

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-DH12R-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; Sadjadpour 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 effector 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 SHIV-MK1 to cause CD4+ T lymphocyte depletion in the jejunum is not as strong as the parental SHIV-KS661.

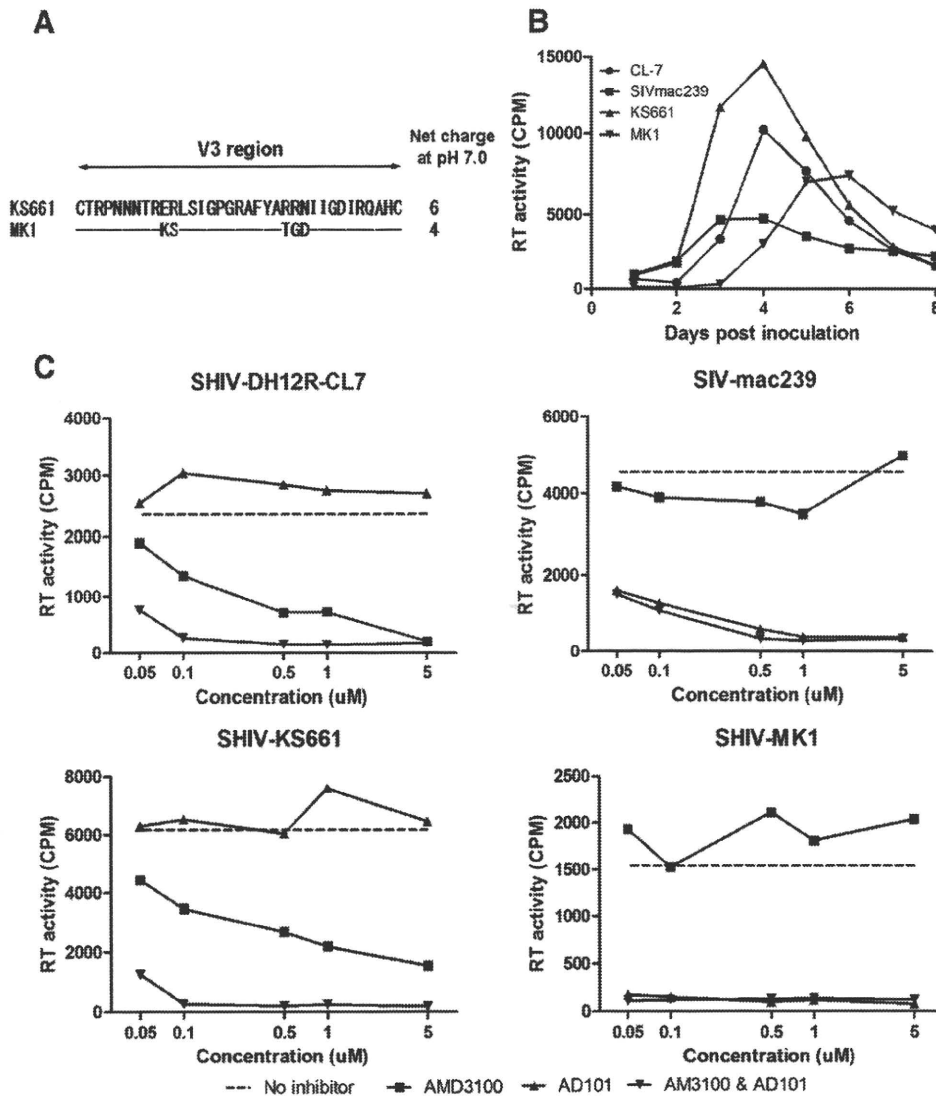


Fig. 1. Construction and *in vitro* analysis of SHIV-MK1. (A) gp120 V3 amino acid alignment of SHIV-MK1. Amino acid substitution positions are indicated under the parental SHIV-KS661 alignment. The net charge at pH 7.0 is indicated beside each amino acid alignment. (B) SHIV and SIV replication in rhPBMCs. 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 μ M. The RT activity on day 5 post-infection was determined by the absence (dashed line) or presence of an inhibitor in the medium.

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-CL7 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 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

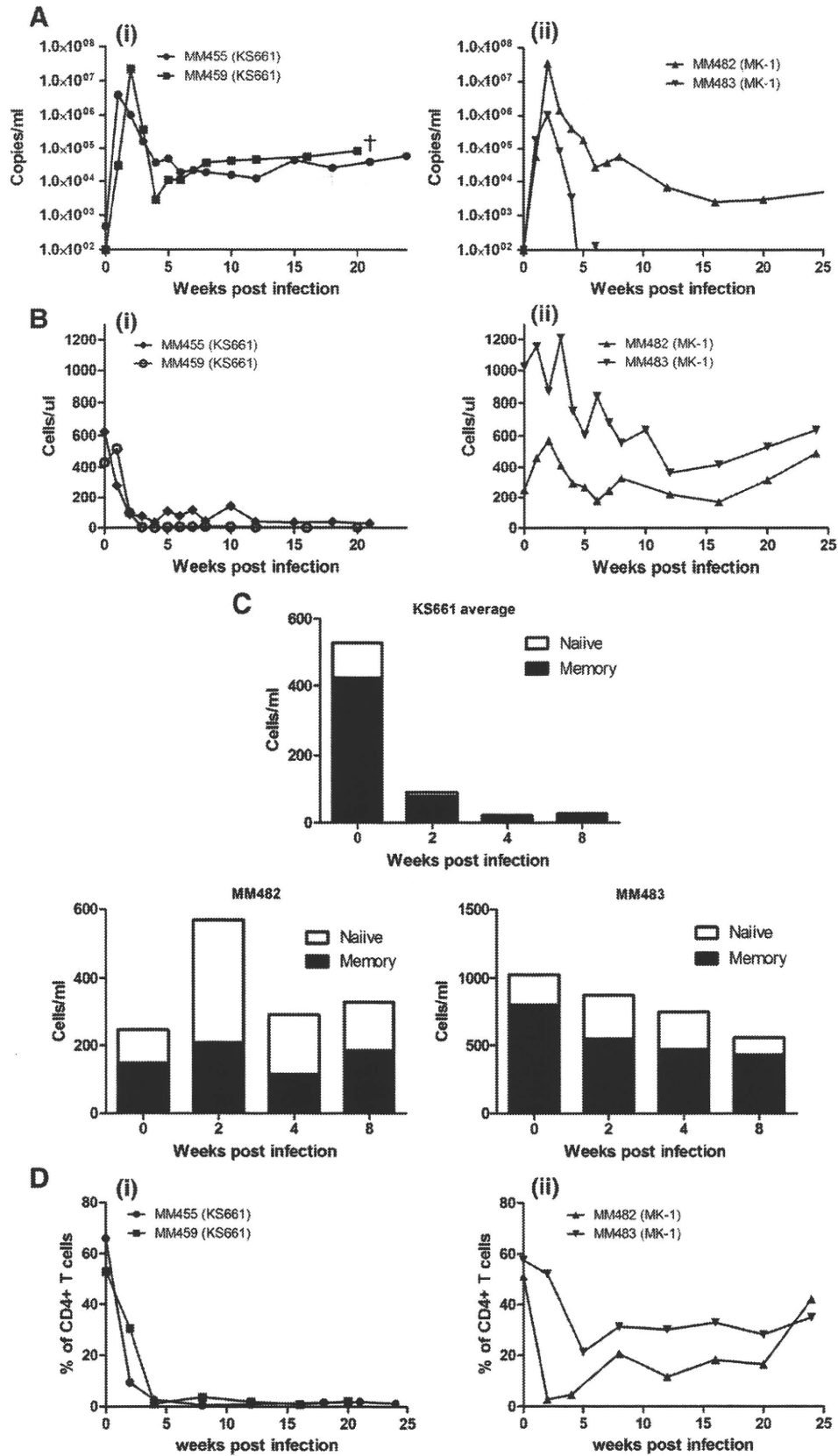


Fig. 2. *In vivo* replication of MK1. (A) Plasma viral RNA loads in SHIV-infected rhesus monkeys were measured at the indicated times. A total of 2000 TCID50 SHIV-KS661 was inoculated intravenously into MM455 and MM459 as a control group (i) and 20,000 TCID50 SHIV-MK1 was inoculated intravenously into 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 endoscope, and were analyzed by FACS.

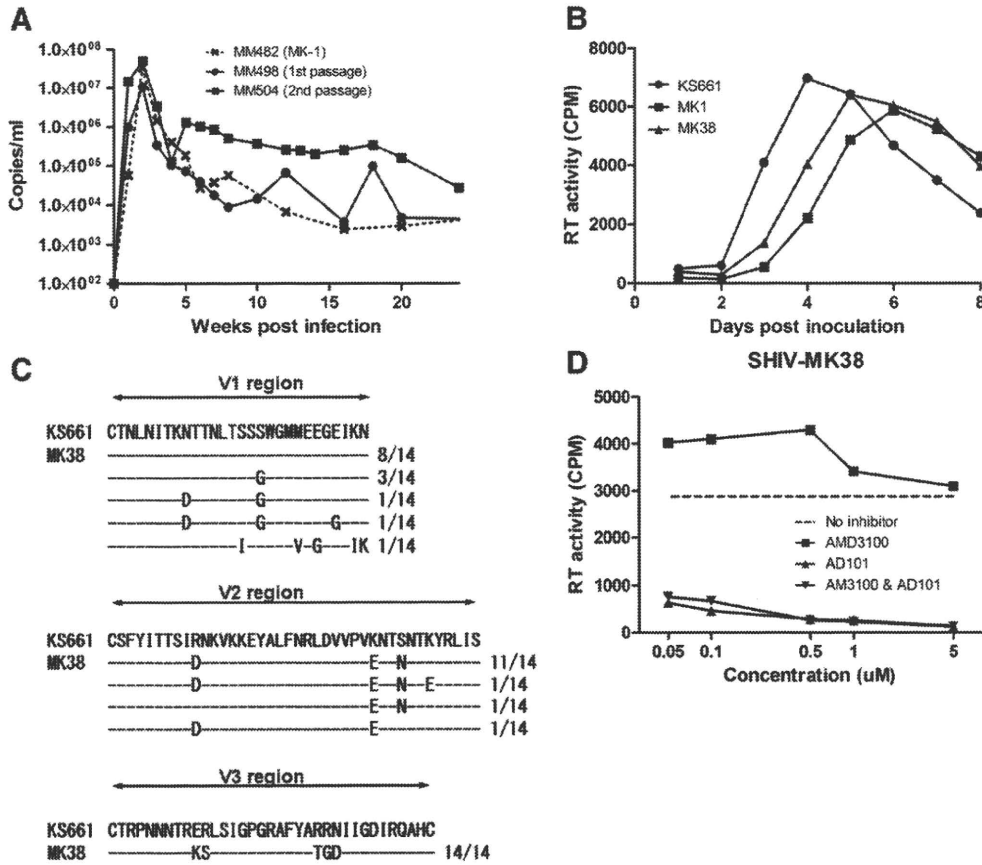


Fig. 3. *In vivo* adaptation of SHIV-MK1, and *in vitro* analysis of reisolated virus. (A) Plasma viral RNA loads of passaged monkeys were measured at the indicated times. The whole blood and dissociated lymph nodes from SHIV-MK1-infected MM482 were transfused into MM498 (first passage) 25 weeks post-inoculation. 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.

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 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

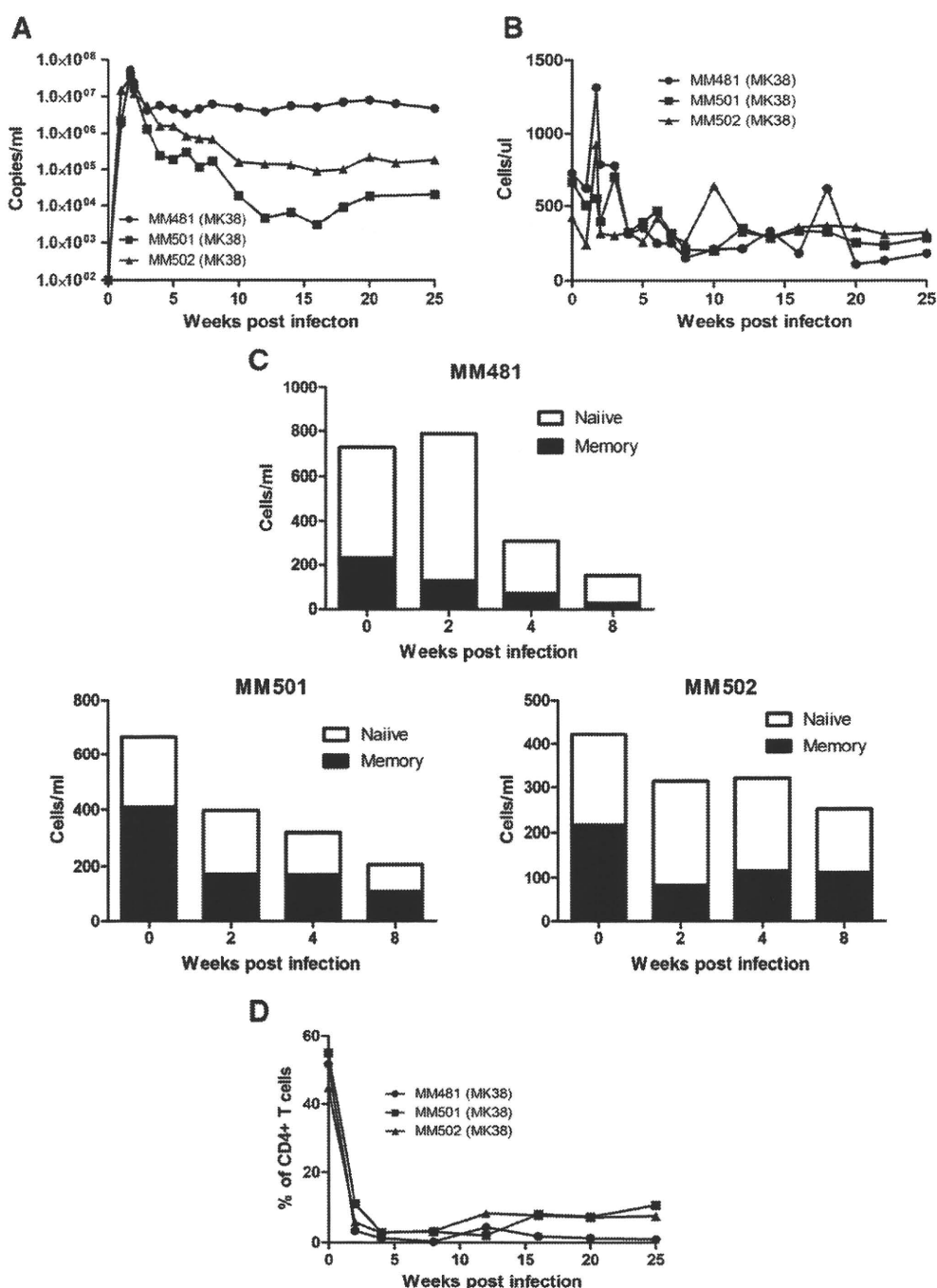


Fig. 4. *In vivo* replication of SHIV-MK38. (A) Plasma viral RNA loads in SHIV-infected rhesus monkeys were measured at the indicated times. A total of 20,000 TCID₅₀ SHIV-MK38 were inoculated into MM481, MM501, and MM502. (B) CD4⁺ T lymphocytes were enumerated using FACS analysis in SHIV-MK38-infected 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.

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 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-GAAAAAGTTTATCTATAGGACCAGGGAGAGCATTATGCAACAGGAGACATAATAGGAG 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-TATGTCTCCTGTTGCATAAAATGCTCTCCCTGGTCCTATAGATAAACTTTTTCTGTATTG 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 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.

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' GTGTAATAACCCACTCTGTG 3' and reverse primer 5' TGGGAGGGGCATACATTGCTTTCC 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⁺ cell reduction and enteropathy in simian/human immunodeficiency virus KS661-infected rhesus macaques in the presence of low viral load

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Human immunodeficiency virus type 1, simian immunodeficiency virus and simian/human immunodeficiency virus (SHIV) infection generally lead to death of the host accompanied by high viraemia and profound CD4⁺ T-cell depletion. SHIV clone KS661-infected rhesus macaques with a high viral load set point (HVL) ultimately experience diarrhoea and wasting at 6–12 months after infection. In contrast, infected macaques with a low viral load set point (LVL) usually live asymptotically throughout the observation period, and are therefore referred to as asymptomatic LVL (Asym LVL) macaques. Interestingly, some LVL macaques exhibit diarrhoea and wasting similar to the symptoms of HVL macaques and are termed symptomatic LVL (Sym LVL) macaques. This study tested the hypothesis that Sym LVL macaques have the same degree of intestinal abnormalities as HVL macaques. The proviral DNA loads in lymphoid tissue and the intestines of Sym LVL and Asym LVL macaques were comparable and all infected monkeys showed villous atrophy. Notably, the CD4⁺ cell frequencies of lymphoid tissues and intestines in Sym LVL macaques were remarkably lower than those in Asym LVL and uninfected macaques. Furthermore, Sym LVL and HVL macaques exhibited an increased number of activated macrophages. In conclusion, intestinal disorders including CD4⁺ cell reduction and abnormal immune activation can be observed in SHIV-KS661-infected macaques independent of virus replication levels.

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INTRODUCTION

The intestinal tract, which is the largest mucosal and lymphoid organ and which contains the majority of the total lymphocytes in the body, is an important port of entry for human immunodeficiency virus type 1 (HIV-1) infection in vertical and homosexual transmission (Smith *et al.*, 2003). Additionally, the intestinal tract is a central site in the interaction between HIV-1 and its host, and suffers profound pathological changes as a result of HIV-1

infection. HIV-1 infection of the intestinal tract is characterized by virus replication (Fackler *et al.*, 1998), CD4⁺ T-cell depletion (Brenchley *et al.*, 2004), opportunistic infection and HIV enteropathy, which is an idiopathic intestinal disorder observed in infected patients with diarrhoea (Kotler, 2005). In particular, CD4⁺ T-cell depletion, which is the immunological hallmark in the development of AIDS, preferentially takes place in the intestinal tract rather than in the peripheral blood throughout the infection (Brenchley *et al.*, 2004). This

observation is based on the following findings: (i) most naturally transmitted HIV-1 strains are chemokine receptor 5 (CCR5)-tropic; and (ii) the intestinal tract, especially the lamina propria, contains a large number of activated memory CCR5⁺ CD4⁺ T cells, which indicates a high susceptibility for HIV-1 infection, whereas the peripheral blood has a relatively small population of these cells (Anton *et al.*, 2000; Lapenta *et al.*, 1999). CD4⁺ T-cell depletion from the intestinal tract by HIV-1 infection is thought to lead to progressive dysfunction of mucosal immunity, which triggers immunodeficiency (Paiardini *et al.*, 2008). In addition to CD4⁺ T-cell depletion in the intestinal tract, HIV-1 infection causes histopathological changes in the intestine, including villous atrophy, crypt hyperplasia and acute/chronic inflammation (Batman *et al.*, 1989).

Chronic disease of the intestinal tract generally manifests as inflammation (Kahn, 1997). Diarrhoea is a major intestinal symptom caused by various stimuli to the intestinal tract such as pathogens, toxins and dysfunction of the immune system (Gibbons & Fuchs, 2007). Because HIV-1 infection weakens the host immune system, AIDS is one of the primary causes of chronic diarrhoea (Sestak, 2005). In developing countries, diarrhoea was a major symptom in advanced HIV-1 infection prior to the establishment of highly active antiretroviral therapy (HAART) (Wilcox & Saag, 2008). Dehydration and malabsorption as a result of chronic diarrhoea can lead to progressive weight loss and can contribute to morbidity and mortality in HIV-1-infected patients (Sharpstone & Gazzard, 1996). Therefore, chronic diarrhoea is one of the most important clinical signs in AIDS patients.

AIDS models using non-human primates have provided many important observations on AIDS pathogenesis. The first finding of early CD4⁺ T-cell depletion from the intestinal tract was reported in a study using simian immunodeficiency virus (SIV)-infected macaques (Veazey *et al.*, 1998). Intestinal CD4⁺ T cells of rhesus macaques predominantly exhibit a CCR5⁺ activated memory phenotype, and CD4⁺ T cells of this phenotype are selectively eliminated in SIV-infected macaques, indicating that the majority of intestinal CD4⁺ T cells are primary targets of SIV infection (Veazey *et al.*, 2000a, b). Accordingly, detailed analysis of the intestinal tract using animal models is essential for an understanding of AIDS pathogenesis.

Simian/human immunodeficiency virus (SHIV)-KS661 is a molecular clone and a pathogenic virus in rhesus macaques. SHIV-KS661 systemically depletes CD4⁺ T cells of rhesus macaques within 4 weeks of infection (Miyake *et al.*, 2006). Based on our observations over a number of years, intravenous infection of rhesus macaques with SHIV-KS661 consistently results in high viraemia and CD4⁺ T-cell depletion, followed by malignant morbidity as a result of severe chronic diarrhoea and wasting after 6–18 months. Generally, the time to clinical morbidity in rhesus macaques infected with pathogenic SHIVs, such as SHIV-89.6P and SHIV-KS661, is considerably shorter than

in HIV-1-infected humans, who take an average of 10 years to progress to AIDS. In addition, all subsets of CD4⁺ T cells including memory and naïve T cells are thoroughly depleted in pathogenic SHIV-infected macaques. However, in the SHIV-KS661 macaque model, diarrhoea and wasting, which are major symptoms in advanced HIV-1 infection, can clearly be recognized and defined in association with disease progression.

Recently, we observed that, in many rhesus macaques infected intrarectally with SHIV-KS661, plasma viral RNA loads decreased gradually to undetectable levels in the chronic phase, which is quite different from the case with intravenous infection. It is well known that pathogenic SIV and SHIV infections in monkeys, like HIV-1 infections in humans, generally lead to high viraemia, profound CD4⁺ T-cell depletion and death. Interestingly, in this study, two out of six intrarectally inoculated macaques with a low plasma viral load experienced malignant morbidity manifest as severe diarrhoea and wasting, similar to what we observed in infected macaques with high viraemia. The purpose of this study was to elucidate why macaques with a low plasma viral load experienced diarrhoea and wasting. As an explanation for this morbidity, we hypothesized that, even if the viral load set-point is suppressed, SHIV-KS661-infected macaques would have the same degree of intestinal abnormalities as infected macaques with high viraemia. To test this hypothesis, we analysed CD4⁺ cell frequencies in lymphoid and intestinal tissues and damage to the intestinal mucosa in infected macaques with high and low viral load set points (HVL and LVL, respectively). Here, we have provided evidence for the development of intestinal disorders in SHIV-KS661-infected macaques irrespective of the plasma viral RNA load.

RESULTS

Diarrhoea and wasting in two macaques despite low viral load

All macaques inoculated intravenously with SHIV-KS661 and one out of seven macaques inoculated intrarectally with SHIV-KS661 exhibited high set points of plasma viral RNA loads, persisting at over 10⁶ copies ml⁻¹ until they needed to be euthanized as a result of diarrhoea and wasting (Fig. 1a). In contrast, in the remaining six macaques inoculated intrarectally with SHIV-KS661, the set points of plasma viral RNA load gradually decreased to undetectable levels (Fig. 1a). We called these macaques showing high and low set points of viral RNA load HVL and LVL macaques, respectively. During an observation period of approximately 1.4 years, two LVL macaques (MM397 and MM399) experienced severe diarrhoea and wasting and required euthanasia at approximately 22 weeks post-infection (p.i.), similar to HVL macaques, whereas the remaining four LVL macaques were asymptomatic (Fig. 1a). We termed the healthy LVL macaques asymptomatic LVL macaques (Asym LVL) and the LVL

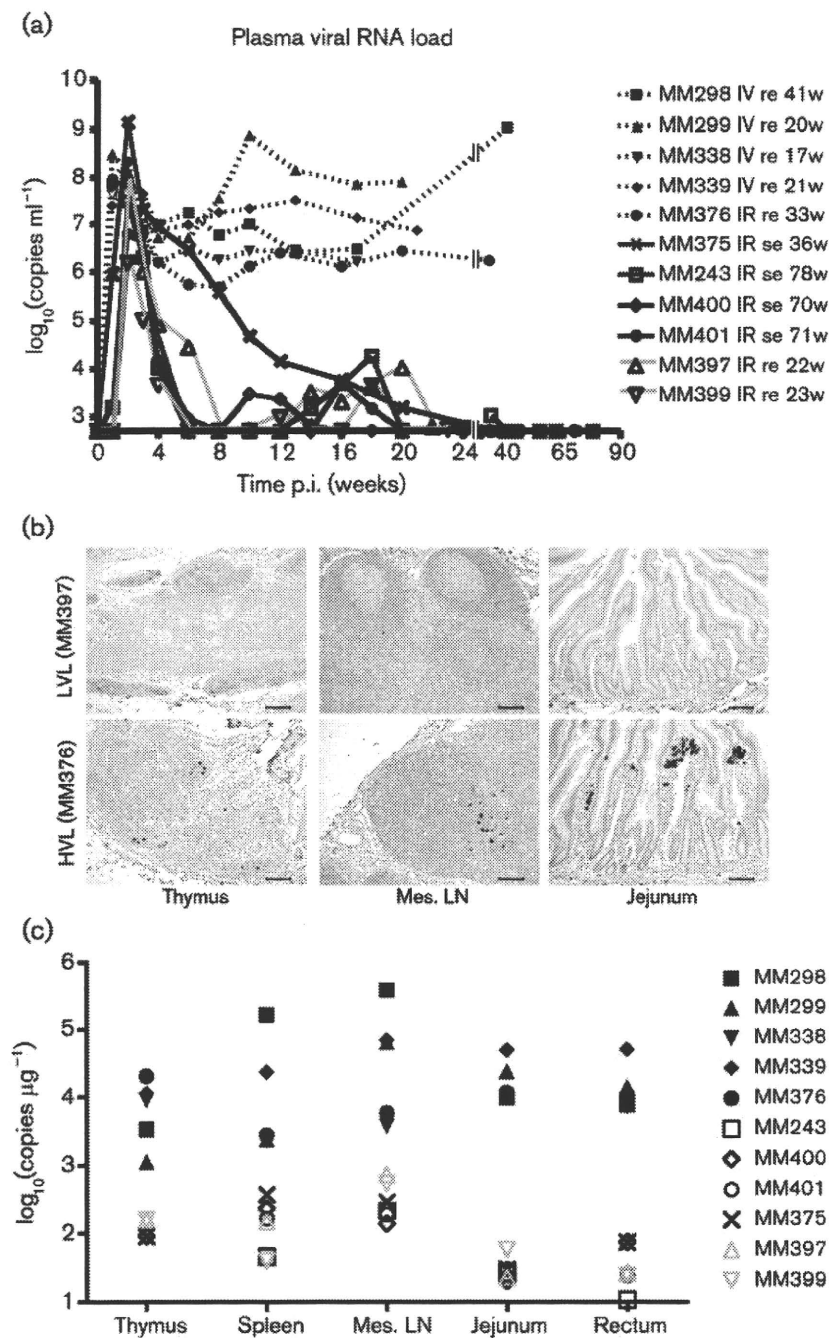


Fig. 1. Distribution of virus in various tissues of SHIV-KS661-infected rhesus macaques. (a) Time course of plasma viral RNA loads as measured by quantitative RT-PCR. The detection limit of plasma viral RNA loads was 500 copies ml⁻¹. The animal ID numbers, infection route and when and how they were euthanized are indicated on the figure. IV, Intravenous inoculation; IR, intrarectal inoculation; re, required euthanasia; se, scheduled euthanasia; w, number of weeks after infection when euthanasia was performed. (b) Immunohistochemical detection of Nef antigen in thymus, mesenteric lymph nodes (Mes. LN) and jejunum. Brown staining indicates Nef⁺ cells. The upper panels show representative tissue sections from a Sym LVL macaque (MM397) and the lower panels show representative tissue sections from an HVL macaque (MM376). Bars, 100 μm. (c) Proviral DNA loads in different tissues of SHIV-KS661-infected macaques, as measured by quantitative PCR. The detection limit of proviral DNA loads was 10 copies μg⁻¹. Filled black symbols indicate HVL macaques, open black symbols indicate Asym LVL macaques and open grey symbols indicate Sym LVL macaques.

macaques with diarrhoea and wasting symptomatic LVL macaques (Sym LVL).

Antibody response against SHIV in infected macaques

The LVL macaques showed antibody responses to SHIV-KS661 at 3–4 weeks p.i. and then developed strong antibody responses that persisted up to 18 weeks p.i. (Table 1). In contrast, two of the HVL macaques (MM298 and MM299) showed no antibody response, whilst the remaining two (MM338 and MM339) showed very low

antibody responses. Among the HVL macaques, only MM376 showed a strong antibody response: the titre reached 1:2048 at 6 weeks p.i., but then decreased to a much lower value. These results showed that LVL macaques succeeded in maintaining a strong antibody response, whilst HVL macaques failed to do so.

Viral levels in tissues from Sym LVL and Asym LVL macaques are not significantly different

To investigate whether the infected macaques had different viral levels in their lymphoid and intestinal tissues, we used

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	16 384	32	64	32	512	512	–	–	–	–
6	8 192	16 384	256	64	4 096	1 024	2 048	–	–	–	–
8	4 096	16 384	1 024	128	1 024	16 384	512	–	–	–	–
10	16 384	16 384	2 048	512	512	16 384	512	–	–	–	–
12	16 384	16 384	256	512	4 096	16 384	512	–	–	–	–
13	–	–	–	–	–	–	–	–	–	–	–
14	16 384	16 384	1 024	512	2 048	–	–	–	–	–	–
16	4 096	8 192	1 024	1 024	1 024	16 384	64	–	–	–	–
17	–	–	–	–	–	–	–	–	–	–	–
18	8 192	16 384	2 048	8 192	4 096	–	–	–	–	–	–

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

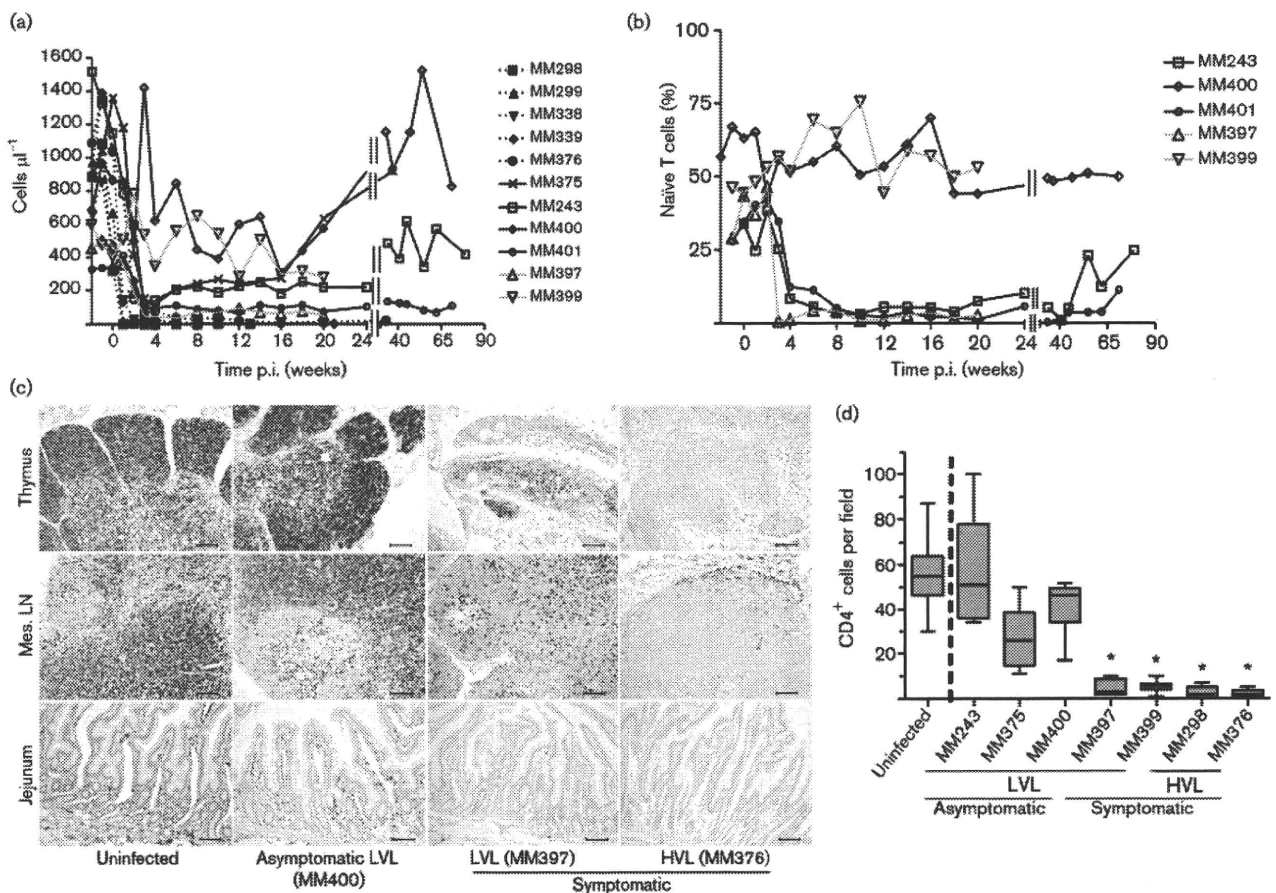


Fig. 2. Counts of circulating CD4⁺ T-cell subsets and CD4⁺ cell frequency in lymphoid and intestinal tissues at the time of euthanasia in SHIV-KS661-infected rhesus macaques. Counts of circulating CD4⁺ T-cell subsets were analysed by flow cytometry and whole-blood counts. (a) Circulating CD4⁺ T-cell 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

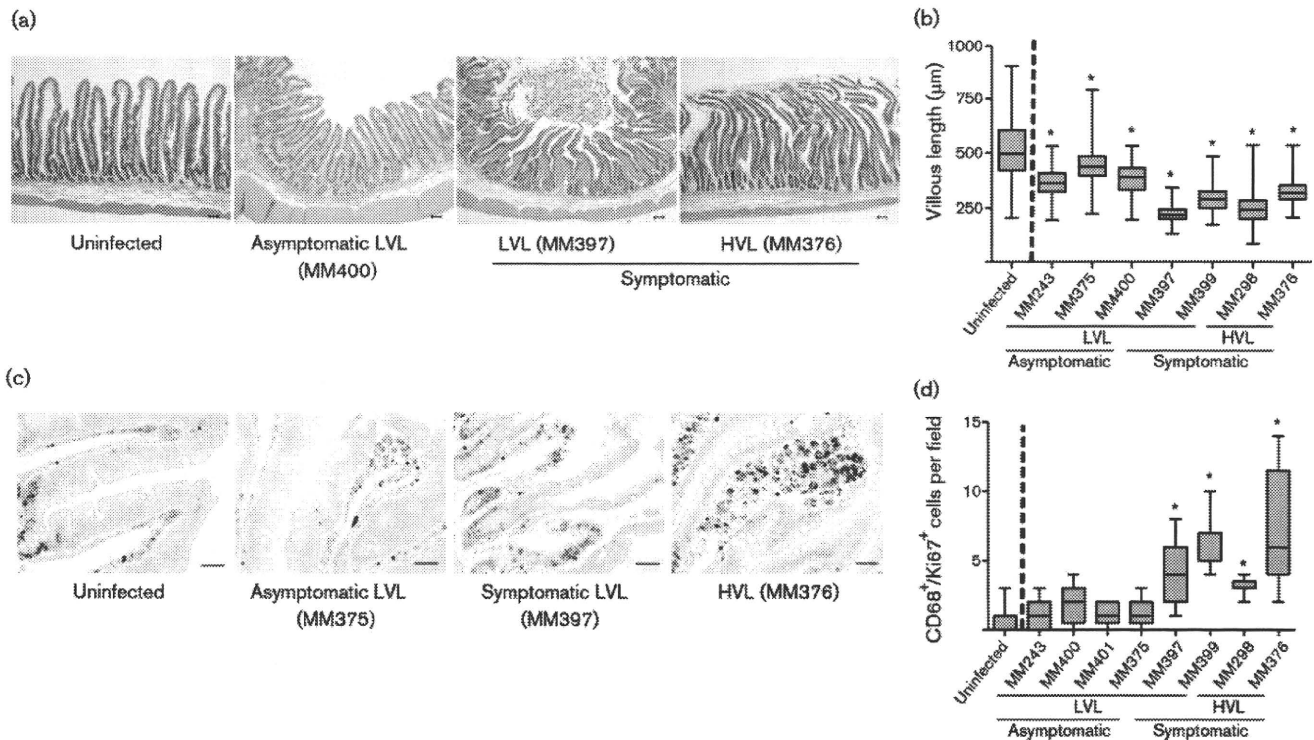


Fig. 3. Villous length in jejunum and counts of activated macrophages in the small intestine at the time of euthanasia in SHIV-KS661-infected rhesus macaques. (a) H&E-stained sections of jejunum of representative uninfected, Asym LVL, Sym LVL and HVL macaques. Bars, 200 µm. (b) Comparison of villous 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/1; 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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Peripheral Blood CD4 and CD8 Double-Positive T Cells of Rhesus Macaques Become Vulnerable to Simian Immunodeficiency Virus by *In Vitro* Stimulation Due to the Induction of CCR5

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ABSTRACT. *In vivo* Simian Immunodeficiency Virus (SIV) challenge of macaques demonstrated the earlier disappearance of CD4 and CD8 double-positive (DP) T cells than CD4 single-positive T cells, although its mechanism remains unclear. Here we found that peripheral DP T cells were readily induced to express CCR5, a secondary receptor for SIV, by *in vitro* stimulation with either concanavalin A or anti-CD3/CD28 monoclonal antibodies. Activated DP T cells were more vulnerable to SIV infection, indicating that the ability of DP T cells to readily express CCR5 after activation may hasten DP T cell death by SIV infection *in vivo*.

KEY WORDS: CCR5, CD4 and CD8 double-positive T cells, Simian Immunodeficiency Virus.

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Simian immunodeficiency virus (SIV) uses the CD4 molecule as the major receptor for infection to target cells [4, 5]. The depletion of CD4-bearing T cells in the body of SIV-infected animals is the hallmark of this viral infection. CD4-bearing T cells include both CD4 single-positive (CD4SP) T cells and CD4 and CD8 double-positive (DP) T cells. DP T cells exist in the intestine and form the large proportion of intestinal T cells in macaque monkeys [10]. *In vivo* SIV challenge demonstrated that intestinal DP T cells are depleted faster and more profoundly than intestinal CD4SP T cells at the early stage of infection. Although this is thought to be caused by higher expression levels of CCR5, the coreceptor for SIV, which are mainly expressed on activated T cells [14, 15], it is still unclear why DP T cells are highly permissive to SIV infection.

Additionally, in macaque monkeys, DP T cells physiologically exist in the peripheral blood [2, 17], the significance of which has not been completely elucidated. However, several investigators have reported that DP T cells exhibit the following characteristics: they proportionally increase in an age-dependent manner [2, 8]; are phenotypically different from thymic DP T cells [1]; have dual immune functions, i.e., low helper and high cytotoxic activity and, freshly isolated, these cells express a high level of interferon γ mRNA [9], which is important for antiviral infection. Although studies have not yet determined whether DP T cells in peripheral blood and the intestine have a common origin, intestinal DP T cells are considered to be from extrathymic lineages [14, 15] and are suggested to have effector functions against viral infection [10]. Thus,

both intestinal and peripheral blood DP T cells are considered to be in the same category of “extrathymic DP T cells”, although their locations are different. In contrast to cells in the intestine, almost all CD4SP and DP T cells in peripheral blood are not in an activated state and have lower CCR5 expression [10]; therefore, these cells are not considered to be suitable target cells for SIV infection in the natural state of peripheral blood. We hypothesized that, upon activation, DP T cells in peripheral blood have the ability to express higher levels of CCR5 and are therefore more vulnerable to SIV infection than peripheral blood CD4SP T cells. To test this hypothesis, and to assess *in vivo* DP T cell vulnerability to SIV infection, we conducted *in vitro* activation of rhesus monkey peripheral blood mononuclear cells (PBMCs) and compared the expression of CCR5 and the vulnerability to SIV infection of DP T cells with those of CD4SP T cells.

We first examined CCR5 expression levels on *in vitro* activated peripheral blood CD4SP and DP T cells in rhesus monkeys. Acid citrate dextrose (ACD) anti-coagulated blood was taken from rhesus monkeys maintained at Kyoto University (*Macaca mulatta*: MM51, MM132, MM450, MM452, MM455, MM464; Samples were randomly selected depending on the availability of the animals). PBMCs were isolated from whole blood by the Ficoll-Paque gradient method. Concanavalin A (ConA) or anti-CD3/CD28mAbs were used to activate T cells *in vitro* [7, 11]. Freshly isolated PBMCs were stimulated with 12.5 $\mu\text{g/ml}$ ConA (Wako, Osaka, Japan) (MM132, MM51 and MM455) or 100 ng/ml anti-CD3 mAb (clone SP34; BD PharMingen) and 1 $\mu\text{g/ml}$ anti-CD28 mAb (clone 28.2; BD PharMingen) (MM132, MM455 and MM464), and cultured in RPMI growth medium (RPMI1640 medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 $\mu\text{g/ml}$ streptomycin, 50 μM 2-mercaptoethanol and 100 U/ml recombi-

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nant interleukin-2). After a stimulation period of 24 hr, cultured cells were washed with RPMI1640, resuspended in RPMI growth medium, and seeded in 96-well cell culture plates (2×10^5 cells/200 μ l/well). The experiments were performed in three monkeys for each treatment, with PBMCs from each animal measured in triplicate. To detect CCR5 expression levels on CD4SP and DP T cell populations, cultured cells were collected at day 2, 4, and 6 post-stimulation and subjected to flow cytometric analysis. Negative controls were prepared in the same manner without mitogen. Cell-surface expression of lymphocyte antigens was identified by flow cytometry using a FACS CantofII (BD PharMingen) and analyzed with BD FACSDiva software. The mAbs used in this study were as follows: anti-CD3 (PE-Cy7; clone SP34, BD PharMingen), anti-CD4 (FITC; clone L200, BD PharMingen), anti-CD8 (RPE-Cy5; clone DK25, Dako, Glostrup, Denmark) and anti-CCR5 (PE; clone 3A9, BD PharMingen). CCR5 expression levels were expressed as the percentage of CCR5-positive cells determined with a gate drawn from unstained controls. From days 4 to 6 post-ConA stimulation, CCR5 expression levels increased both on CD4SP T cells and DP T cells. Of note, DP T cells expressed higher levels of CCR5 ($90.47 \pm$

3.40% at day 6) than CD4SP T cells ($38.39 \pm 13.98\%$ at day 6) (Fig. 1A and 1B). In anti-CD3/CD28 mAbs-stimulated PBMC, almost identical results were obtained; DP T cells exhibited higher levels of CCR5 expression than CD4SP T cells, from days 4 to 6 post-stimulation (Fig. 1C and 1D). DP T cells exhibited higher levels of CCR5 expression than CD4SP T cells, although 4 to 6 days were required to observe a statistical difference. In previous reports on human peripheral blood lymphocytes, CCR5 expression levels increased by *in vitro* activation [12, 16]. In these reports, CCR5 was expressed on memory T cells, not on naive T cells. Freshly isolated DP T cells from the peripheral blood of rhesus monkeys contain a large population of memory cells (CD95+) compared to CD4SP T cells [10]; therefore, it is possible that CCR5 is quickly induced on DP T cells because of the higher percentage of memory T cells in DP T cells than CD4SP T cells.

Next, we compared the vulnerability to SIV infection of *in vitro* activated DP T cells to that of CD4SP T cells. It was previously reported that CD4+CCR5+ T cells were selectively decreased in SIV-inoculated rhesus monkeys *in vivo* [14, 15]. Because *in vitro* activated DP T cells expressed CCR5 at higher levels than CD4SP T cells (Fig. 1), we

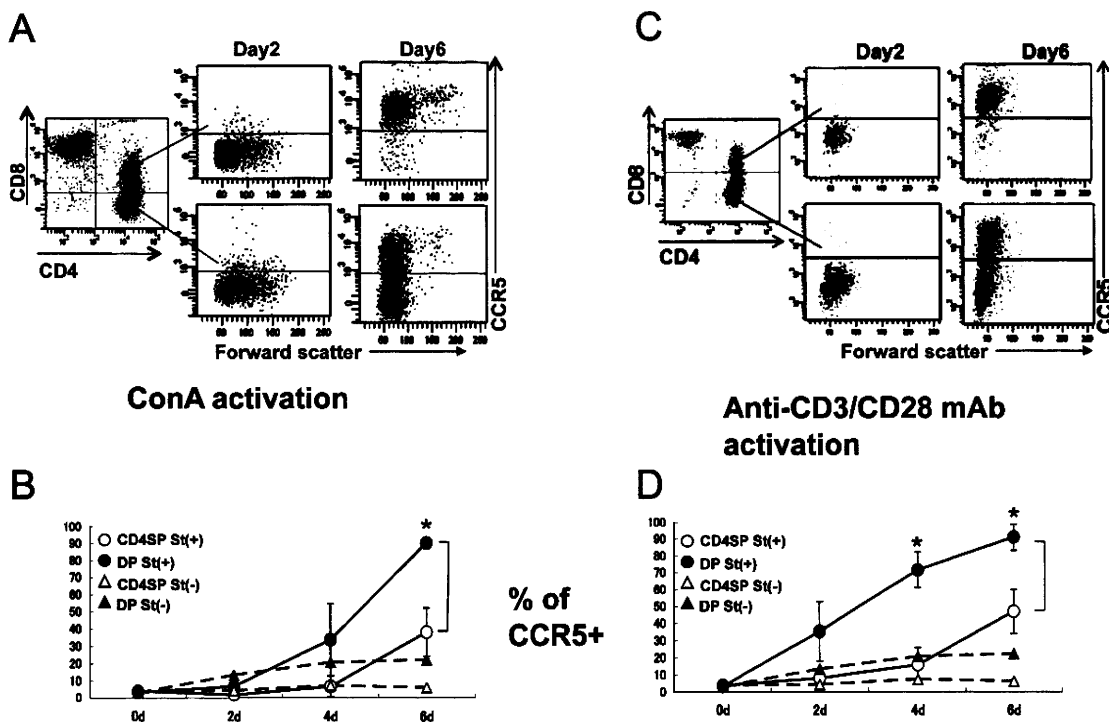


Fig. 1. CCR5 expression on CD4SP and DP T cells after ConA or anti-CD3/CD28mAb activation. (A) Flow cytometric analysis of CCR5 expression on CD4SP and DP T cells at days 2 and 6 after ConA activation, and (C) after anti-CD3/CD28 mAb activation. [(A) and (C) show representative data from one monkey, MM132.] (B) The kinetics of CCR5 expression levels on CD4SP and DP T cells post-ConA activation (circles). Vertical axis is the percentage of CCR5-positive cell population (CCR5 expression level). (D) Kinetics of CCR5 expression on both CD4SP and DP T cells after anti-CD3/CD28 mAb activation (circles). Triangles represent control cells (no mitogen stimulation). [(B) and (D) show the average and standard deviation (SD) of the data from three individual monkeys.] Statistical analyses were performed with a two-tailed unpaired Student's *t*-test using Microsoft Excel. * Statistically significant ($P < 0.05$).