Table 1. Anti-HIV antibody titres in infected monkeys

– indicates a titre of <32.

Time (weeks)	Intrarectal inoculation						Intravenous inoculation				
	LVL						HVL				
	MM243	MM397	MM399	MM400	MM401	MM375	MM376	MM298	MM299	MM338	MM339
0	–	–	–	–	–	–	–	–	–	–	–
1	–	–	–	–	–	–	–	–	–	–	–
2	–	–	–	–	–	–	–	–	–	64	64
3	32	–	32	–	–	128	–	–	–	32	32
4	32	16384	32	64	32	512	512	–	–	–	–
6	8192	16384	256	64	4096	1024	2048	–	–	–	–
8	4096	16384	1024	128	1024	16384	512	–	–	–	–
10	16384	16384	2048	512	512	16384	512	–	–	–	–
12	16384	16384	256	512	4096	16384	512				
13								–	–	–	–
14	16384	16384	1024	512	2048						
16	4096	8192	1024	1024	1024	16384	64				
17								–	–	–	–
18	8192	16384	2048	8192	4096						

the Nef antigen as a marker of virus infection using immunohistochemistry and quantitative analysis of proviral DNA in lymphoid and intestinal tissues. Nef$^+$ cells were detected in large numbers in the tissues of HVL macaques, but were undetectable in both Sym LVL (Fig. 1b) and Asym LVL (data not shown) macaques.

In the HVL macaques, high proviral DNA loads (>1000 copies μg^{-1}) were found in all of the tissues examined (Fig. 1c). In contrast, the proviral DNA loads in the tissues of the LVL macaques were only several tens to several hundreds of copies μg^{-1} (Fig. 1c). Furthermore, Sym LVL and Asym LVL macaques exhibited comparably low proviral DNA loads in these tissues (Fig. 1c). The low viral levels in lymphoid and intestinal tissues in the LVL macaques were consistent with their set points of plasma viral RNA loads. The viral levels in lymphoid and intestinal tissues were not significantly different between Sym LVL and Asym LVL macaques.

Diarrhoea and wasting in LVL macaques correlate with CD4$^+$ cell frequency in lymphoid and intestinal tissues, but not in peripheral blood

Because CD4$^+$ T-cell depletion is the hallmark of AIDS, we first examined CD4$^+$ T-cell counts in peripheral blood. Whilst peripheral CD4$^+$ T cells were completely and irreversibly depleted in HVL macaques throughout the infection, they displayed various kinetics in LVL macaques (Fig. 2a). MM397 (Sym LVL) and MM401 (Asym LVL) had very low CD4$^+$ T-cell counts (<150 cells ml^{-1}) at all times at which they were examined after infection, whereas MM399 (Sym LVL) and MM400 (Asym LVL) maintained moderate CD4$^+$ T-cell counts (>300 cells ml^{-1}) throughout the experiment (Fig. 2a).

Naïve CD4$^+$ T cells of MM397 (Sym LVL), MM243 (Asm LVL) and MM401 (Asym LVL) were depleted as early as 4 weeks p.i., whereas those of MM399 (Sym LVL) and MM400 (Asym LVL) remained at moderate levels (Fig. 2b). The HVL macaques were not examined because their peripheral CD4$^+$ T cells were depleted.

In addition to evaluating CD4$^+$ T cells in the blood, we evaluated CD4$^+$ cells in lymphoid and intestinal tissues using CD4 staining. The HVL macaques showed severe depletion of CD4$^+$ cells in all lymphoid tissues and intestine compared with the uninfected macaques (Fig. 2c, d). Interestingly, the CD4$^+$ cell frequencies in the tissues were clearly lower in Sym LVL macaques than in uninfected macaques (Fig. 2c, d). However, the CD4$^+$ cell frequencies in the tissues of Asym LVL macaques were comparable to those in uninfected macaques. These findings indicated that the emergence of diarrhoea and wasting in LVL macaques correlated with the low CD4$^+$ cell frequency in lymphoid tissues and the intestines, but not with the counts of peripheral CD4$^+$ T-cell subsets.

Infected animals exhibit significantly shorter villi

Symptomatic animals (Sym LVL and HVL macaques) exhibited diarrhoea. To examine whether the jejunum of symptomatic animals exhibited the histopathological changes that suggest AIDS-related enteropathy, we measured villous length on haematoxylin and eosin (H&E)-stained samples of jejunum in uninfected and infected macaques. Surprisingly, villous length was significantly

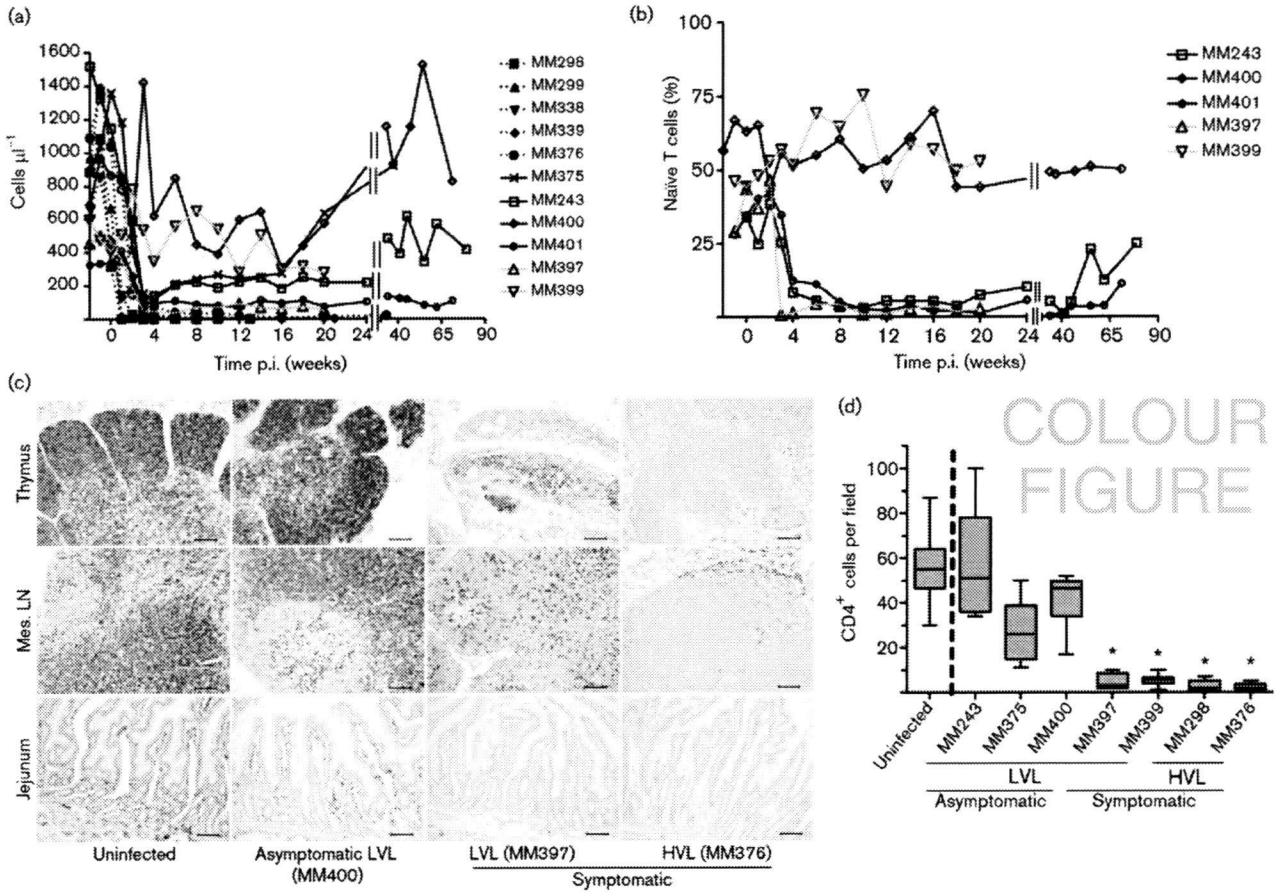

Fig. 2. Counts of circulating CD4$^+$ T-cell subsets and CD4$^+$ cell frequency in lymphoid and intestinal tissues at the time of euthanasia in SHIV-KS661-infected rhesus macaques. Counts of circulating CD4$^+$ T-cell subsets were analysed by flow cytometry and whole-blood counts. (a) Circulating CD4$^+$ T-cell counts. The ID numbers of the macaques are indicated on the figure. (b) Proportion of CD95$^+$ naïve cells in circulating CD4$^+$ T cells of LVL macaques. Solid black lines indicate Asym LVL macaques and solid grey lines indicate Sym LVL macaques. (c) CD4$^+$ cell frequencies in thymus, mesenteric lymph nodes (Mes. LN) and jejunum of representative uninfected, Asym LVL, Sym LVL and HVL macaques. Bars, 100 μm. (d) Quantification of jejunum CD4$^+$ cells in uninfected and infected macaques. The numbers of CD4$^+$ cells were enumerated in at least ten fields of the tissues at a magnification of 200×. Statistical analysis was performed using Student's *t*-test for the data from five uninfected and each infected macaque (*, $P<0.0001$). Data for MM299, MM338, MM339 and MM401 were not available.

shorter in all of the infected animals than in uninfected animals ($P<0.0001$) (Fig. 3a, b). This suggested that SHIV-infected animals develop villous atrophy, irrespective of viral load.

Increased number of activated macrophages in the jejunum of symptomatic animals

Macrophages appeared to be more abundant in H&E-stained jejunal sections in symptomatic animals. This was confirmed by CD68 staining: the frequency of CD68$^+$ macrophages in the jejunum was considerably higher in symptomatic animals than in uninfected animals, but was not significantly different between uninfected animals and Asym LVL macaques (data not shown). Furthermore, CD68$^+$ macrophages in the small intestine of Sym LVL and HVL macaques appeared to be activated because their size was increased. To examine whether the number of activated CD68$^+$ macrophages increased in the small intestine, we double stained for CD68 and Ki67 in the small intestine sections by immunohistochemistry. The frequency of CD68$^+$ Ki67$^+$ macrophages in the jejunum of all symptomatic animals examined was significantly higher than that of uninfected animals ($P<0.0001$) (Fig. 3c, d). This suggested that abnormal activation of intestinal macrophages occurred in symptomatic animals irrespective of viral load.

DISCUSSION

It is important to discuss initially why some SHIV-infected macaques had an HVL at the late stage, whilst others had

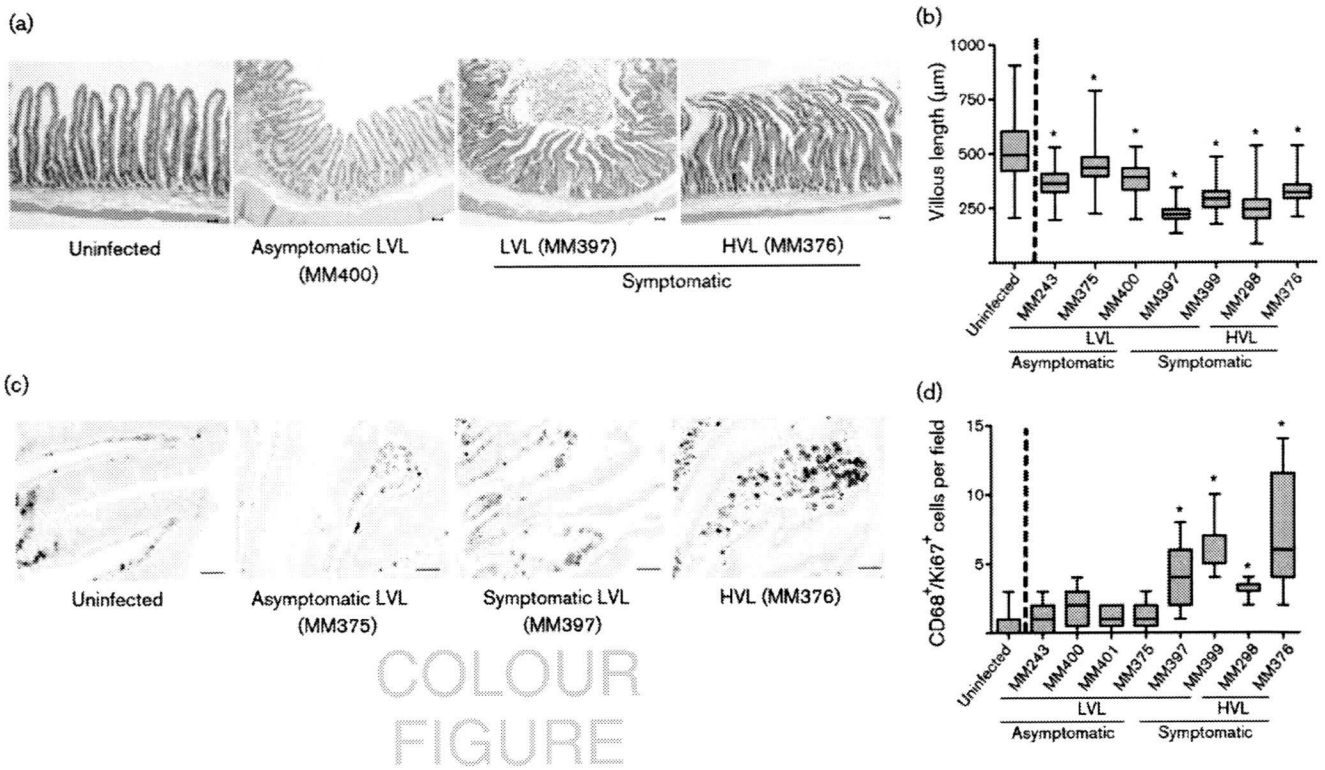

Fig. 3. Villous length in jejunum and counts of activated macrophages in the small intestine at the time of euthanasia in SHIV-KS661-infected rhesus macaques. (a) H&E-stained sections of jejunum of representative uninfected, Asym LVL, Sym LVL and HVL macaques. Bars, 200 μm. (b) Comparison of villous length in uninfected and infected macaques. The lengths of at least 100 villi were measured in each macaque. Statistical analysis was performed using Student's *t*-test for the data from four uninfected and each infected macaque (*, $P<0.0001$). Data for MM299, MM338, MM339 and MM401 were not available. (c) Ki67 and CD68 staining in the small intestine of representative uninfected, Asym LVL, Sym LVL and HVL macaques. Brown staining indicates $Ki67^+$ cells and blue staining indicates $CD68^+$ cells. Bar, 50 μm. (d) Comparison of $CD68^+$ $Ki67^+$ cell counts in uninfected and infected macaques. The numbers of $CD68^+$ $Ki67^+$ cells were enumerated in at least ten fields of the tissues at a magnification of 200×. Statistical analysis was performed using Student's *t*-test for the data from seven uninfected and each infected macaque (*, $P<0.0001$). Data for MM299, MM338 and MM339 were not available.

an LVL. The LVL macaques had much stronger antibody responses than the HVL macaques (Table 1). SHIV-89.6P is easily controlled by the antibody response (Montefiori *et al.*, 1998). SHIV-KS661, which shares its genetic origin with SHIV-89.6P, might be strongly affected by the antibody response. Virus replication during the primary phase clearly occurred later in the intrarectally inoculated macaques than in the intravenously inoculated macaques. Therefore, this delay might contribute to the continuous and strong antibody response in the intrarectally inoculated macaques, consequently resulting in a low viral load in most of the intrarectally inoculated macaques.

The purpose of this study was to elucidate why LVL macaques experience diarrhoea and wasting. A comparison of circulating $CD4^+$ T-cell counts (Fig. 2a) and relative levels of naïve T-cells (Fig. 2b) in LVL macaques did not reveal a substantial difference between Sym LVL (which showed diarrhoea and wasting) and Asym LVL (which were healthy) macaques. The villous length in the intestine also did not affect the level of malignancy of the disease condition, as all infected monkeys showed significant villous atrophy, suggesting a high sensitivity to infection itself. However, Sym LVL and HVL macaques exhibited two findings that Asym LVL macaques did not: (i) $CD4^+$ cell reduction in intestinal and lymphoid tissues (Fig. 2c, d), a hallmark of AIDS; and (ii) abnormal innate immune activation, which was reflected by an increased number of activated macrophages within the intestines (Fig. 3c, d). Ki67 serves as a proliferation marker and proliferation of macrophages may seem unlikely. However, there are some reports about local macrophage proliferation in inflammation sites, indicating the infiltration of activated macrophages associated with tissue damage (Isbel *et al.*, 2001; Norton, 1999). These observations indicated the existence of immunopathological disorders in the intestines not only in HVL macaques but also in Sym LVL macaques.

Many studies have shown positive correlations between the development of AIDS and some characteristic features in

the intestinal tracts of HIV-1-infected humans and pathogenic SIV- or SHIV-infected monkeys: continuous $CD4^+$ T-cell depletion (Brenchley *et al.*, 2004; Ling *et al.*, 2007), abnormal and chronic immune activation (Brenchley *et al.*, 2006; Hazenberg *et al.*, 2003) and enteropathy (Kotler, 2005). Immune activation (as shown by an increased number of intestinal activated macrophages) and intestinal $CD4^+$ cell depletion in Sym LVL macaques strongly suggest the presence of an AIDS-like disease in this subset of animals. Hence, these results suggest that an AIDS-like intestinal disease can occur in LVL macaques despite their low viral load, as well as in HVL macaques.

Some HIV-1-infected patients experience poor recovery of circulating $CD4^+$ T cells, even when their plasma HIV-1 RNA load is suppressed by HAART (Kaufmann *et al.*, 2003; Marchetti *et al.*, 2006; Piketty *et al.*, 1998). These individuals are called immunological non-responders (Marchetti *et al.*, 2006), and have been found to have increased plasma lipopolysaccharide levels, suggesting that bacteria had been translocated from the intestines into the circulation with concomitant activation of T-cell compartments (Marchetti *et al.*, 2006, 2008). Furthermore, some patients who maintain an undetectable or nearly undetectable plasma viral RNA load in the absence of HAART also develop AIDS disease progression (Madec *et al.*, 2005) and have abnormal immune activation and increased plasma lipopolysaccharide levels (Hunt *et al.*, 2008). These observations may indicate that disease progression in a subset of HIV-1-infected individuals is independent of viraemia. Accordingly, the disease progression under conditions of low viral load that we observed in SHIV-KS661-infected macaques can also occur in HIV-1-infected individuals.

Consistent with the fact that intestinal $CD4^+$ cell depletion triggers mucosal immune dysfunction, a notable difference observed between Sym LVL and Asym LVL macaques was the low $CD4^+$ cell frequency in the intestines of the Sym LVL macaques. We propose that the intestinal $CD4^+$ cells in Sym LVL macaques were not able to recover after intestinal $CD4^+$ cell reduction during the early phases of infection. We reported previously that SHIV-KS661 infection of rhesus macaques caused early intestinal $CD4^+$ T-cell depletion (Fukazawa *et al.*, 2008; Miyake *et al.*, 2006). Although we did not examine the macaques during the early phases of infection, the intestinal $CD4^+$ T cells of both Sym LVL and Asym LVL macaques should have been depleted at this time, as even moderately pathogenic SHIV can cause intestinal $CD4^+$ cell reduction during the early phase of infection (Fukazawa *et al.*, 2008). Therefore, the near-normal frequency of intestinal $CD4^+$ cells in Asym LVL macaques would be the result of $CD4^+$ cell recovery after intestinal $CD4^+$ cell reduction during the early phase of infection. In contrast, intestinal $CD4^+$ cells in Sym LVL macaques may be unable to recover, even though virus replication has been controlled. Similarly, intestinal $CD4^+$ cell recovery was found to be important for halting disease progression in SIVmac239-infected rhesus macaques (Ling *et al.*, 2007). Accordingly, one of the important determinants for disease progression in SHIV-KS661-infected macaques may be $CD4^+$ cell recovery in the intestines.

We further hypothesize that this inappropriately low level of $CD4^+$ cells within the intestines of the SHIV-KS661-infected animals (and phenotypically similar humans) is permissive to the excessive activation of resident tissue macrophages. One implication of these studies is that regulatory T-cell subsets of $CD4^+$ cells may be especially vulnerable to this depletion, thus allowing this macrophage activation in view of the well-known role of regulatory T cells in inhibiting innate immune responses (Maloy *et al.*, 2003). This hypothesis will be important to assess in future studies to understand the pathophysiology in the intestines during the chronic phase of HIV-1 infection.

Taken together, the present results suggest that $CD4^+$ cell reduction and enteropathy can occur in SHIV-KS661-infected rhesus macaques even when the viral load is low. The ability or inability to restore intestinal $CD4^+$ cells may be a key factor determining disease progression, irrespective of virus replication levels in the chronic phase of SHIV-KS661 infection. The reason that the recovery of intestinal $CD4^+$ cells is impeded is unknown, although we can speculate on some possibilities such as the co-existence of other infectious microbial agents or impaired T-cell reconstitution caused by damage during thymopoiesis at an early phase of SHIV infection (Motohara *et al.*, 2006). We demonstrated comparable proviral DNA loads in the examined tissues between Sym and Asym LVL macaques, although the $CD4^+$ cell frequencies in the tissues were clearly reduced in Sym LVL macaques. Therefore, the quantity of provirus per CD4 cell in the tissues of Sym LVL macaques is considered to be relatively higher than that of Asym LVL macaques, and low-level replication that may be undetectable in the plasma viral load might be maintained in Sym LVL but not in Asym LVL macaques. Identifying the mechanisms of poor recovery of intestinal $CD4^+$ cells is needed to understand AIDS pathogenesis, because, as stated above, some HIV-1-infected patients have low $CD4^+$ T-cell counts even when viraemia is controlled. One useful approach is comparative and periodical analysis, including cellular immunology data, of the intestinal tract of the same animals from the early to the chronic phases using Sym LVL and Asym LVL macaques in this SHIV infection macaque model.

METHODS

Virus, animals and sample collection. Highly pathogenic SHIV-KS661 is a molecular clone of SHIV-C2/1 (GenBank accession no. AF217181), which was derived through *in vivo* passages of SHIV-89.6 (Shinohara *et al.*, 1999). The virus stock was prepared from the supernatant of virus-infected CEMx174 and M8166 human lymphoid cell lines.

All rhesus macaques used in this study were treated in accordance with the institutional regulations approved by the Committee for

Experimental Use of Non-human Primates in the Institute for Virus Research, Kyoto University, Japan. All macaques were inoculated with 2×10^3 50 % tissue culture infectious dose of SHIV-KS661 measured with CEMx174. The animal ID numbers, infection route and when they were euthanized are provided in Fig. 1(a).

Blood was collected periodically using sodium citrate as an anti-coagulant and examined by flow cytometry and for quantification of plasma viral RNA load. Tissue samples were obtained at the time of euthanasia and were used for quantification of proviral DNA and histopathology.

Determination of plasma viral RNA and proviral DNA loads. The viral loads in plasma and proviral DNA loads in lymphoid and intestinal tissues were determined by quantitative RT-PCR and quantitative PCR, respectively, as described previously (Motohara *et al.*, 2006). DNA samples were extracted directly from frozen tissue sections of each monkey using a DNeasy Tissue kit (Qiagen) according to the manufacturer's protocol.

Determination of antibody titres. Anti-HIV antibody titres were determined using a commercial particle agglutination kit (Serodia-HIV1/2; Fujirebio). Isolated plasma samples were serially diluted and assayed. The end point of the highest dilution giving a positive result was determined as the titre.

Flow cytometry. Flow cytometry was performed as described previously (Motohara *et al.*, 2006). Briefly, $CD4^+$ T cells were analysed by a combination of fluorescein isothiocyanate (FITC)-conjugated anti-monkey CD3 (clone FN-18; BioSource) and phycoerythrin-conjugated anti-human CD4 (clone NU-TH/I; Nichirei), and subsets of naïve and memory $CD4^+$ cells were analysed by a combination of FITC-conjugated anti-human CD95 (clone DX2; BD Pharmingen) and allophycocyanin-conjugated anti-human CD4 (clone L200; BD Pharmingen). $CD95^-$ $CD4^+$ cells were defined as naïve $CD4^+$ T cells and $CD95^+$ $CD4^+$ cells were defined as memory $CD4^+$ T cells. Labelled lymphocytes were examined on a FACSCalibur analyser using CellQuest software (BD Biosciences).

Histology and immunohistochemistry. Tissue samples were fixed in 4 % paraformaldehyde in PBS at 4 °C overnight and embedded in paraffin wax. Sections (4 μm) were dewaxed using xylene, rehydrated through an alcohol gradient, and stained with H&E. The villous length of the jejunum was measured with a micrometer. At least 40 villi from each section were measured.

For immunohistochemistry, sections were rehydrated and processed for 10 min in an autoclave in 10 mM citrate buffer (pH 6.0) to unmask the antigens, sequentially treated with TBS/Tween 20 (TBST) and aqueous hydrogen peroxide, left at 4 °C overnight or at room temperature for 30 min or 1 h for primary antibody reactions, washed with TBST, incubated at room temperature for 1 h with an Envision+ kit (a horseradish peroxidase-labelled anti-mouse immunoglobulin polymer; Dako), visualized using diaminobenzidine (DAB) substrate (Dako) as a chromogen, rinsed in distilled water, counterstained with haematoxylin and analysed by light microscopy (Biozero BZ-8000: Keyence).

For double staining (CD68 and Ki67) of sections, appropriately processed sections were incubated at room temperature for 1 h with unlabelled anti-Ki67 antibody at a dilution of 1 : 2000, the highly sensitive tyramide amplification step (CSAII; Dako) was performed, the slides were reacted with DAB to visualize the results and incubated with unlabelled anti-CD68 antibody at 4 °C overnight followed by incubation at room temperature for 1 h with Histofine Simple Stain AP (an alkaline phosphatase-labelled anti-mouse immunoglobulin polymer (Nichirei), and the results were visualized with a Blue Alkaline Phosphatase Substrate kit III (Vector Laboratories). Measurements of $CD68^+$ $Ki67^+$ cell counts were performed in ten fields at a magnification of 200 × by light microscopy.

Primary antibodies used in immunohistochemistry were anti-human CD4 (diluted 1 : 30; clone NCL-CD4; Novacastra Laboratories), anti-SIV Nef (diluted 1 : 500; FIT Biotech), anti-human CD68 (diluted 1 : 50; clone KP-1; Dako) and anti-human Ki67 (Ki-S5; Dako).

Statistical analysis. The significance of $CD4^+$ or $CD68^+$ $Ki67^+$ cell frequency measurements and villous length in the jejunum of infected monkeys compared with uninfected monkeys was analysed using an unpaired Student's *t*-test (two-tailed) using GraphPad Prism 4.0E software (Varsity Wave).

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In vivo analysis of a new R5 tropic SHIV generated from the highly pathogenic SHIV-KS661, a derivative of SHIV-89.6

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ABSTRACT

Although X4 tropic SHIVs have been studied extensively, they show distinct infection phenotypes from those of R5 tropic viruses, which play an important role in HIV-1 transmission and pathogenesis. To augment the variety of R5 tropic SHIVs, we generated a new R5 tropic SHIV from the highly pathogenic X4 tropic SHIV-KS661, a derivative of SHIV-89.6. Based on consensus amino acid alignment analyses of subtype B R5 tropic HIV-1, five amino acid substitutions in the third variable region successfully changed the secondary receptor preference from X4 to R5. Improvements in viral replication were observed in infected rhesus macaques after two passages, and reisolated virus was designated SHIV-MK38. SHIV-MK38 maintained R5 tropism through *in vivo* passages and showed robust replication in infected monkeys. Our study clearly demonstrates that a minimal number of amino acid substitutions in the V3 region can alter secondary receptor preference and increase the variety of R5 tropic SHIVs.

Introduction

Simian immunodeficiency virus (SIV) macaque models for AIDS have been used extensively to elucidate the pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection. Although SIV is an excellent model virus that has contributed to various virological discoveries, SIV has many limitations as an HIV-1 model. Because the antigenicity of SIV is different from that of HIV-1, it is difficult to evaluate HIV-1 vaccines in animal models by employing SIV as a challenge virus. This is especially true for evaluating the induction of neutralizing antibodies by HIV-1 vaccine candidates (Baba et al., 2000; Dey et al., 2009; Mascola et al., 2000). In addition to CCR5, SIV utilizes secondary receptors such as GPR1, GPR15 (Bob), and STRL-33 (Bonzo), which are scarcely used by HIV-1 (Clapham and McKnight, 2002). Although there have been no reports that have directly demonstrated the significance of these receptors for *in vivo* pathogenesis, possible influences of these minor receptors cannot be denied.

To supplement the limitations of the SIV model, a simian and human immunodeficiency virus (SHIV) macaque model has been generated. SHIVs were constructed by exchanging the envelope gene and other accessory genes of SIV with that of HIV-1 (Shibata et al., 1991). Therefore, SHIVs share the same envelope antigenicity and receptor usage with HIV-1. In early studies of HIV-1, isolated viruses were mostly X4 or dual tropic because they were isolated from AIDS patients using T-cell lines expressing CXCR4. Because envelope genes from X4 or dual tropic viruses were introduced to generate the chimeric virus, most SHIVs utilize CXCR4 as a secondary receptor. X4 tropic viruses infect distinct subsets of lymphocytes and the mode of viral replication during the acute phase of infection is different from that of R5 tropic viruses (Nishimura et al., 2004). During the acute phase of infection, X4 tropic SHIVs rapidly deplete circulating CD4 positive (+) T cells (Reimann et al., 1996; Sadjadpour et al., 2004). Most infected monkeys fail to seroconvert, because rapid depletion of helper T cells typically occurs within 4 weeks of infection. In contrast, R5 tropic viruses do not show such a catastrophic reduction in CD4+ T cells. The phenotypes observed during X4 SHIV infection are rare during actual HIV-1 infection, and it has been suggested that R5 tropic viruses are mainly involved in HIV-1 transmission and pathogenesis (Margolis and Shattock, 2006). Therefore, there is a demand for R5 tropic SHIVs in this field of research.

There are some R5 tropic SHIVs that have already been used in various experiments, including analyses on the efficacy of broadly neutralizing antibodies (Hessell et al., 2009). Due to the paucity of available R5 tropic SHIVs, however, it is difficult to conduct comparative analyses on the efficacy of neutralizing antibodies between different strains of SHIVs. *In vivo* analyses of neutralizing antibodies should be conducted with more than one or even a mixture of several strains of R5 tropic virus to reflect the wide variety of HIV-1

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envelope genes that are found worldwide. Therefore, our primary aim was to generate a new R5 tropic SHIV, which carries a different *env* from that of other existing R5 SHIVs.

Currently available R5 SHIVs were constructed by introducing the envelope gene and other accessory genes from R5 tropic HIV-1 into the SIV backbone (Humbert et al., 2008; Luciw et al., 1995). There is one report that demonstrated the construction of an R5 tropic SHIV by exchanging the whole third variable region (V3) of an X4 tropic SHIV with that of an R5 SHIV (Ho et al., 2005). This study clearly indicated that the V3 region of the envelope gene determines the secondary receptor preference *in vivo*. Although other studies have indicated that there are specific amino acids within the V3 region that are responsible for receptor preference (Cardozo et al., 2007; Yamaguchi-Kabata et al., 2004), there have been no reports demonstrating the generation of R5 tropic SHIV by the introduction of specific amino acid substitutions to the V3 region. Therefore, our secondary aim in this study was to alter the receptor usage of a well-studied X4 tropic SHIV by introducing a minimal number of amino acid substitutions in the *env* V3 region. The consensus amino acid alignment of subtype B R5 tropic HIV-1, which is strongly correlated with secondary receptor usage (Cardozo et al., 2007; Yamaguchi-Kabata et al., 2004), was introduced to the V3 region of a highly pathogenic SHIV-KS661 that possesses the typical infection phenotype of X4 tropic SHIV (Fukazawa et al., 2008; Miyake et al., 2006). SHIV-KS661 is a molecular clone constructed from the consensus sequence of SHIV-C2/1 (Gen Bank accession number AF21718) (Shinohara et al., 1999), a derivative of the non-pathogenic SHIV-89.6

Results

Generation of R5 tropic SHIV-MK1 from the highly pathogenic X4 tropic SHIV-KS661

The X4 tropic virus SHIV-KS661, a derivative of SHIV-89.6, depletes CD4+ T lymphocytes in systemic tissues within weeks of infection and causes AIDS-like symptoms in macaque monkeys (Fukazawa et al., 2008; Miyake et al., 2006). To convert the virus into an R5 tropic virus, we introduced five amino acid substitutions in the V3 region of SHIV-KS661 by site-directed mutagenesis. The positions of the substitutions were selected using information from alignment of the V3 amino acids of R5 tropic HIV-1 (Cardozo et al., 2007; Yamaguchi-Kabata et al., 2004). All five substitutions (E305K, R306S, R318T, R319G, and N320D) were accompanied by changes in electrical charge. As a result, the net charge of the V3 region shifted towards being more acidic (Fig. 1A). To determine whether this mutant, designated SHIV-MK1, was capable of replication within monkey cells, we spinoculated SHIV-MK1 on rhPBMCs at an MOI of 0.1. The RT activity in the supernatant was monitored daily. The X4 tropic SHIV-DH12R-CL-7 and parental SHIV-KS661 actively replicated on rhPBMCs, reaching its peak RT activity level 4 days after inoculation. The R5 tropic SIVmac239 reached its peak RT value at the same time point; however, the peak value was less than 50% of that of SHIV-DH12R-CL-7 and SHIV-KS661. SHIV-MK1 also replicated on rhPBMCs, but it took 2 days longer to reach peak RT activity levels, and the peak RT value was significantly lower than that of the parental SHIV-KS661 (Fig. 1B).

Next, to determine whether SHIV-MK1 was capable of utilizing CCR5, but not CXCR4, we conducted a small molecule inhibitor assay. Briefly, SIVmac239, SHIV-DH12R-CL-7, SHIV-KS661, or SHIV-MK1 was spinoculated on rhPBMCs that were preincubated with AD101 (R5 inhibitor), AMD3100 (X4 inhibitor), or both inhibitors at various concentrations. The supernatant RT activities were measured 5 days post-inoculation. The replication of X4 tropic SHIV-DH12-CL-7 was inhibited with AMD3100 in a dose-dependent manner; however, it was not restrained with AD101 as described previously (Igarashi et al., 1999, 2003; Sadjapour et al., 2004). The same pattern was observed in SHIV-KS661-infected rhPBMCs, thus indicating that this virus is also an X4 tropic virus. In contrast, there was no replication inhibition of R5 tropic SIVmac239 in the presence of AMD3100; however, dose-dependent inhibition was observed in the presence of AD101. This result is consistent with other reports (Marcon et al., 1997; Zhang et al., 2000). SHIV-MK1 exhibited the same inhibition profile as SIVmac239, indicating that this virus predominantly utilizes CCR5, but not CXCR4, as an entry secondary receptor.

R5 tropic SHIV-MK1 can replicate in rhesus macaques

To determine whether SHIV-MK1 is capable of replication in rhesus macaques, we intravenously inoculated two monkeys (MM482 and MM483) with 20,000 TCID50 SHIV-MK1. Large amount of virus was inoculated to this group of monkey because *in vitro* replication of SHIV-MK1 was significantly weak compared with that of parental SHIV-KS661. As a control, two other monkeys (MM455 and MM459) were infected with 2000 TCID50 SHIV-KS661, a sufficient amount of virus to induce AIDS-like symptoms (Fukazawa et al., 2008; Miyake et al., 2006). Plasma viral RNA loads were monitored periodically using quantitative RT-PCR. Both groups of infected monkeys exhibited viremia, which reached peak plasma viral RNA loads of 10^6–10^8 copies/ml 2 weeks post-infection. In SHIV-KS661-infected monkeys, the set point of plasma viral RNA loads was between 10^4 and 10^6 copies/ml (Fig. 2Ai). In contrast, the plasma viral RNA load in one of the two monkeys infected with SHIV-MK1 was undetectable by 6 weeks post-infection, although 10-fold more virus was inoculated. The other monkey maintained 10^3–10^4 copies/ml plasma viral RNA for more than 25 weeks post-infection (Fig. 2Aii).

Next, circulating CD4+ T lymphocytes were analyzed by fluorescence activated cell sorting (FACS) to elucidate the impact of infection on lymphocyte subsets. As previously reported, X4 tropic SHIV-KS661 caused a massive depletion of circulating CD4+ T lymphocytes within 4 weeks post-infection (Fig. 2Bi). In contrast, circulating CD4+ T lymphocytes transiently decreased in monkeys infected with SHIV-MK1; however, they tended to recover by 24 weeks post-infection (Fig. 2Bii).

Because X4 tropic viruses preferably target naive CD4+ T lymphocytes, and R5 tropic viruses preferably target memory CD4+ T lymphocytes, circulating memory and naive CD4+ T lymphocytes were analyzed. The ratios of memory and naive CD4+ T cells were monitored 0, 2, 4, and 8 weeks post-SHIV-MK1 infection (Fig. 2C). Consistent with previous reports (Nishimura et al., 2004), X4 tropic SHIV-KS661 preferentially depleted naive T lymphocytes by 2 weeks post-infection. Although there was a subtle reduction in CD4+ T lymphocytes, the ratio of memory and naive CD4+ T lymphocytes did not change in SHIV-MK1-infected monkeys. This result indicates that a reduction in CD4+ T cells during SHIV-MK1 infection was not sufficient to alter the ratio of memory T cells, at least in circulating T lymphocytes.

The intestine is an effecter site where most CD4+ T lymphocytes are memory cells, and is the primary target for R5 tropic viruses (Harouse et al., 1999; Veazey et al., 1998). To elucidate the impact of viral infection in the intestine, tissue samples from the jejunum were obtained periodically and CD4+ T lymphocyte subsets were analyzed (Fig. 2D). As reported previously, CD4+ T lymphocytes of KS661-infected monkeys were depleted by 4 weeks post-infection (Fukazawa et al., 2008; Miyake et al., 2006). Although CD4+ T lymphocyte depletion was observed in one of the SHIV-MK1-infected monkeys (MM482) within 4 weeks post-infection, CD4+ T lymphocytes recovered as plasma viral RNA loads decreased. Another SHIV-MK1 infected monkey (MM483) whose plasma viral RNA load dropped below detectable levels showed only a transient reduction in CD4+ lymphocytes 5 weeks after infection. Taken together, these results suggest that, although the magnitude of jejunal CD4+ T-cell reduction was greater than that of circulating CD4+ T cells, the capability of

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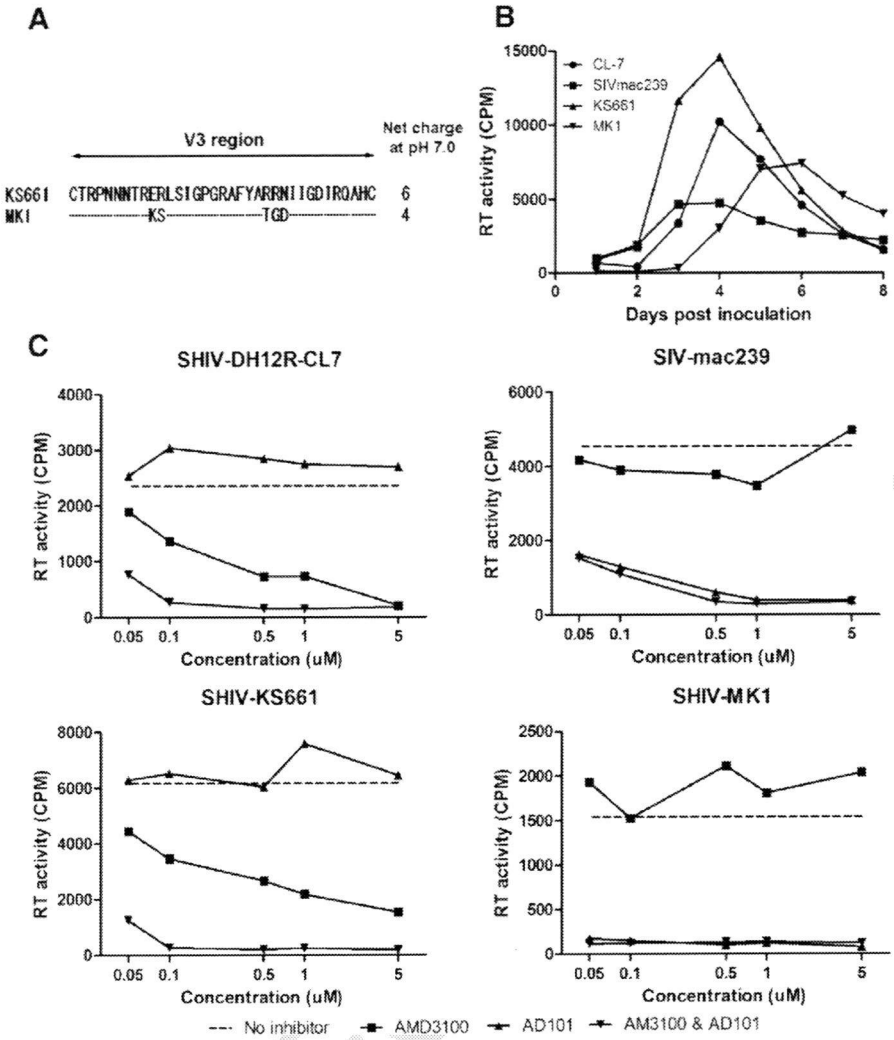

Fig. 1. Construction and *in vitro* analysis of SHIV-MK1. (A) gp120 V3 amino acid alignment of SHIV-MK1. Amino acid substitution positions are indicated under the parental SHIV-KS661 alignment. The net charge at pH 7.0 is indicated beside each amino acid alignment. (B) SHIV and SIV replication in rhPBMCs. The replication of control viruses (SIVmac239, SHIV-DH12R-CL7, and SHIV-KS661) and the mutant virus (SHIV-MK1) are shown. Culture supernatants were collected at the indicated time points, and RT activity was determined. Representative results of three independent experiments are shown. (C) Secondary receptor inhibitor sensitivity of the three SHIV inocula and an SIV control. The inoculum viruses SHIV-DH12R-CL7, SIVmac239, SHIV-KS661, and SHIV-MK1 were spinoculated on rhPBMCs in the presence of the indicated small molecule inhibitors. The inhibitor concentrations used were 0.05, 0.1, 0.5, 1, and 5 μM. The RT activity on day 5 post-infection was determined by the absence (dashed line) or presence of an inhibitor in the medium.

SHIV-MK1 to cause CD4+ T lymphocyte depletion in the jejunum is not as strong as the parental SHIV-KS661.

In vivo passage and characterization of the reisolated virus, SHIV-MK38

To adapt SHIV-MK1, we conducted *in vivo* passages. Briefly, disaggregated lymphocytes from inguinal lymph nodes and fresh blood collected from SHIV-MK1-infected MM482, were mixed and intravenously inoculated into an uninfected monkey, MM498. During the first passage, MM498 showed a plasma viral RNA load peak and set point equal to that of SHIV-MK1-infected MM482. During the second passage, disaggregated lymphocytes from inguinal lymph nodes and fresh blood collected from MM498 were mixed and intravenously inoculated into an uninfected monkey, MM504. MM504 showed a peak plasma viral RNA load of 5×10^7 copies/ml, which is slightly higher than that of MM482 and MM498. Furthermore, the set point of the viral load ranged from 10^4 to 10^6 copies/ml, which is approximately 10 times higher than that of MM482 and MM498 (Fig. 3A).

Although the inoculum doses were different in passaged monkeys, this result suggests that SHIV-MK1 acquired a better replicative capacity through *in vivo* passage. Therefore, we decided to reisolate the virus from MM504 for *in vitro* characterization. Briefly, CD8-depleted PBMCs from MM504 and an uninfected monkey were co-cultured for 2 weeks. The culture supernatant with the highest RT activity was stored in liquid nitrogen. This virus stock was designated SHIV-MK38.

First, we examined the replication kinetics of SHIV-MK38 in rhPBMCs. The infection assay revealed that although SHIV-MK38 could not replicate as fast or as efficiently as the parental KS661, there was a slight improvement in replication capacity compared with the original SHIV-MK1 (Fig. 3B). This result indicates that mutations that arose through *in vivo* passage increased replication ability in rhPBMCs.

As shown in Fig. 1B, however, X4 tropic viruses (SHIV-DH12R-CL-7 and SHIV-KS661) usually show fast and efficient replication in PBMCs compared with that of R5 tropic viruses (SIVmac239 and SHIV-MK1). Hence, there is the possibility of reversion in the V3 region, which may give SHIV-MK38 the appearance of having better replication capacity

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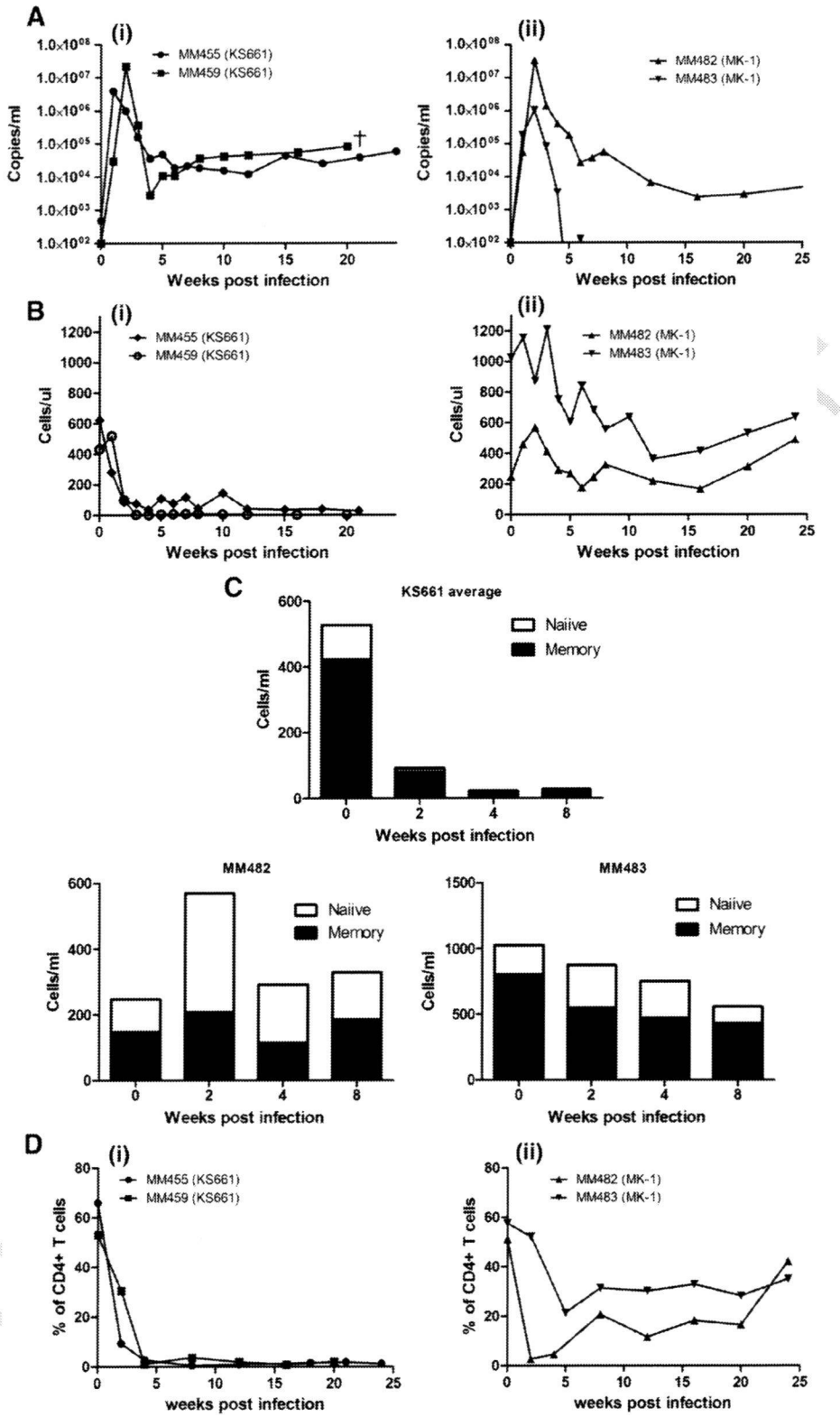

Fig. 2. *In vivo* replication of MK1. (A) Plasma viral RNA loads in SHIV-infected rhesus monkeys were measured at the indicated times. A total of 2000 TCID50 SHIV-KS661 was inoculated intravenously into MM455 and MM459 as a control group (i) and 20,000 TCID50 SHIV-MK1 was inoculated intravenously into MM482 and MM483 (ii). (B) CD4+ T lymphocytes were enumerated using FACS analysis in the SHIV-KS661 infected group (i) and the SHIV-MK1 infected group (ii) over the course of infection. (C) Changes in naive (open bar) and memory (black bar) CD4+ T cells in rhesus macaques inoculated with SHIV-KS661 (average of two infected monkeys) and SHIV-MK1 (MM482 and MM483) 0, 2, 4, and 8 weeks post-inoculation. (D) Percentage of CD4+ T lymphocytes in the jejunum. Tissues from the jejunum were collected from SHIV-KS661 infected monkeys (i) and SHIV-MK1 infected monkeys (ii) with a pediatric enteroscope, and were analyzed by FACS.

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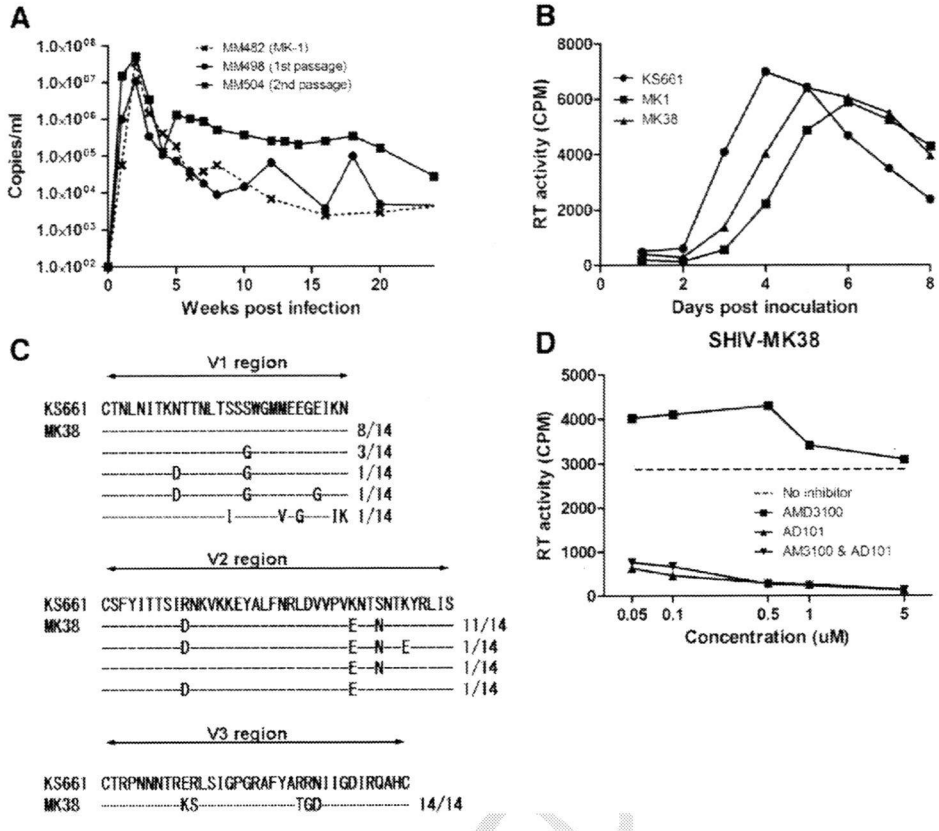

Fig. 3. *In vivo* adaptation of SHIV-MK1, and *in vitro* analysis of reisolated virus. (A) Plasma viral RNA loads of passaged monkeys were measured at the indicated times. The whole blood and dissociated lymph nodes from SHIV-MK1-infected MM482 were transfused into MM498 (first passage) 25 weeks post-inoculation. The whole blood and disaggregated lymph nodes from MM498 were transfused into MM504 (second passage) 5 weeks post-inoculation. (B) SHIV replication in rhPBMCs. The replication of control viruses (SHIV-KS661 and SHIV-MK1) and a passaged virus (SHIV-MK38) is shown. Culture supernatants were collected at the indicated time points, and RT activity was determined. Representative results of three independent experiments are shown. (C) gp120 V1, V2, and V3 amino acid alignment of SHIV-KS661 and 14 clones of SHIV-MK38. The positions of the amino acid substitutions in the 14 clones are indicated under the SHIV-KS661 sequence. (D) Secondary receptor inhibitor sensitivity of the SHIV-MK38 inoculum. RT activity 5 days post-infection was determined in the absence (dashed line) or presence of an inhibitor in the medium.

in rhPBMCs (Cho et al., 1998). Therefore, we examined the viral genome sequence to rule out the presence of reversions in the V3 region. Indeed, there were no back mutations in the V3 region of SHIV-MK38 when the V1 to V3 regions of the *env* sequences from 14 clones were analyzed (Fig. 3C). Nonetheless, we found mutations in the V1 and V2 regions of SHIV-MK38. These mutations have the potential to affect secondary receptor usage.

To confirm whether SHIV-MK38 maintains R5 tropism, we conducted a small molecule inhibitor assay, which revealed that SHIV-MK38 could not replicate in rhPBMCs in the presence of AD101 but could replicate in the presence of AMD3100. This indicates that SHIV-MK38 maintains R5 tropism in the primary cell (Fig. 3D).

In vivo analysis of SHIV-MK38

To evaluate whether SHIV-MK38-infected monkeys show stable infection phenotypes compared with that of SHIV-MK1-infected monkeys, we inoculated three monkeys with 20,000 TCID50 SHIV-MK38. All three infected monkeys possessed a peak plasma viral RNA load of approximately 10^7 copies/ml 12 days after infection. Although the peak plasma viral RNA load was at the same level in these monkeys, set points varied widely (Fig. 4A). That of MM501 was 10^3–10^4 copies/ml, which is similar to that of SHIV-MK1-infected MM482. MM502 had a slightly higher set point of 10^4–10^5 copies/ml, which is similar to that of MM504. Finally, MM481 had the highest set point, at 10^6–10^7 copies/ml. No monkey showed a decrease in viral RNA load under the detectable level, indicating that SHIV-MK38 robustly replicates in rhesus macaques.

Next, reductions in circulating CD4+ T cells were analyzed. Unlike SHIV-MK1 infection, all of the SHIV-MK38-infected monkeys exhibited a continuous reduction in CD4+ T cells without signs of recovery (Fig. 4B). The impact of infection on ratios of circulating memory and naive CD4+ T cells was also analyzed. Compared with monkeys infected with SHIV-MK1, SHIV-MK38 preferentially reduced memory fractions of CD4+ T cells (Figs. 2C and 4C).

To elucidate how improvements in viral replication affect the reduction of CD4+ T cells at effector sites, tissue samples from the jejunum were obtained periodically and CD4+ T lymphocyte subsets were analyzed. In SHIV-MK38-infected monkeys, CD4+ T cells were rapidly reduced by 2 weeks post-infection, as seen in SHIV-MK1 infection. Furthermore, recovery of CD4+ T cells was not observed in infected monkeys. In particular, CD4+ T cells in MM481 were depleted throughout the observation period (Figs. 2D and 4D). These data indicate that SHIV-MK38 has an increased ability to reduce CD4+ T cells and maintain higher plasma viral RNA loads in infected monkeys compared with pre-adapted SHIV-MK1.

Discussion

Based on the analysis of consensus amino acid alignments of subtype B R5 viruses, five amino acid substitutions (E305K, R306S, R318T, R319G, and N320D) were introduced into the V3 region of the pathogenic SHIV-KS661 *env* gene by site-directed mutagenesis. These

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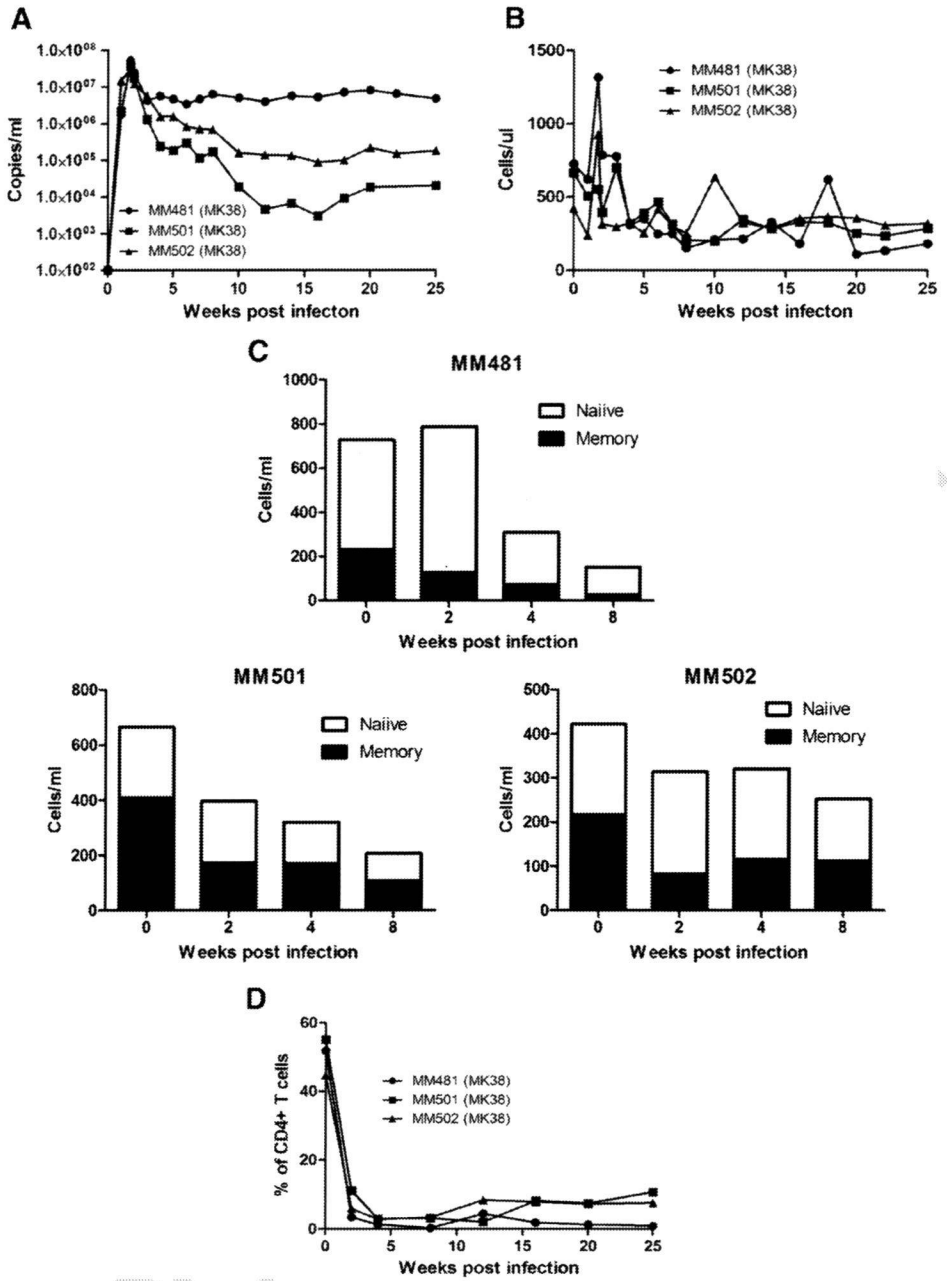

Fig. 4. *In vivo* replication of SHIV-MK38. (A) Plasma viral RNA loads in SHIV-infected rhesus monkeys were measured at the indicated times. A total of 20,000 TCID50 SHIV-MK38 were inoculated into MM481, MM501, and MM502. (B) CD4+ T lymphocytes were enumerated using FACS analysis in SHIV-MK38-infected monkeys over the course of infection. (C) Changes in naïve (open bar) and memory (filled bar) CD4+ T cells in rhesus macaques inoculated with SHIV-MK38 0, 2, 4, and 8 weeks post-inoculation. (D) Percentage of CD4+ T lymphocytes in the jejunum. Tissues from the jejunum were collected from SHIV-MK38-infected monkeys with a pediatric enteroscope, and analyzed by FACS.

substitutions included the 11/24/25th amino acid of the V3 region, which are strongly correlated with secondary receptor usage (Cardozo et al., 2007; Yamaguchi-Kabata et al., 2004). As expected, these substitutions successfully altered the secondary receptor usage of SHIV-KS661 from X4 to R5 tropic. This result clearly demonstrates for the first time that specific V3 amino acid alignment information from HIV-1 can be applied to SHIV to alter secondary receptor usage, at least in the context of the subtype B envelope. The prediction of viral secondary receptor tropism in HIV-1-infected people prior to the prescription of CCR5 antagonists has important economic and practical implications. There are at least six algorithms that predict viral tropism from the V3 sequence; however, the accuracy of these algorithms must be improved (de Mendoza et al., 2008; Dorr et al., 2005; Fätkenheuer et al., 2005; Mefford et al., 2008). For example, the Web PSSM algorithm (Jensen et al., 2003) predicts that SHIV-MK1 exclusively utilizes CCR5, while the Geno2pheno algorithm (Sing et al., 2007) suggests that it may also utilize CXCR4. In this study, we demonstrated that specific amino acids in the V3 region are responsible for secondary receptor usage both *in vitro* and *in vivo*. Accumulation of this type of information will provide important data that can be used to improve predictions and increase the genotype sensitivity of algorithms.

Although minimal numbers of amino acid substitutions were introduced to change secondary receptor usage, SHIV-MK1 showed

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relatively inefficient replication compared with that of parental SHIV-KS661, both *in vitro* and *in vivo*. SHIV-MK1 caused measurable levels of viremia in infected monkeys; however, plasma viral RNA levels dropped below detectable levels in one of two infected monkeys 6 weeks after inoculation, despite the fact that enormous amount of virus was inoculated. When evaluating the efficacy of passively administered neutralizing antibodies, or those induced by candidate anti-HIV-1 vaccines, this variability in viral replication is not desirable for the assessment of efficacy, because it is impossible to determine whether the virus was controlled by natural immune responses or by vaccine-induced immune responses. However, an improvement in viral replication was observed in rhPBMCs after *in vivo* passage of SHIV-MK1. This outcome suggests that, as in the case of other existing R5 tropic SHIVs, *in vivo* adaptation is required regardless of the minimal number of amino acid substitutions (Humbert et al., 2008; Tan et al., 1999).

Because various reports have demonstrated the emergence of the X4 tropic virus from the R5 tropic virus after serial passages (Ho et al., 2007; Pastore et al., 2000), there was a concern over the emergence of the X4 tropic virus through two *in vivo* passages. Although there were only five amino acid substitutions, no reversions in any of the substituted amino acids in the V3 region were observed. Some mutations were accompanied by amino acid substitutions in V1 and V2 regions. Previous reports suggest that these two variable regions may influence secondary receptor preference (Cho et al., 1998); however, a small molecule inhibitor assay revealed that SHIV-MK38 maintained R5 tropism after passage. The V1 and V2 regions also play a role in sensitivity against neutralizing antibodies (Laird et al., 2008; Wei et al., 2003). Although further investigations are required, SHIV-MK38 could have developed mutations in the V1 and V2 regions to modify antigenicity in an attempt to evade neutralizing antibodies (Sagar et al., 2006). Indeed, neutralization assay on TZM-BL cells revealed that neutralizing antibody from an MK1-infected monkey can neutralize SHIV-KS661 and SHIV-MK1, but fail to neutralize SHIV-MK38. On the other hand, plasma from the monkey in which SHIV-MK38 was isolated could neutralize all three viruses. Thus, the antigenicity was changed through *in vivo* passages (Supplementary Figure). Taken together, these results suggest that the improved replication of SHIV-MK38 over MK1 was not due to the re-emergence of X4 tropic viruses. Furthermore, the acquisition of mutations outside the V3 region is most likely attributable to the improved replication of SHIV-MK38 *in vivo*.

To confirm the replication advantage of SHIV-MK38 over SHIV-MK1, SHIV-MK38 was intravenously inoculated into three uninfected monkeys. Despite the fact that the same amount of SHIV-MK38 was inoculated, higher peaks and set points of plasma RNA loads were observed in SHIV-MK38 compared with SHIV-MK1 infection. Although SHIV-MK38-infected monkeys showed no obvious signs of AIDS-like symptoms during the observation period, none of these monkeys was able to control viral replication. A greater reduction in the memory portion of circulating CD4+ T cells was observed in SHIV-MK38-infected monkeys. This preferential reduction of circulating memory CD4+ T cells was well defined in MM481, which correlates with the maintenance of high plasma viral RNA loads throughout the observation period. Reductions of CD4+ T cells in the jejunum of SHIV-MK38-infected monkeys were greater than that of SHIV-MK1-infected monkeys, and there was no obvious recovery during the observation period. These infection phenotypes are characteristic of an R5 tropic virus, which is distinct from the infection of X4 tropic SHIVs such as parental SHIV-KS661 (Fukazawa et al., 2008; Miyake et al., 2006).

Harous et al. clearly demonstrated that R5 tropic virus preferentially reduces mucosal CD4+ T cells where memory CD4+ T cells are abundant, whereas X4 tropic virus preferentially reduces peripheral CD4+ T cells where naive CD4+ T cells are abundant (Harouse et al., 1999). From this observation, it is clear that the receptor preference has strong impact on tissue specific CD4+ T-cell reductions. However, in some cases, systemic and irreversible reduction of CD4+ T cells was observed in highly pathogenic X4 SHIV infection (Fukazawa et al., 2008; Nishimura et al., 2004). It has been suggested that highly pathogenic X4 SHIV preferentially targets naive CD4+ T cells but eventually reduces memory CD4+ T cells (Nishimura et al., 2004). The depletion of CD4+ T cells at the effector site in SHIV-KS661 infected monkeys supports this suggestion (Fig. 2D).

The envelope gene of SHIV-MK38 belongs to subtype B, which can be compared with other subtype B or C R5 tropic SHIVs (Humbert et al., 2008; Tan et al., 1999). Comparing the efficacy of passively administered neutralizing antibodies and their induction by candidate HIV-1 vaccines against a variety of R5 tropic SHIVs would provide a more precise evaluation against a variety of HIV-1 strains worldwide (Wei et al., 2003). Furthermore, despite the fact that SHIV-MK38 is derived from SHIV-KS661, and mutations were obtained through the alteration of secondary receptor usage and passage, SHIV-MK38 is still genetically homologous to SHIV-89.6P, because they both originate from the same molecular clone, SHIV-89.6. Highly pathogenic X4 tropic SHIV-89.6P has been used extensively in various experiments, including vaccine concept evaluations (Shiver et al., 2002). There are claims, however, that the utilization of X4 tropic SHIVs as challenge viruses has led to overestimation of vector-based vaccines (Feinberg and Moore, 2002). Therefore, SHIV-MK38 can be useful in the future to determine whether such overestimations are truly caused by using X4 SHIVs or are due to using an SHIV derived from the specific lineage of SHIV-89.6.

Based on our observations, it can be concluded that R5 tropic SHIV-MK38 can robustly replicate, and we successfully generated a new R5 tropic SHIV by a new method. Although infected monkeys showed no signs of AIDS-like symptoms during the observation period, and further characterization such as neutralization profiles must be conducted, SHIV-MK38 has the potential to be a new R5 SHIV model.

Materials and methods

Virus production

Non-synonymous nucleotide substitutions in the V3 domain of the SHIV-KS661 *env* gene were introduced by site-directed mutagenesis for substitution of amino acids. A 5.9 kb DNA fragment containing the *env* V3 domain was subcloned into a pUC119 vector following digestion with restriction enzymes Sse8387I and XhoI. The resulting vector was designated pKS661v3, and was used as the template for two sets of polymerase chain reaction (PCR). All amplifications were performed as follows: one cycle of denaturation (98 °C, 5 min), 32 cycles of amplification (98 °C, 10 s/60 °C, 30 s/72 °C, 2 min), and an additional cycle for final extension (72 °C, 10 min) using iProof High-Fidelity Master Mix (Bio-Rad Laboratories, Hercules, CA). The following primers were used for the first set of PCR; 5′ CAATACAA-GAAAAAGTTTATCTATAGGACCAGGGAGAGCATTTTATGCAACAGGAGA-CATAATAGGAG 3′ (forward primer corresponding to the 7250–7317th nucleotides of SHIV-KS661; positions of mismatches are underlined) and 5′ GCTGAAGAGGCACAGGCTCCGC 3′ (reverse primer corresponding to the 8633–8612th nucleotide of SHIV-KS661; no mismatches). The following primers were used for the second set of PCR: 5′ CTCCTAT-TATGTCTCCTGTTGCATAAAATGCTCTCCCTGGTCCTATAGA-TAAACTTTTTCTTGTATTG 3′ (reverse primer corresponding to the 7317–7250th nucleotide of SHIV-KS661; positions of mismatches are underlined) and 5′ CTCCAGGACTAGCATAAATGG 3′ (forward primer corresponding to the 5617–5637th nucleotide of SHIV-KS661; no mismatches). The products from these two sets of PCR were mixed, and overlap PCR was performed using primers 5′ GCTGAAGAGGCA-CAGGCTCCGC 3′ and 5′ CTCCAGGACTAGCATAAATGG 3′. The PCR product was then digested with the restriction enzymes BsaBI and NcoI. The resulting fragment was introduced back into the pKS661v3

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vector, and designated pKS661v3m. Then pKS661v3m DNA with mutations was digested by Sse8387I and XhoI, and the fragment was introduced back into the KS661 full genome plasmid, and designated pMK1.

SHIV-MK1 was prepared by transfecting pMK1 into the 293T cell line using the FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) and the culture supernatant 48 h after transfection, and was stored in liquid nitrogen until use. The same procedures were conducted to prepare SIVmac239 (Kestler et al., 1991), SHIV-KS661 (Shinohara et al., 1999), and SHIV-DH12R-CL7 (Igarashi et al., 1999). The 50% tissue culture infectious dose (TCID50) was measured using the C8166-CCR5 cell line (Shimizu et al., 2006).

Viral replication on rhPBMCs

Rhesus macaque PBMCs (rhPBMCs), prepared from an uninfected monkey, were suspended in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), 2 mM L-glutamine, and 1 mM sodium pyruvate, and then stimulated for 20 h with 25 μg/ml Concanavalin A (Sigma-Aldrich, St. Louis, USA), followed by an additional 2-day cultivation with 100 units/ml IL-2 (Shionogi, Osaka, Japan). On day 3, 5×10^4 cells were dispensed into 96-well round-bottom plates in triplicate. The cells were then inoculated with virus at a multiplicity of infection (MOI) of 0.1 using the spinoculation method (O'Doherty et al., 2000). Virion-associated reverse transcriptase (RT) activity of the culture supernatant was monitored periodically (Willey et al., 1988).

Inhibition of viral replication by a small molecule inhibitor

A small molecule inhibitor assay was conducted as described previously (Igarashi et al., 2003), with minor modifications. Briefly, uninfected rhesus PBMCs were prepared as described above. On day 3, 5×10^4 cells were dispensed into 96-well round-bottom plates. Various concentrations (0, 0.05, 0.1, 0.5, 1, and 5 μM) of a small molecule CCR5-specific receptor antagonist (AD101 was provided by Dr. Julie Strizki, Schering Plough Research Institute, Kenilworth, NJ) (Trkola et al., 2002) and/or a CXCR4-specific receptor antagonist (AMD3100; Sigma-Aldrich, St. Louis, MO) (Donzella et al., 1998) were added to duplicate wells and incubated for 1 h at 37 °C. Then each test virus was spinoculated at 1200×g for 1 h at an MOI of 0.1. On day 5 post-infection, virus replications were assessed by RT assay of the culture supernatants.

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. Collection of blood, biopsies, and i.v. virus inoculations (2000 TCID50 of SHIV-KS661, 20000 TCID50 of SHIV-MK1, 20000 TCID50 of SHIV-MK38) were performed on monkeys under anesthetization with ketamine hydrochloride (Daiichi-Sankyo, Tokyo, Japan). Plasma viral RNA loads were determined by quantitative RT-PCR as described previously (Kozyrev et al., 2002). Plasma viral RNA loads under 100 copies/ml were characterized as undetectable levels.

Jejunal biopsy

Tissue samples from the jejunum were collected with a pediatric enteroscope (Olympus GIF type XP260N, Olympus Medical System Corp., Tokyo, Japan). Five pieces (samples) of fresh jejunal tissue were placed on a shaker for 2 h at room temperature in 40 ml RPMI 1640 medium containing 10% FBS and 0.01 g collagenase from *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, MO). Disaggregated cells were filtered through glass wool loaded in a 20 ml disposable syringe. Cells were prepared from the filtrate by centrifugation at a speed of 1200 rpm for 10 min. Subsets of lymphocytes in the resuspended cells were analyzed by flow cytometry.

Flow cytometry

To analyze CD4+ T lymphocytes, whole blood and jejunal samples were stained with two fluorescently labeled mouse monoclonal antibodies, fluorescein isothiocyanate (FITC) conjugated anti-monkey CD3 (Clone FN-18, BioSource Intl, Camarillo, CA) and phycoerythrin (PE) conjugated anti-human CD4 (Clone Nu-TH/I; Nichirei, Tokyo, Japan). To analyze memory and naive CD4+ T lymphocytes, whole blood and jejunal samples were stained with three fluorescently labeled mouse monoclonal antibodies, FITC conjugated anti-human CD95 (Clone DX2; BD Pharmingen, Tokyo, Japan), PE conjugated anti-human CD28 (Clone CD28.2; Coulter Immunotech, Marseille, France), and allophycocyanin (APC) conjugated anti-human CD4 (Clone L200; BD Pharmingen). After hemolysis of whole blood and jejunal samples using a lysing solution (Beckton Dickinson, Franklin Lakes, NJ), each type of labeled lymphocyte was examined on a FACScalibur analyzer using Cellquest (BD Biosciences, San Jose, CA). CD95+$CD4^{high}$+ cells were considered memory T lymphocytes, and CD95-CD28+$CD4^{high}$+ cells were considered naive T lymphocytes (Pitcher et al., 2002). The absolute number of lymphocytes in the blood was determined using an automated blood counter, KX-21 (Sysmex, Kobe, Japan).

In vivo passage

Inguinal lymph nodes were aseptically collected from MM482 25 weeks after infection. The lymph nodes were minced with scissors, disaggregated using an 85-ml Bellco Tissue Sieve Kit (Bellco Glass, Inc., Vineland, NJ), and filtered through a 100-μm pore cell strainer (REF 35-2360, BD Falcon, Franklin Lakes, NJ). Filtrates were centrifuged and then washed four times with phosphate-buffered saline (PBS). These disaggregated cells were mixed with 2 ml frozen plasma (collected from the animal 8 weeks post-infection and stored at −80 °C) and 20 ml fresh blood from MM482, and then transfused into an uninfected monkey (MM498) intravenously. During the second passage, inguinal lymph nodes were aseptically collected from MM498 5 weeks after infection. The disaggregated inguinal lymph node was mixed with 2 ml frozen plasma (collected 2 weeks post-infection), 5×10^7 cells inguinal lymphocytes (collected 16 days post-infection and stored at −80 °C), and 15 ml fresh blood, and then transfused into an uninfected monkey (MM504).

Reisolation of virus

Fresh blood was obtained from the uninfected monkey, and PBMCs were isolated. These cells were incubated for 30 min with PE labeled anti-CD8 antibody (SK1 clone, BD Pharmingen), then washed once with PBS. Next, cells were incubated with anti-PE MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and CD8− cells were negatively selected with a magnetic column. CD8− PBMCs were cultured as described above.

On day 0, fresh blood was obtained from MM504 (16 weeks post-infection) and CD8 cells were depleted as described above. CD8+ cells were also depleted from frozen PBMCs (obtained from MM504 8 weeks post-infection and stored at −80 °C). These CD8− PBMCs from uninfected and infected monkeys were co-cultured in PBMC culture medium (described above) at a concentration of 2×10^6 cells/ml at 37 °C. Medium was replaced daily for 16 days and culture supernatants were stored at −80 °C. The culture supernatant with the highest RT value was stored in liquid nitrogen. This virus stock was designated SHIV-MK38.

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Sequence of V1, V2, and V3 regions of SHIV-MK38

SHIV-MK38 viral stock was used as a template for RT-PCR to amplify the V1 to V3 regions of the *env* gene. The forward primer 5′ GTGTAAAATTAACCCCACTCTGTG 3′ and reverse primer 5′ TGGGAGGGGCATACATTGCTTTTCC 3′ were used for RT-PCR. The amplified DNA fragment was cloned into the pCR2.1 vector using a TA Cloning Kit (Invitrogen, Carlsbad, CA), and 14 clones were sequenced.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2010.01.008.

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Small Intestine CD4$^+$ T Cells Are Profoundly Depleted during Acute Simian-Human Immunodeficiency Virus Infection, Regardless of Viral Pathogenicity$^\nabla$

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To analyze the relationship between acute virus-induced injury and the subsequent disease phenotype, we compared the virus replication and CD4$^+$ T-cell profiles for monkeys infected with isogenic highly pathogenic (KS661) and moderately pathogenic (#64) simian-human immunodeficiency viruses (SHIVs). Intrarectal infusion of SHIV-KS661 resulted in rapid, systemic, and massive virus replication, while SHIV-#64 replicated more slowly and reached lower titers. Whereas KS661 systemically depleted CD4$^+$ T cells, #64 caused significant CD4$^+$ T-cell depletion only in the small intestine. We conclude that SHIV, regardless of pathogenicity, can cause injury to the small intestine and leads to CD4$^+$ T-cell depletion in infected animals during acute infection.

The highly pathogenic simian-human immunodeficiency virus (SHIV) SHIV-C2/1-KS661 (KS661), which was derived from SHIV-89.6 (23), replicates to high titers and causes the irreversible depletion of the circulating CD4$^+$ T cells during the acute phase of intravenous infection, followed by AIDS-like disease within 1 year (23). We previously reported that KS661 massively replicates and depletes CD4$^+$ T cells in both peripheral and mucosal lymphoid tissues during the initial 4 weeks postinfection (16). On the other hand, the isogenic SHIV-#64 (#64), which was derived from SHIV-89.6P, is moderately pathogenic. The genomic sequences of the two SHIVs differ by only 0.16%, resulting in a total of six amino acid changes in the products of the *pol*, *env-gp41*, and *rev* genes. The intravenous inoculation of rhesus macaques with #64 induces plasma viral burdens comparable to those induced by KS661 during the acute phase of infection and causes a transient reduction of the circulating CD4$^+$ T lymphocytes (10). After the acute phase, the viral loads decline to undetectable levels and the populations of CD4$^+$ T cells recover to preinfection levels.

To clarify the relationship between acute viral replication kinetics and subsequent clinical courses for these isogenic SHIVs with distinct pathogenicities, we examined proviral DNA, infectious-virus-producing cells (IVPCs), and CD4$^+$ T-cell depletion in peripheral and mucosal lymphoid tissues of 17 infected (Table 1) and 7 uninfected adult rhesus macaques (*Macaca mulatta*). Both Chinese and Indian rhesus monkeys were randomly assigned to these groups. The monkeys 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. The animals were inoculated via intrarectal infusion as described previously (17). Following serial euthanasia, tissues were collected and analyzed up to 27 days postinfection (dpi) as described previously (16, 17).

Gross virus replication was assessed by measuring plasma viral loads by reverse transcriptase PCR (16). By 6 dpi, plasma viral RNA levels became detectable in all the KS661-infected macaques (Fig. 1A) and three of seven #64-infected macaques (animals MM372, MM391, and MM374) (Fig. 1B). Although the plasma viral loads of the two groups at 13 dpi, when the virus loads reached their initial peaks, were not significantly different ($P = 0.1673$), the average load (± the standard deviation) in KS661-infected monkeys ($9.3 \times 10^8 \pm 15.9 \times 10^8$ copies/ml) was about 10 times higher than that in #64-infected monkeys ($6.3 \times 10^7 \pm 11.6 \times 10^7$ copies/ml). These results suggest that KS661 spread faster and reached a somewhat higher titer than did #64 when the viruses were inoculated intrarectally.

Levels of peripheral blood CD4$^+$ T lymphocytes in all the KS661-infected monkeys decreased substantially within 4 weeks (Fig. 1C). On the other hand, the reductions in the levels of CD4$^+$ T cells varied among the #64-infected monkeys (Fig. 1D). For example, MM378 did not exhibit any appreciable changes, even though the plasma viral RNA load in this monkey reached 2.6×10^7 copies/ml by 21 dpi (Fig. 1 B and D).

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TABLE 1. Experimental schedule for individual monkeys[a]

Virus (inoculum size)	Monkeys examined at:		
	6 dpi	13 dpi	27 dpi
KS661 (2×10^3 $TCID_{50}$)	MM300, MM309	MM313, MM334, MM392, MM393	MM308, MM310, MM394, MM395
#64 (2×10^5 $TCID_{50}$)	MM379, MM390	MM372, MM373*, MM391	MM374, MM378

[a] $TCID_{50}$, 50% tissue culture infective doses; *, MM373 received 2×10^3 $TCID_{50}$ of #64.

These data suggest that the decline in circulating $CD4^+$ T cells in KS661-infected animals was more severe and more reproducible than that in the #64-infected monkeys.

Another highly pathogenic SHIV, SHIV-DH12R, is known to cause systemic and synchronous replication events in animals following intravenous inoculation (6). To reveal the spread of virus in monkeys following intrarectal infection, we measured proviral DNA loads in a variety of tissues as described previously (16). KS661 proviral DNA was detected not only in samples from the rectums, the site of virus inoculation, but also in peripheral blood mononuclear cells and some lymph nodes (LN) at 6 dpi (Fig. 2A), suggesting that the virus was already spreading systemically. At 13 dpi, when the viral RNA loads in peripheral blood increased to the highest titers, proviral DNA levels in all of the tissues examined also increased, with levels in most monkeys exceeding 10^4 copies/μg of DNA. The levels of proviral DNA in all the tissues declined remarkably by 27 dpi. In contrast, #64 proviral DNA was detected only in the rectum of one (MM390) of the two monkeys examined at 6 dpi (Fig. 2A). At 13 dpi, the amount of proviral DNA in each tissue sample from #64-infected monkeys ($<10^4$ copies/μg of DNA) was considerably smaller than

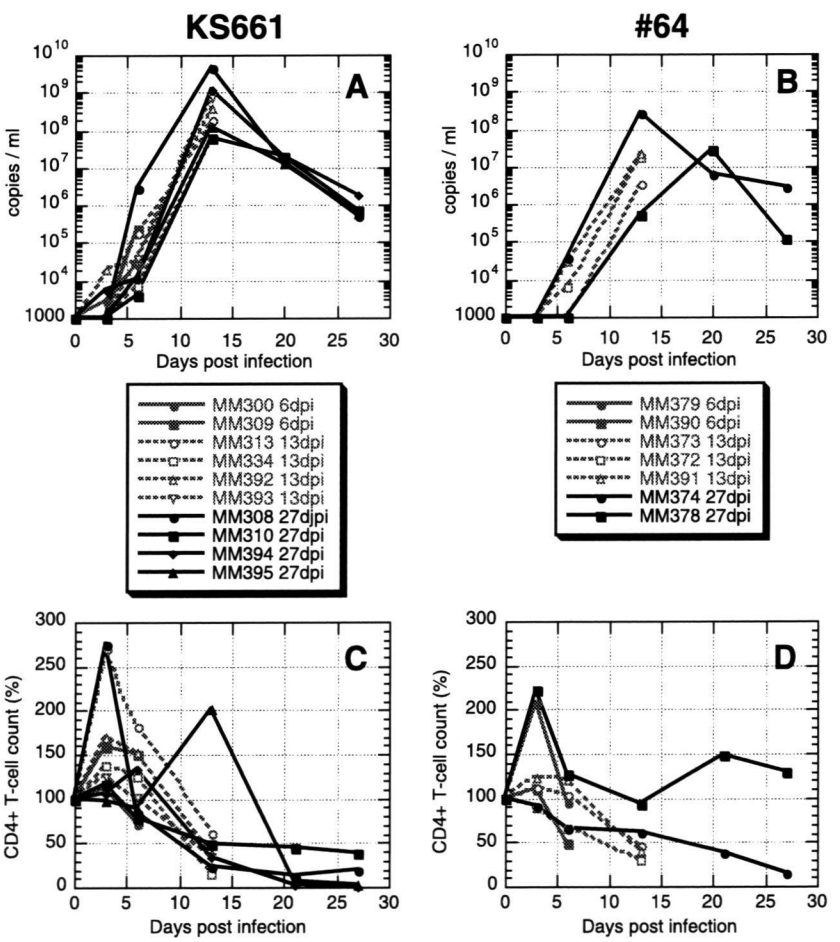

FIG. 1. Plasma viral RNA loads and profiles of circulating $CD4^+$ T cells for monkeys intrarectally infected with highly pathogenic KS661 and moderately pathogenic #64. (A and B) Plasma viral RNA loads were measured by quantitative reverse transcriptase PCR. The detection limit of this assay was 10^3 copies/ml. (C and D) Levels of $CD4^+$ T cells in peripheral blood samples from monkeys infected with KS661 and #64. The absolute number of $CD3^+$ $CD4^+$ cells in peripheral blood immediately before infection (day 0 postinfection) was defined as 100% for each monkey.

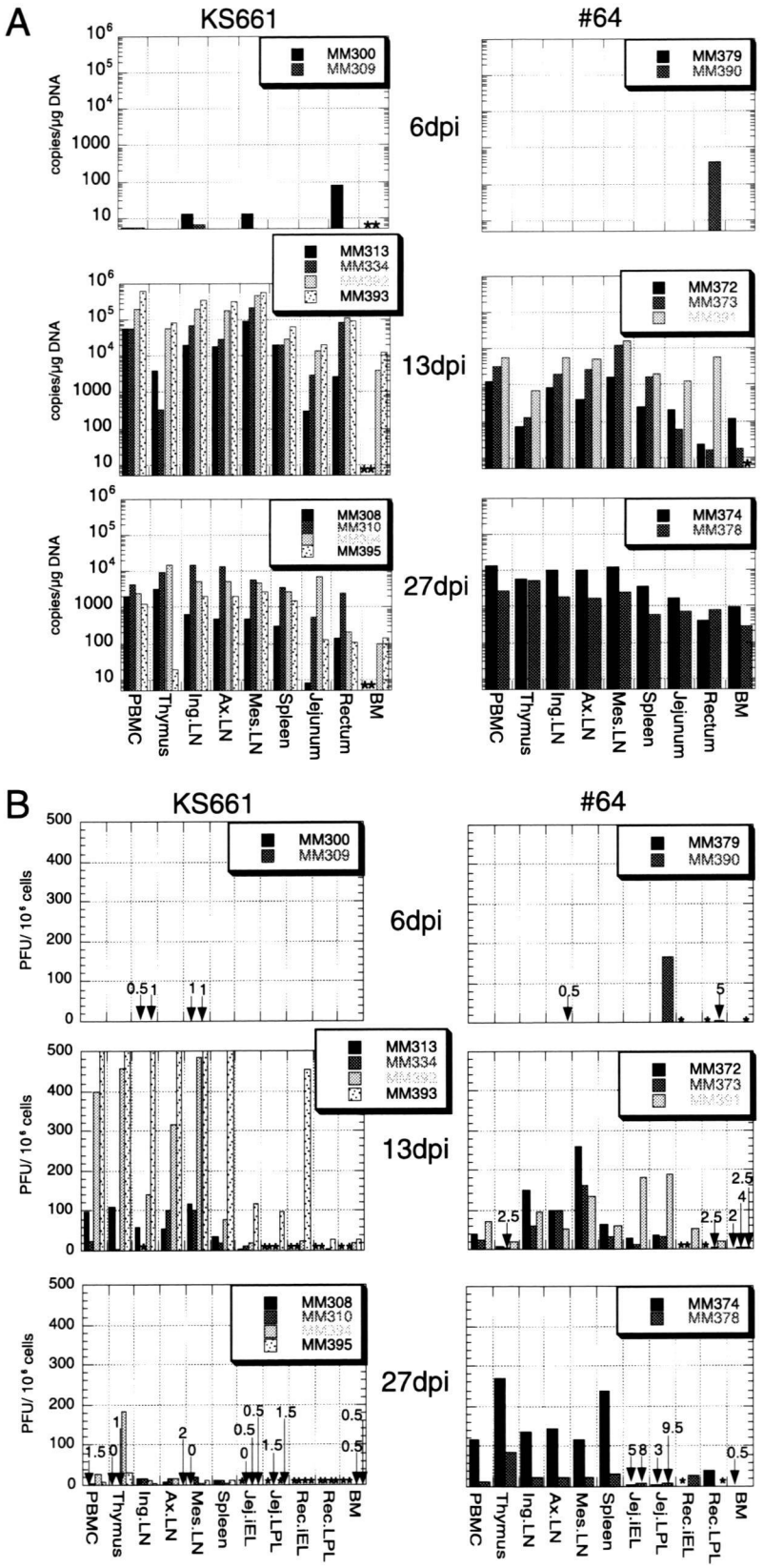

FIG. 2. (A) Proviral DNA loads in tissues of KS661- and #64-infected monkeys at 6, 13, and 27 dpi. Viral burdens were determined by quantitative PCR and expressed as the numbers of viral DNA copies per microgram of total DNA extracted from tissue homogenates. PBMC, peripheral blood mononuclear cells; Ing., inguinal; Ax., axillary; Mes., mesenteric; BM, bone marrow; *, not done. (B) Numbers of IVPCs in tissues of KS661- and #64-infected monkeys at 6, 13, and 27 dpi. Numbers of IVPCs were determined by an infectious plaque assay and were expressed as the numbers of PFU per 10^6 cells. Jej., jejunum; Rec., rectum; iEL, intraepithelial lymphocytes; *, not done.