africa known to exhibit broad and potent neutralizing activity against acute clade C isolates were also tested. All three SHIVs demonstrated moderate sensitivity or were resistant to neutralization by this panel of clade-matched Ig. IC50 titers of these mAbs and Ig reagents against a panel of four acute/early clade C tier 2 HIV-1 Env pseudoviruses are shown in Table 2 for comparative reference. Finally, we investigated whether the six rhesus monkeys infected with either SHIV KB9 C3, SHIV KB9 C4, or SHIV KB9 C5 developed autologous or heterologous neutralizing antibody responses against these isolates. Plasma samples from eight timepoints spanning one to twelve months post-infection were tested from each animal against all three strains of SHIV. Surprisingly, no plasma neutralizing activity was detected for up to one year post-infection, even against the autologous infecting strain (data not shown). Plasma samples from all animals did exhibit potent neutralizing activity against a sensitive clade C Tier1A HIV-1 Env pseudovirus (MW965.26), indicating that the animals did develop neutralizing antibodies following challenge. Together, these data indicate that, in the TZM.bl neutralization assay, SHIV KB9 C3, SHIV KB9 C4, and SHIV KB9 C5 exhibit a neutralization phenotype that is on the more resistant side of the sensitivity spectrum for primary HIV-1 isolates (Seaman et al., 2010).

Determination of 50% Monkey Infectious Doses (MID_{50}) of SHIV KB9 C3 and SHIV KB9 C5 Challenge Stocks

We also sought to determine the 50% monkey infectious doses (MID_{50}) for SHIV KB9 C3 and SHIV KB9 C5 challenge stocks, as these two viruses were neutralized by several monoclonal antibodies more efficiently than SHIV KB9 C4.

First 1:100 and 1:50 dilutions of the SHIV KB9 C3 stock were inoculated into two naïve, Indian-origin rhesus monkeys each by the intra-rectal route. At these dilutions, all four monkeys remained uninfected (data not shown). Next, three monkeys were challenged with a 1:10 dilution and two monkeys were challenged with a 1:5 dilution of the virus intra-rectally. Two of the three monkeys (monkeys R553, R566) challenged with the 1:10 dilution of the virus became infected at day 14 post-infection with a peak plasma viremia of $10^{7–8}$ log/ml, while monkey R552 remained uninfected (Fig. 6A). Both monkeys (R294, R546) challenged with the 1:5 dilution of the stock became infected with a peak viremia of $10^{6–7}$ log/ml. Monkey R294 became infected as early as day 7 post-infection, but exhibited a peak of viremia at day 14 (Fig. 6A). This shows the consistency of *in vivo* infectivity of the SHIV KB9 C3 challenge stock by the intra-rectal route. At a 1:5 dilution, this virus challenge stock is 100% infectious administered intra-rectally.

As the SHIV KB9 C5 challenge stock has a lower $TCID_{50}$ than the SHIV KB9 C3 stock, we challenged monkeys (n=2/dilution) with undiluted or with 1:10, 1:5, 1:2 dilutions of SHIV KB9 C5 intra-rectally. As shown in Fig 6B, both monkeys challenged with the 1:2 dilution of SHIV KB9 C5 became infected. Peak viremia in all four monkeys (R923, R545, R284, R544) infected with either the 1:2 dilution or undiluted virus was observed at day 14 with 10^7 log plasma viral RNA copies/ml. However, one of the two monkeys challenged with either the 1:10 or 1:5 dilution of the virus remained uninfected. The monkeys that became infected (R556 and R557) had a lower peak plasma viremia of 10^6 log copies/ml that was also delayed at day 21 post-infection (Fig 6B). These data show that the SHIV KB9 C5 challenge stock can infect 100% of the monkeys consistently by the intra-rectal route at a 1:2 dilution and 50% of the monkeys at a 1:5 or 1:10 dilution.

DISCUSSION

Nonhuman primate studies are extremely important for determining the efficacies of different vaccine vectors, immunogens, adjuvants and vaccination strategies before proceeding towards phase I clinical trials. Recent clinical trials in humans and preclinical studies in rhesus monkeys have suggested that antibodies against HIV-1 Env may have some protective effects (Florese et al., 2009; Hessell et al., 2009; Hidajat et al., 2009; Letvin et al.; Rerks-Ngarm et al., 2009). Furthermore, studies of mucosal transmission of HIV-1 infection have suggested that most transmission events are founded by a single or very limited number of viral variants (Abrahams et al., 2009; Keele et al., 2008). These two findings prompted us to undertake the development of a SHIV encoding a transmitted/founder Env from Clade C HIV-1 isolates, which are responsible for the majority of HIV-1 infections worldwide.

We produced 7 replication-competent SHIVs containing Envs from Clade C T/F HIV-1. The Envs encoded in these viruses were directly cloned from HIV-1-infected subjects at Fiebig stage I/II of infection, and correspond to the phylogenetic root of the swarm of early circulating viruses. The Envs in these SHIVs thus correspond precisely to those of T/F HIV-1. Based on prior experience, we assumed that many HIV-1 *env* genes, when inserted into an SIV backbone, would not yield a pathogenic SHIV. Thus, we used a novel approach to simultaneously generate and assess *in vivo* multiple SHIVs with different HIV-1 Envs. To

increase the efficiency with which a pathogenic SHIV might be isolated, we pooled multiple recombinant viruses and inoculated them as a pool of viruses into rhesus monkeys. Our hypothesis that only a small minority of HIV-1 Envs would produce a viable SHIV was confirmed, as only three viable viruses could be isolated. Even a close phylogenetic relationship between viruses in the inoculum did not guarantee persistence in the monkeys. Our results establish that at least some Clade C T/F HIV-1 Env exhibit the ability to utilize rhesus macaque receptors efficiently. Recently, a SHIV expressing HIV-1 clade B T/F Env has been developed and reported (Del Prete et al., 2014). Although the same cloning and selection strategies that we describe in our study were used in that recent report, for *in vivo* selection of an infectious clone from a pool of SHIVs, the monkeys were depleted of their CD8+ T lymphocytes by pre-treatment with anti-CD8 antibody prior to inoculation with the pool of viruses. Depletion of CD8+ T lymphocytes *in vivo* may enhance infection and thereby selection of viruses. Because we did not manipulate the immune status of our monkeys, our study provides a more direct evaluation of the ability of a T/F SHIV to establish infections in macaques.

We confirmed that three SHIVs containing diverse clade C T/F Env can reliably establish infection in rhesus monkeys after intravenous or intrarectal inoculation. Viral loads in different animals infected with these SHIVs show very consistent peaks and sustained viremia for a period of at least 150 days. This course is comparable to that observed after infection with SIVmac251. All three SHIVs that established persistent infections in the monkeys exhibited low Env reactivity, and were relatively resistant to soluble CD4 and neutralizing antibodies (Tier 2 phenotype). Thus, low Env reactivity is compatible with the establishment of persistent infections *in vivo* (Haim et al., 2011). A lower level of inter-animal variability in post-infection viral loads could potentially simplify the interpretation of vaccination effects on post-transmission peak and set point viremia, and increase the power to detect differences between vaccine groups for given cohort sizes. The challenge stocks of these three T/F viruses were prepared from acutely infected monkeys, minimizing i*n vivo* passaging that can cause changes in genetic and biological properties of the viruses.

The low Env reactivity of T/F HIV-1 (Keele et al., 2008; Haim et al., 2011) likely contributes to the ability of viruses to avoid the host neutralizing antibody responses. Like most T/F HIV-1, the three Clade C SHIVs are relatively resistant to antibody neutralization. In the monkeys infected with these SHIVs, only very limited levels and types of neutralizing antibodies were elicited. Remarkably, no autologous neutralizing antibodies were detected in any of the Clade C T/F SHIV-infected monkeys for up to one year after infection. However, antibodies capable of neutralizing a heterologous Clade C HIV-1 with a highly reactive, Tier 1 Env were elicited in these monkeys. This same pattern of antibody generation was observed in monkeys infected with a Clade B SHIV, and shown to be related to the low Env reactivity, with respect to soluble CD4 and cold, of the infecting virus (McGee et al., 2014). Apparently, low Env reactivity not only influences virus sensitivity to neutralizing antibodies (Haim et al., 2011), but also modulates the structure of the Env antigens presented to the humoral immune system during natural infection. Presumably, Env elements commonly targeted by strainspecific neutralizing antibodies during early infection are less accessible and/or immunogenic in the Clade C T/F Envs in the three successful

SHIVs in this study. However, these Clade C T/F Envs still present entry-relevant conformations to the host immune system, allowing the generation of neutralizing antibodies with some breadth. Understanding the structural basis of these immunogenic properties of Clade C T/F Envs may guide the design of improved vaccine candidates.

Although significant declines in peripheral blood CD4+ T lymphocytes have not occurred to date in the monkeys infected with these SHIVs, it is premature to reach conclusions about the pathogenicity of these viruses. It is not unusual for monkeys that are persistently viremic with SHIV infections to succumb to AIDSlike illness 3–4 years after inoculation, and the monkeys in this study have been observed for only approximately 1.5 years. About 2/3 of the monkeys infected with these Clade C T/F SHIVs exhibit persistent viremia, so some of these monkeys may develop disease in time.

Development of these three viruses provides novel information about the *in vivo* behavior of viruses with Envs from well-documented T/F HIV-1 from Clade C. Clade C HIV-1 variants constitute the majority of viruses infecting humans in the current pandemic. Therefore, the behavior of Clade C T/F SHIVs *in vivo* is of great relevance to our understanding of the majority of new HIV-1 transmissions occurring in the world today. Thus, the availability of the clade C T/F SHIVs enables studies of virus transmission and immunogenicity, and should expedite the development of prophylactic approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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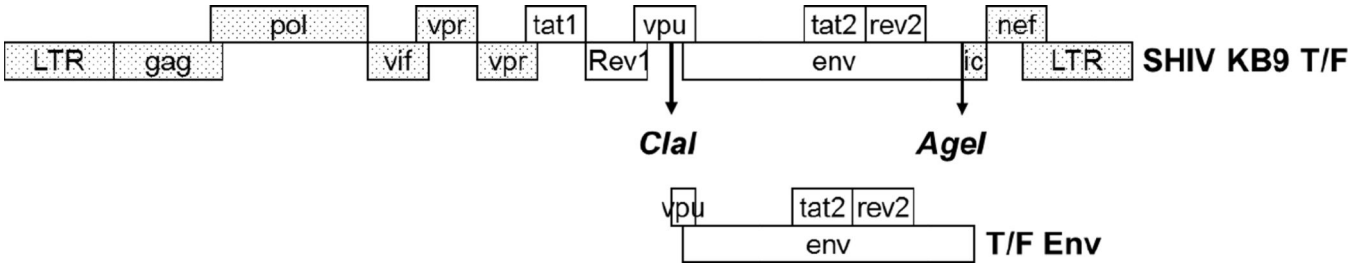

Figure 1.

Schematic diagram of T/F SHIVs constructs. T/F *env* genes were cloned into the SHIV KB9 backbone. A *Cla1* restriction site was introduced immediately upstream of the *env* ATG and an *Age1* restriction site upstream of the 3' gp41 HIV-SIV recombination junction of SHIV KB9. *Cla1* and *Age 1* restriction sites were introduced into the T/F *env* sequences as well to facilitate cloning of the T/F *env* in SHIV KB9. As a result of this cloning strategy, exon 2 of *tat, rev* and 3' half of *vpu* of SHIV KB9 were replaced by those of the new T/F virus and exon 1 of *tat, rev* as well as the 5'end of *vpu*, which lie outside *env*, remained from SHIV KB9. Genes from SHIV KB9 are shown in "Dotted" boxes and the cloned genes are shown in "White" boxes.

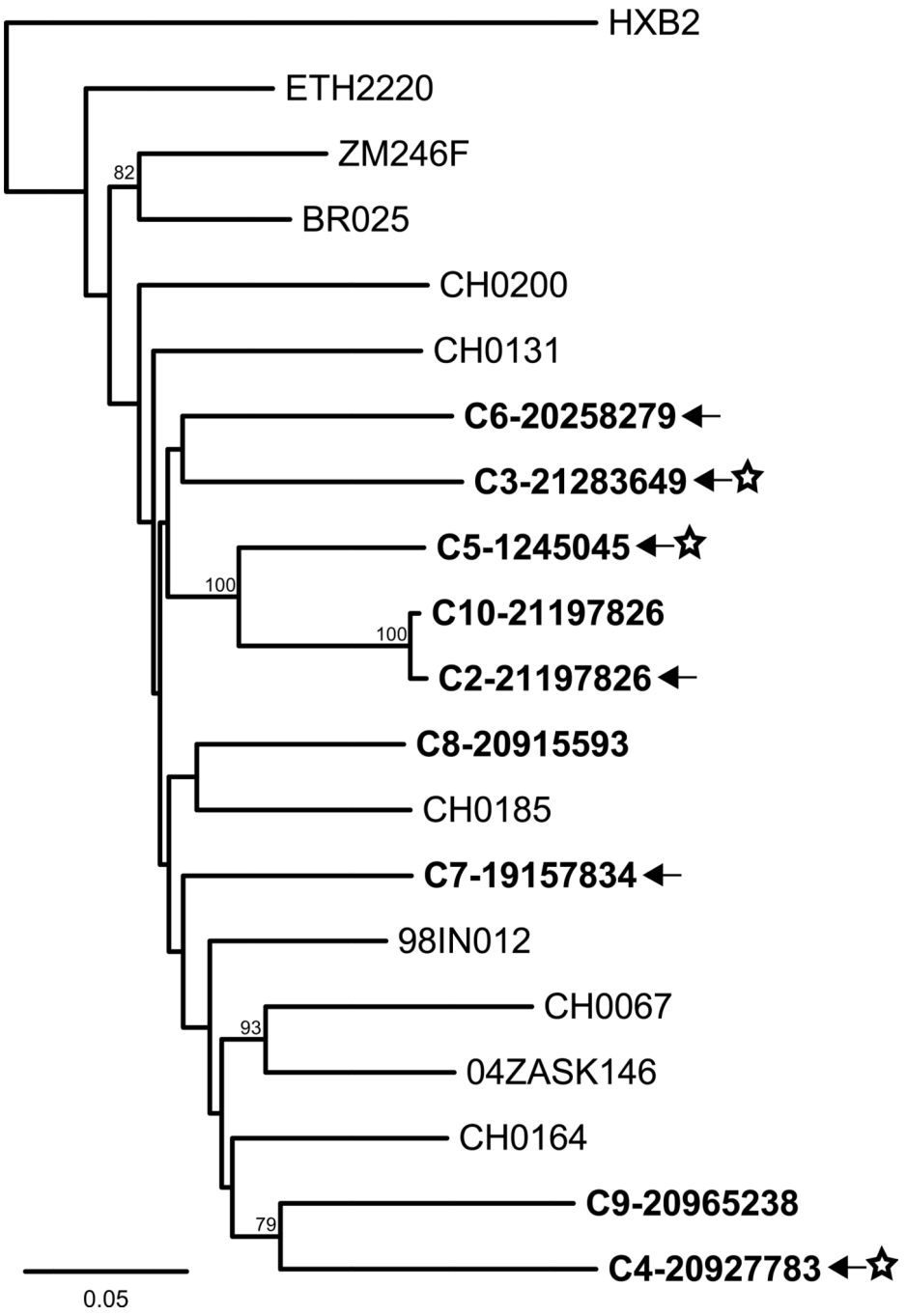

Figure 2.

Phylogenetic relationships of clade C T/F Envs used for SHIV construction. A maximum likelihood tree is shown that depicts the position of nine subtype C T/F *env* sequences (C2-C10; bold) in relation to each other and reference strains (made using PhyML version 3 with a TVM+I+G model chosen using Modeltest version 2.1.4). A tenth *env* sequence (C1-19912872) was identified as an A/C recombinant and is thus not included in the analysis. Reference sequences included subtype C [BR025 (accession # U52953), ETH2220 (U46016), 98IN012 (AF286231), 04ZASK146 (AY772699), ZM246F (FJ496192), CH0131

(KC894107), CH0185 (KC156129), CH0067 (KC156125), CH0200 (KC149183), and CH0164 (KC894125)] and subtype B [HXB2 (K03455)] strains. C2-C7 *env* genes (indicated by arrows), along with C1-19912872 *env* (not shown), were cloned into the SHIV KB9 proviral backbone; SHIVs containing C3, C4 and C5 *env* genes (stars) were able to initiate a productive infection in monkeys. Bootstrap support values greater than 70% are shown at nodes in the tree. The scale bar represents 0.05 substitutions/site.

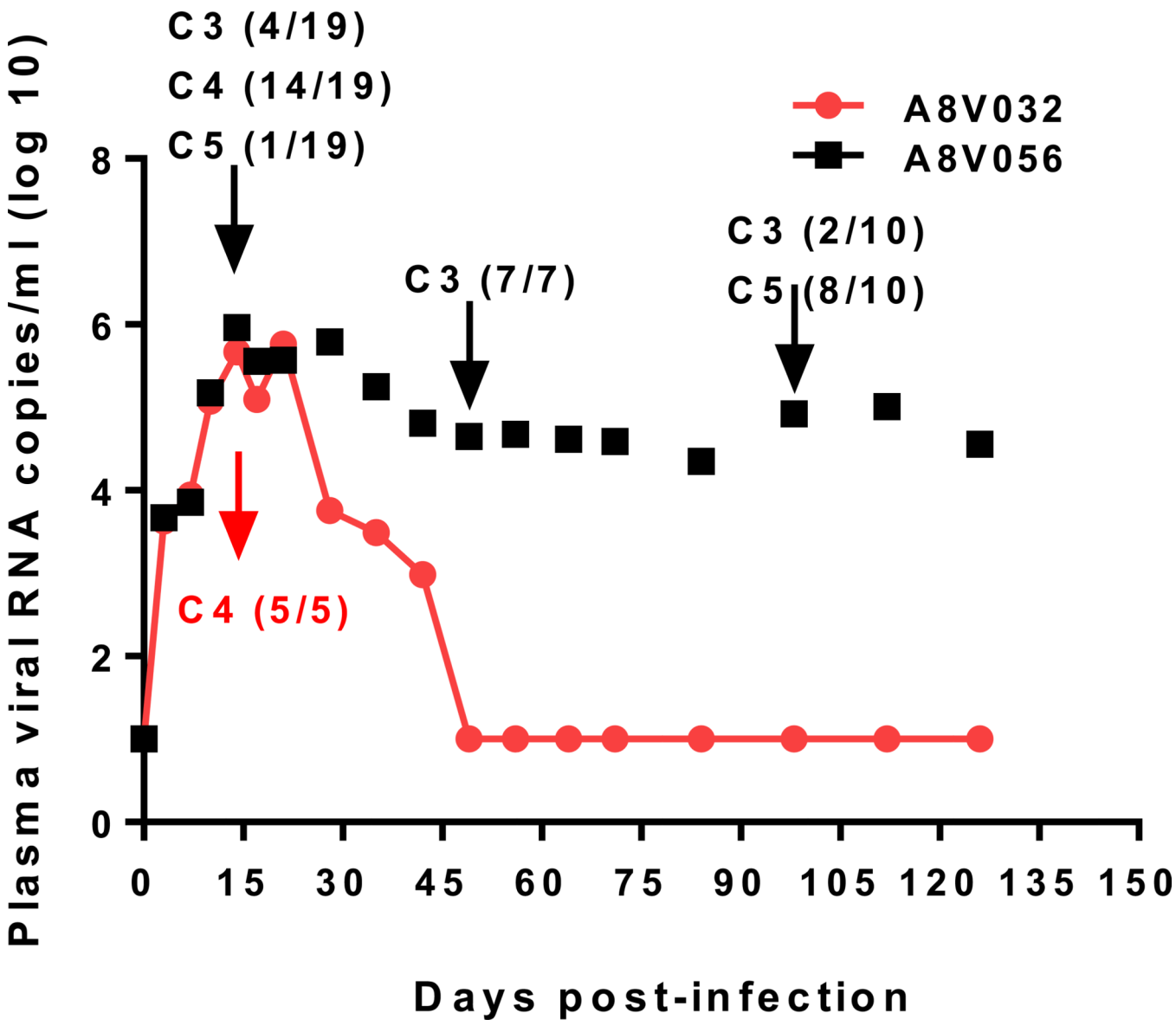

Figure 3.

In vivo selection of clade C T/F SHIVs. Seven clade C T/F *env* genes, C1 through C7, were cloned into the SHIV KB9 backbone to generate seven different clade C SHIVs. These seven clade C SHIVs were combined in equal ratios (100 $TCID_{50}$ of each of the viruses) to make a pool of viruses and were inoculated in two naïve rhesus monkeys intravenously. Infecting clones of SHIVs were isolated from the monkeys at various timepoints during the course of infection as indicated by the arrows. Clones isolated from monkey A8V032 are shown in “Red” letterings and the time point is indicated by “Red” arrow, whereas clones isolated from monkey A8V056 are shown in “Black” letterings and timepoints are shown in “black” arrows. The number of clones present in the total number of isolated clones at each timepoint is noted in parentheses next to each of the clones. Among nineteen clones isolated from monkey A8V056 at day 14 post-infection, four clones matched with SHIV KB9 C3, fourteen matched with SHIV KB9 C4 and one matched with SHIV KB9 C5 sequences. A

total of seven clones of SHIV KB9 C3 were isolated from monkey A8V056 at day 49 post-infection and, at day 98 post-infection, two SHIV KB9 C3 and eight SHIV KB9 C5 clones were detected in ten isolated clones. All five clones isolated from monkey A8V032 at day 14 post-infection matched the sequence of SHIV KB9 C4.

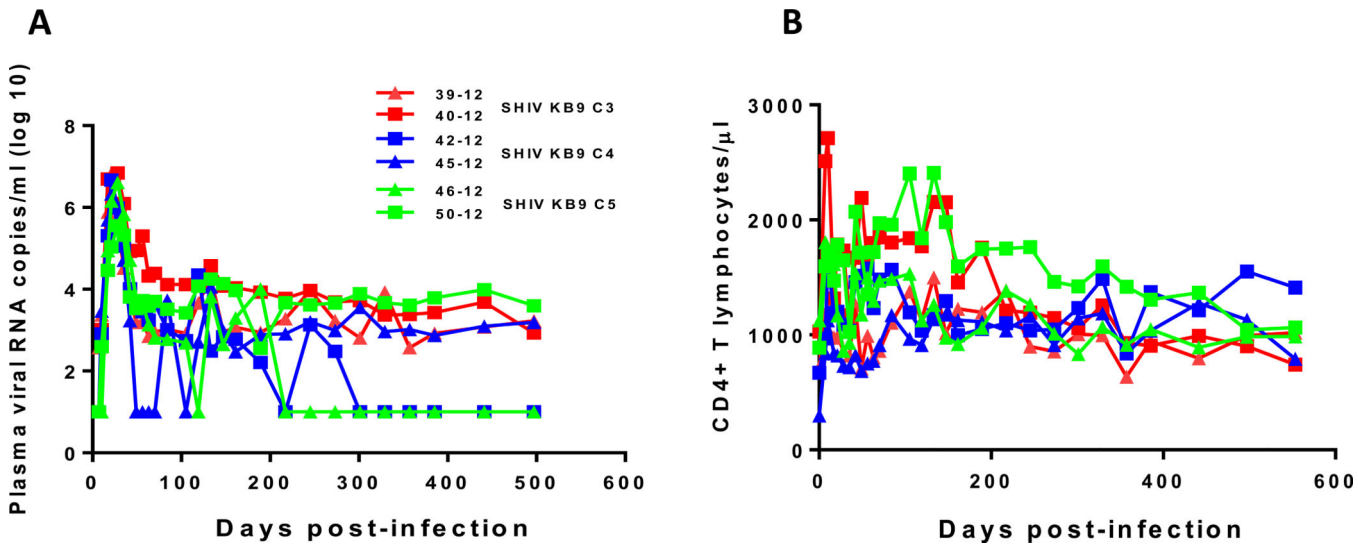

Figure 4.

In vivo infectivity of the individual clones of SHIVs. Culture supernatants containing 1000 TCID50 of SHIV KB9 C3, SHIV KB9 C4 or SHIV KB9 C5 were used to infect two naïve rhesus monkeys each by the intravenous route. (A) Plasma viral RNA level in monkeys. The data associated with SHIV KB9 C3 are shown in red, SHIV KB9 C4 in blue and SHIV KB9 C5 in green symbols and lines. All three viruses were able to establish infection with high peak viremia of 10^{6-7} RNA copies/ml, with sustained viremia up to 400 days post-infection. (B) CD4+ T lymphocyte subsets were determined by multi-channel flow cytometry for CD3, CD4, CD8, CD28, CD95, CCR5 and CCR7. Total $CD4^+$ T lymphocyte counts were calculated by multiplying the total lymphocyte count by the percentage of $CD3^+$ $CD4^+$ T cells. CD4+ T lymphocyte counts were monitored for all six monkeys post-infection and are shown using same color scheme as in (A). No significant decline in CD4+ T lymphocyte counts was seen.

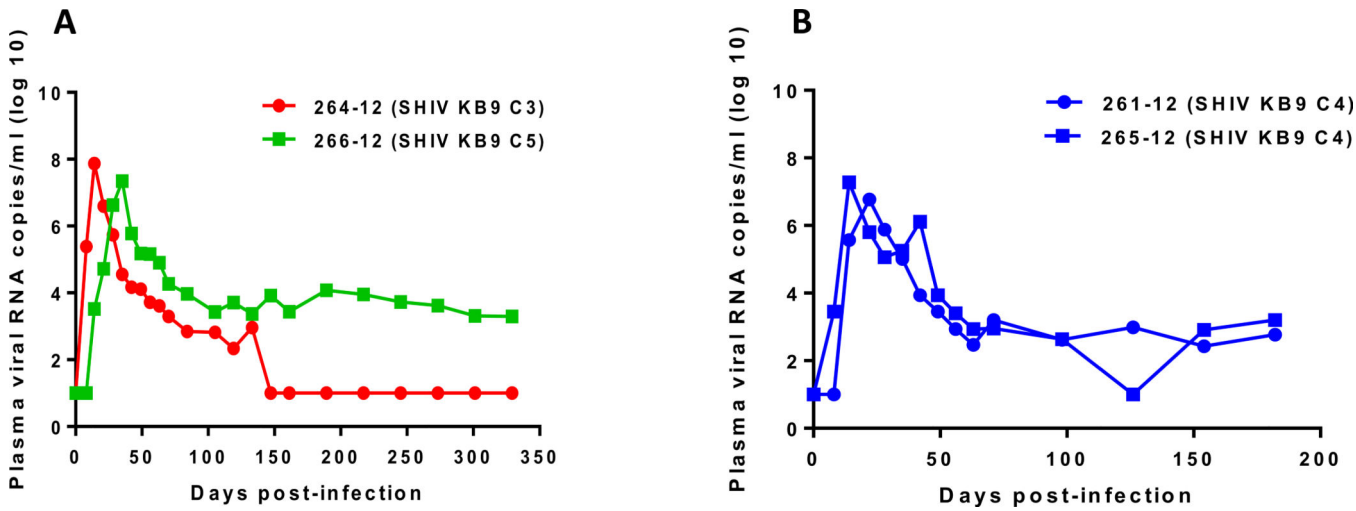

Figure 5.

Mucosal transmissibility of the SHIV KB9 C3, SHIV KB9 C4, and SHIV KB9 C5 challenge stocks. Undiluted stocks of SHIV KB9 C3 and SHIV KB9 C5 (A) SHIV KB9 C4 (B) were inoculated into naïve rhesus monkeys by the intrarectal route. Challenge stocks of all three clade C T/F SHIVs were able to establish infection in the monkeys by the mucosal route. Infections resulted in high peak viremia of $10^{6–8}$ RNA copies/ml at 14–22 days post-infection. Persistent viremia of 10^3 RNA copies/ml was observed in the SHIV KB9 C3-infected monkey up to 130 days post-infection. For the SHIV KB9 C5-infected monkey, plasma viral RNA of 10^3 copies/ml was detected up to 330 days post-infection. For the SHIV KB9 C4-infected monkeys, viremia of 10^3 RNA copies/ml was observed up to 180 days post-infection.

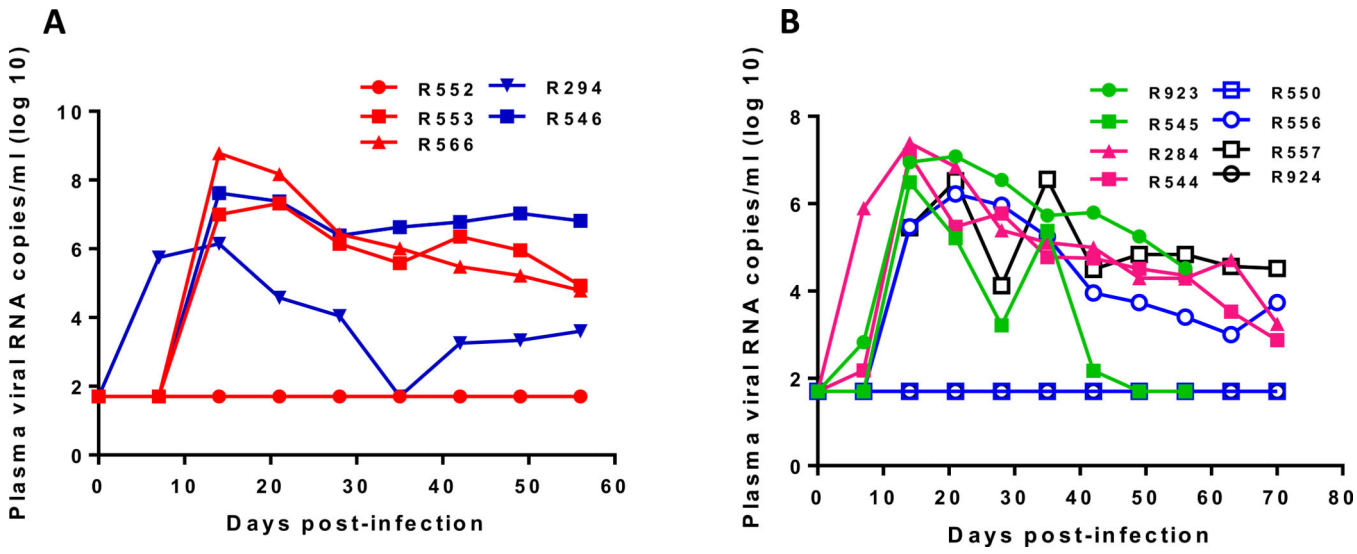

Figure 6.

Determination of 50% monkey infectious doses (MID_{50}) of SHIV KB9 C3, and SHIV KB9 C5 challenge stocks. (A) Two different dilutions of SHIV KB9 C3 were inoculated into a total of five naïve rhesus monkeys by the intra-rectal route. Monkeys inoculated with a 1:10 dilution of the virus are shown in “Red” lines and symbols and those with a 1:5 dilution are shown in “Blue” lines and symbols. One of the three monkeys (monkey R552) receiving a 1:10 dilution of the virus remained uninfected. (B) Four different dilutions of SHIV KB9 C5 were inoculated into a total of eight naïve rhesus monkeys by the intra-rectal route. Monkeys inoculated with the undiluted virus stock or with, 1:2, 1:5 and 1:10 dilutions of the virus are shown in “Green”, “Magenta”, “Blue” and “Black” lines and symbols, respectively. One of the monkeys receiving 1:5 (monkey R550) and 1:10 (monkey R924) dilutions of the virus remained uninfected.

Table 1

SHIV KB9 C3, SHIV KB9 C4 and SHIV KB9 C5 Challenge Stock

	Viral load of stock	TCID50/ml
SHIV KB9 C3	2.38 × 10^8 copies/ml	267,185
SHIV KB9 C4	2 × 10^8 copies/ml	53,437
SHIV KB9 C5	2.12 × 10^8 copies/ml	18,275

Table 2

Neutralization Assessment of SHIV KB9 C3, SHIV KB9 C4, SHIV KB9 C5

		IC50 Titer in TZM.bl cells (μg/ml)[a]						
		Primary SHIV Isolates			Acute/Early Clade C Tier 2 Pseudoviruses			
Reagent	**Description**	**SHIV KB9 C3**	**SHIV KB9 C4**	**SHIV KB9 C5**	**Ce1172_H1**	**Ce0393_C3**	**CAP45.2.00.G3**	**ZM233M.PB6**
sCD4	soluble huCD4	**0.71**	**4.65**	**2.91**	**4.96**	**21.50**	**26.00**	**2.90**
b12	mAb-CD4bs	**11.2**	>25	>50	>50	>50	**0.70**	>50
VRC01	mAb-CD4bs	**2.26**	>25	**34.84**	>10	**0.62**	>10	**1.67**
3BNC117	mAb-CD4bs	**29.42**	>25	>50	>50	0.20	**3.88**	**0.13**
CH31	mAb-CD4bs	>50	>50	>50	NT	NT	NT	NT
4E10	mAb-MPER	>50	>25	>50	**0.32**	**2.40**	**2.60**	**1.20**
2F5	mAb-MPER	>50	>25	>50	>50	>50	>50	>50
10E8	mAb-MPER	**5.74**	**7.93**	**5.27**	**0.72**	**1.18**	**1.82**	**0.44**
2G12	mAb-V3 Glycan	>50	>25	>50	>50	>50	**0.70**	>50
PGT128	mAb-V3 Glycan	>50	**1.5**	>50	**0.01**	>50	>50	>50
PGT121	mAb-V3 Glycan	>50	**0.178**	>50	**0.01**	>50	**1.63**	**3.69**
CH01	mAb-V1/V2	>50	>50	>50	NT	NT	NT	NT
PG9	mAb-V1/V2	>50	>25	**1.84**	**0.07**	**0.02**	**0.01**	**0.01**
PG16	mAb-V1/V2	>50	>25	**0.23**	**0.01**	**<0.01**	**<0.01**	**<0.01**
PGT145	mAb-V1/V2	>50	>50	**0.165**	**0.26**	**0.11**	**<0.01**	**0.03**
PGT151	mAb-gp41	>50	>50	>50	>50	>50	**0.01**	**0.03**
HIVIG-B	Clade B HIV+ polyclonal Ig	>2,500	>2,500	>2,500	**850**	**1,800**	**270**	**462**
HIVIG-C	Clade C HIV+ polyclonal Ig	**256**	>625	**440**	**61**	**85**	**12**	**94**
SA-C2	S. Africa clade C HIV+ Ig	**2,485**	**2,419**	**2,420**	**280**	**967**	**51**	**364**
SA-C8	S. Africa clade C HIV+ Ig	>2,500	**599**	>2,500	**749**	**45**	**108**	**132**
SA-C44	S. Africa clade C HIV+ Ig	>2,500	**1,801**	>2,500	**627**	**228**	**23**	**48**
SA-C62	S. Africa clade C HIV+ Ig	**1,146**	**2,168**	>2,500	**48**	**122**	**210**	**55**
SA-C72	S. Africa clade C HIV+ Ig	**1,099**	**2,223**	>2,500	**2,183**	**256**	**67**	**399**

		IC50 Titer in TZM.bl cells (μg/ml)[a]						
		Primary SHIV Isolates			Acute/Early Clade C Tier 2 Pseudoviruses			
Reagent	**Description**	**SHIV KB9 C3**	**SHIV KB9 C4**	**SHIV KB9 C5**	**Ce1172_H1**	**Ce0393_C3**	**CAP45.2.00.G3**	**ZM233M.PB6**
SA-C74	S. Africa clade C HIV+ Ig	**1,349**	**904**	>2,500	**820**	**42**	**508**	**646**

[*] Positive neutralization titers indicated in bold

NT: Not tested