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エイズ多剤併用療法中のリザーバーの特定および選択的障害に関する研究

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I. 厚生労働科学研究費補助金（エイズ・肝炎・新興再興感染症研究事業）
（総括・分担）研究報告書

エイズ多剤併用療法中のリザーバーの特定および選択的障害に関する研究

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研究要旨

サルエイズモデルを用いて多剤併用療法中もウイルスを保持し続けるリザーバーを同定する目的で、抗HIV-1剤５剤からなる多剤併用療法をSIV感染サルに対し一年間適用し安楽殺、全身の主要組織中のウイルスRNA量の定量、および組織化学的検索を行った。ウイルスRNAはエフェクターサイト及びリンパ系組織でのみ検出され、後者でより高値であった。リンパ系組織切片を多重組織化学染色した所、SIV Nef抗原陽性，またはウイルスRNA陽性細胞が低頻度で検出されたが、それらはCD3陽性ではなく、CD68（マクロファージマーカー）陽性であった。長期間にわたる多剤併用療法下においてマクロファージでウイルス複製が進行している事が示唆された。

A. 研究目的

抗 HIV-1 多剤併用療法の確立により、HIV-1 感染症は死に至る病ではなくなったが、この療法は未だ完全なものではなく、問題も認識されている。多剤併用療法の根本的な問題は感染者からウイルスを完全に排除する事が出来ない点であり、この為、感染者は薬剤を一生服用せねばならず、薬剤の副作用、変異ウイルスの出現、高額な医療費等の問題が派生する。本研究計画では多剤併用療法中にウイルスを保持し続けるリザーバーを特定、そのリザーバーを選択的に傷害する方法の開発を最終目的とする。リザーバーの全身レベルでの検索を行う為、サルエイズモデルである SIVmac/アカゲザル実験系を用い，検索をおこなった。このモデル系においてリザーバーの検索に応用できる様な強力な化学療法は 20 年度に既に確立した。22 年度は感染サルに確立した多剤併用療法を一年間にわたり適用し安楽殺し、各組織を RT-PCR 及び多重組織化学により検索した。

B. 研究方法

20 年度に確立した多剤併用療法は以下の通りである。逆転写阻害剤としてジドブジン(AZT)、ラミブジン（3TC）、テノフォビル（TDF）を選択した。プロテアーゼ阻害剤としてロピナビル(LPV)およびリトナビル(RTV)を選択した。投薬は選択した抗ウイルス剤（市販、成人感染者推奨服薬量の半量）を粉砕、同じく粉砕したサル飼料（PMI 社製 Certified animal diet 5048）30 グラムとバナナ 75 グラムを混合、成形し、1 日 2 回(TDF のみ 1 日 1 回)、10 時間および 14 時間間隔で自由摂食させた。ウイルスは病原性 SIVmac239 を 2000 $TCID_{50}$ 静脈内接種した。サルはインド産アカゲザル 6 頭を用い、2 頭は非治療対照とし、4 頭に多剤併用療法を感

染８週後から61-65週後まで適用した。治療群個体は化学療法適用下で安楽殺し、全身のリンパ系組織及び主要組織を PCR 検索及び組織学的検索用に採材した。PCR 検索にはRNA 安定化溶液に浸漬後凍結保存した各組織より総 RNA を抽出し、SIV gag 特異的プライマー/プローブを用いリアルタイム RT-PCR により遺伝子の増幅・定量を行った。非治療個体から上記に準じ組織を採取、対照とした。組織学的検索は、２種の多重組織化学染色を行った。ウイルス RNA 陽性細胞 (in situ hybridization) と細胞マーカーの検索及びウイルスタンパク陽性細胞 (免疫組織化学) と細胞マーカーの検索である。各組織を 10% 中性ホルマリン（免疫組織化学検索用）および 4%パラホルムアルデヒド（用）固定後、包埋・薄切した。組織切片はウイルス RNA プローブまたは SIV Nef 特異的単クローン抗体を反応させた後、細胞マーカーとして CD3 (T 細胞)および CD68（マクロファージ）に対する単クローン抗体を反応させ、異なる蛍光色素で可視化後、共焦点顕微鏡で検索した。また、ウイルス接種後、経時的に採取した血液から血漿を調整、ウイルス RNA 量を PCR により定量した。

（倫理面への配慮）

実験は「研究機関等における動物実験等の実施に関する基本指針」に基づく「京都大学における動物実験の実施に関する規定」を遵守し行った。

C. 研究結果

1. 多剤併用療法によるSIV感染アカゲザル血漿中ウイルスRNA量の抑制

６頭のアカゲザルに SIV239 を静脈内接種し、血漿中ウイルス RNA 量を経時的に検索した。全てのサルで接種１または２週後に 8×10^6–3×10^7 コピー/ml のピークに達し、その後、接種５週後に 10^5 コピー/ml 台まで低下したが再び上昇を始めた。接種８週後からこのうちの４頭に対して多剤併用療法を適用した。治療開始時の血漿中ウイルス RNA 量の高い個体ほど検出限界以下まで抑制するのに長い期間を要したが、全ての個体で接種 22 週後までには血漿注ウイルス RNA 量は検出限界（200 コピー/ml）未満まで抑制された。この後安楽殺までの１年間（53–57 週間）全てのサルでウイルス RNA は検出限界以下に抑制され、一過性の上昇は見られなかった。

2. 各組織におけるウイルス RNA 量

治療個体の非リンパ系主要臓器（心、肝、腎)におけるウイルス RNA 量は検出限界(2.9 $\times10^3$ コピー/μg 総 RNA）以下であった。非治療個体ではこれら臓器で 1.3×10^5–3.3×10^6 コピー/μg 総 RNA のウイルス RNA が検出された。

HIV-1 および SIV 感染急性期には、腸管等のエフェクターメモリーT 細胞が多く存在する、所謂「エフェクターサイト」でウイルスが爆発的に複製し、リンパ球を著減させる事が知られている。非治療個体の膣、肺および消化管（空腸、回腸、結腸および直腸）は、それぞれ 3.2×10^5、1.2×10^6、6×10^4–5×10^7 コピー/μg 総 RNA のウイルス RNA が検出された。治療群のこれら組織から 10^3–10^4 コピ

ー/μg総RNAのウイルスRNAが検出された。これら個体の血漿中ウイルス RNA 量が検出限界以下であった事から、血中ウイルス量が必ずしも体内ウイルス複製を正確に反映しない事が示唆された。

中枢神経系は現行の抗HIV-1剤が到達せず、ウイルスの聖域と考えられている。SIV 感染非治療個体では中枢神経系ウイルス RNA 量は大脳、小脳、脳幹においてそれぞれ 2.6、3.9、3.7×10^4コピー/μg 総RNAであったが、治療群個体では全頭検出限界以下であった。

HIV-1およびSIVはリンパ球指向性である事から、リンパ系組織は非治療個体においてはウイルス量が多い。そこでこの組織における多剤併用療法のウイルス抑制効果を解析した。非治療個体では 7×10^5-5×10^8 コピー/μg 総 RNA と非常に高いウイルス量が検出された。治療群個体では、これより低いものの、検索したほぼ全てのリンパ系組織でウイルスRNA が検出された（5×10^3–4×10^5コピー/μg 総RNA）。

3. リンパ系組織における感染細胞の検出

PCR検索から一年間にわたる治療によってもウイルス RNA 発現が抑制されない組織としてリンパ系組織が同定された。この組織に絞ってウイルス感染細胞の多重組織化学染色による検出を試みた。はじめにPCR検索と同様にウイルス RNA を検出した所、非常に低い頻度ながら陽性細胞が検出された。同時に行った CD3 及び CD68 染色により、ウイルス RNA 陽性細胞はほぼ全て CD68 陽性、即ちマクロファージであった。次に、ウイルス感染細胞において大量の発現が見られる Nef タンパクを指標に免疫組織化学を実施した。ウイルス RNA 陽性細胞よりも更に低い頻度でNef陽性細胞が検出された。これら細胞はRNA陽性細胞と同様、マクロファージマーカー陽性であった。

D.考察

長期間の治療が成功している感染者においてもresidual viremiaが報告されており、多剤併用療法下でのウイルス複製が示されている。サルエイズモデルを用いた、一年間にわたる多剤併用療法を適用した本研究結果から、そのウイルスは主にエフェクターサイトとリンパ系組織で産生されている事、さらにマクロファージがウイルス産生をしている事が示唆された。このモデル系において血中薬物濃度のトラフ値は感染者で推奨されている量の約10倍であり、投薬量が不足していた可能性は排除できる。耐性ウイルスの出現の可能性に関しては現在ウイルスの塩基配列を検索している。本研究ではプロウイルスよりも治療下でウイルス複製環が維持されている、即ちウイルスRNA発現のある細胞，更に転写のみならずウイルスタンパク発現細胞を検索した所、リンパ球よりもマクロファージが同定された。感染マクロファージの半減期は2週間ほどと見積もられている事から、今回検出したNef陽性マクロファージは治療下での新規感染を示すものと考えられる。昨年度報告した短期治療個体ではT細胞がウイルスタンパク発現細胞として同定されたが、長期治療群ではマクロファージがウイルス抗原陽性細胞として同定されたことから、マクロファージには治療下でもウイルス感染が拡大しうる可能性が

考えられる。多剤併用療法により、一般に新規感染阻止が不可能なのか、マクロファージへの新規感染が特に抑制困難か明らかにする必要がある

今回の検索に用いた SIV239/アカゲザルエイズモデルは現在、最も広く用いられているエイズ動物モデルであるが、

1.ウイルスが分子クローンである事、

2. T 細胞指向性である事から、実際の HIV-1 感染を再現していない可能性がある事も忘れてはならない。

E.結論

SIV239/アカゲザルエイズモデルにおいて多剤併用療法下でリンパ系組織及びそこに存在するマクロファージでは新規感染が維持されている事が示唆された。

F.健康危険情報

なし

G.研究発表

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H.知的財産権の出願・登録状況（予定を含む）

なし

II. 研究成果の刊行に関する一覧表

書籍　なし

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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III. 研究成果の刊行物・別刷

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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 envelope genes that are found worldwide. Therefore, our primary aim

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

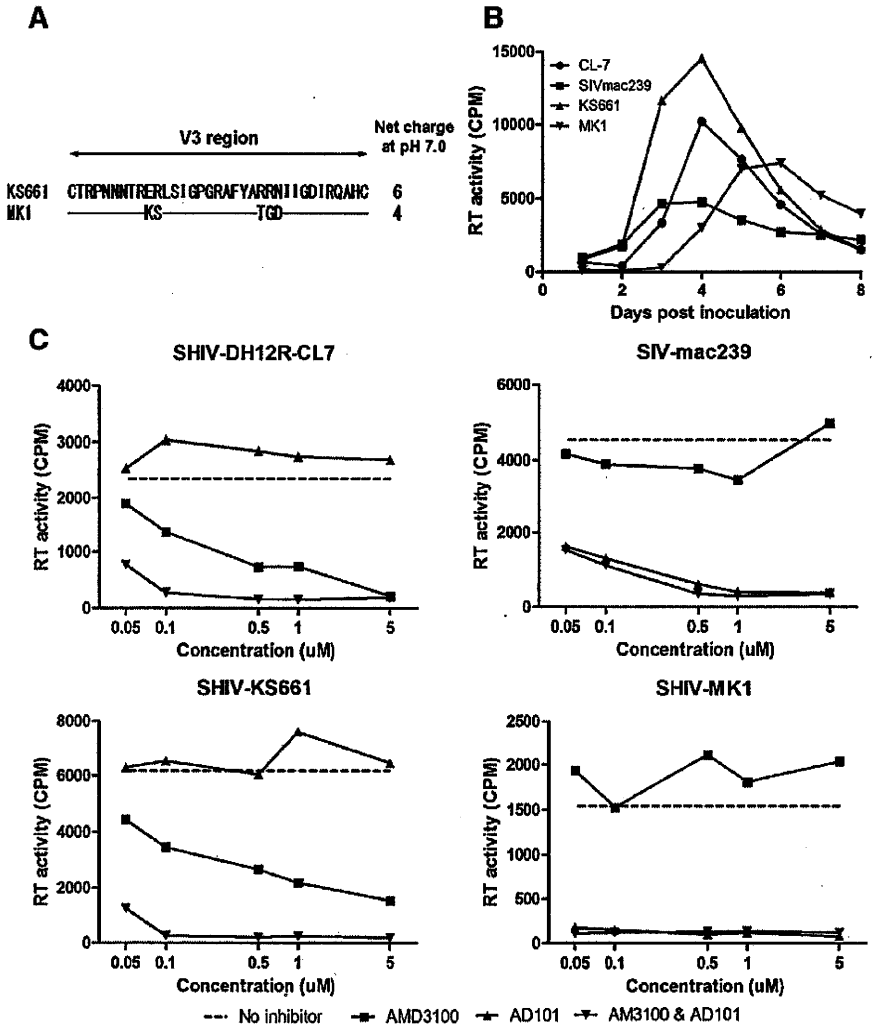

Fig. 1. Construction and *in vitro* analysis of SHIV-MK1. (A) gp120 V3 amino acid alignment of SHIV-MK1. Amino acid substitution positions are indicated under the parental SHIV-KS661 alignment. The net charge at pH 7.0 is indicated beside each amino acid alignment. (B) SHIV and SIV replication in rhPBMCs. 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 μM. 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-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 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

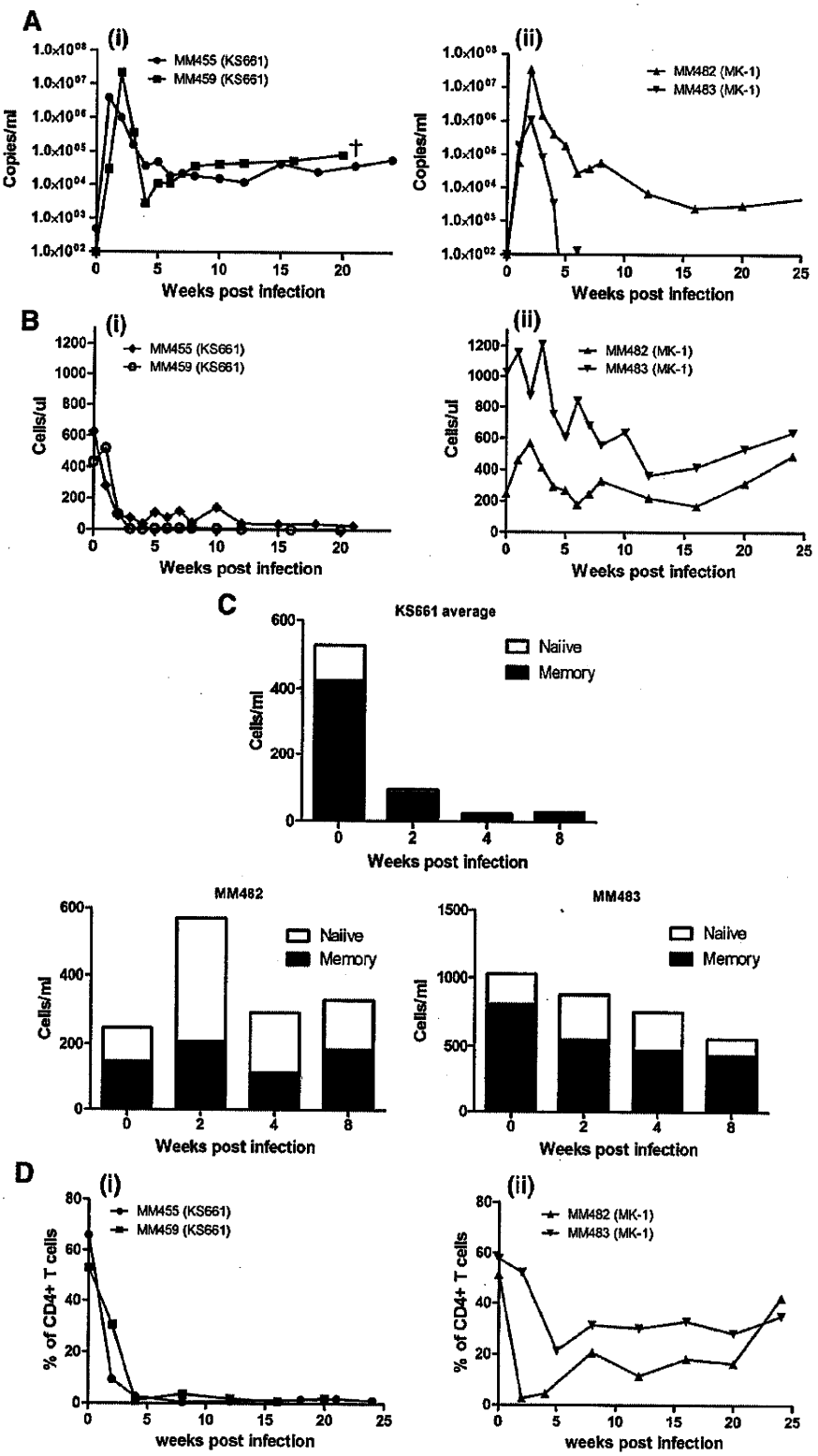

Fig. 2. *In vivo* replication of MK1. (A) Plasma viral RNA loads in SHIV-infected rhesus monkeys were measured at the indicated times. A total of 2000 TCID50 SHIV-KS661 was inoculated intravenously into MM455 and MM459 as a control group (i) and 20,000 TCID50 SHIV-MK1 was inoculated intravenously into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

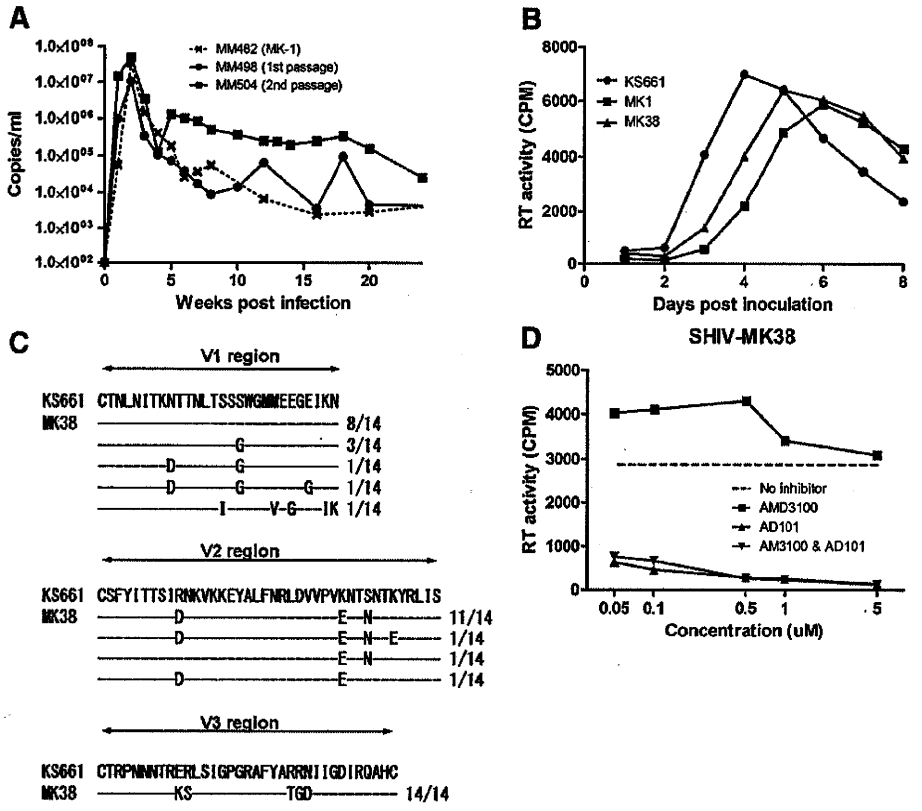

Fig. 3. *In vivo* adaptation of SHIV-MK1, and *in vitro* analysis of reisolated virus. (A) Plasma viral RNA loads of passaged monkeys were measured at the indicated times. The whole blood and dissociated lymph nodes from SHIV-MK1-infected MM482 were transfused into MM498 (first passage) 25 weeks post-inoculation.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

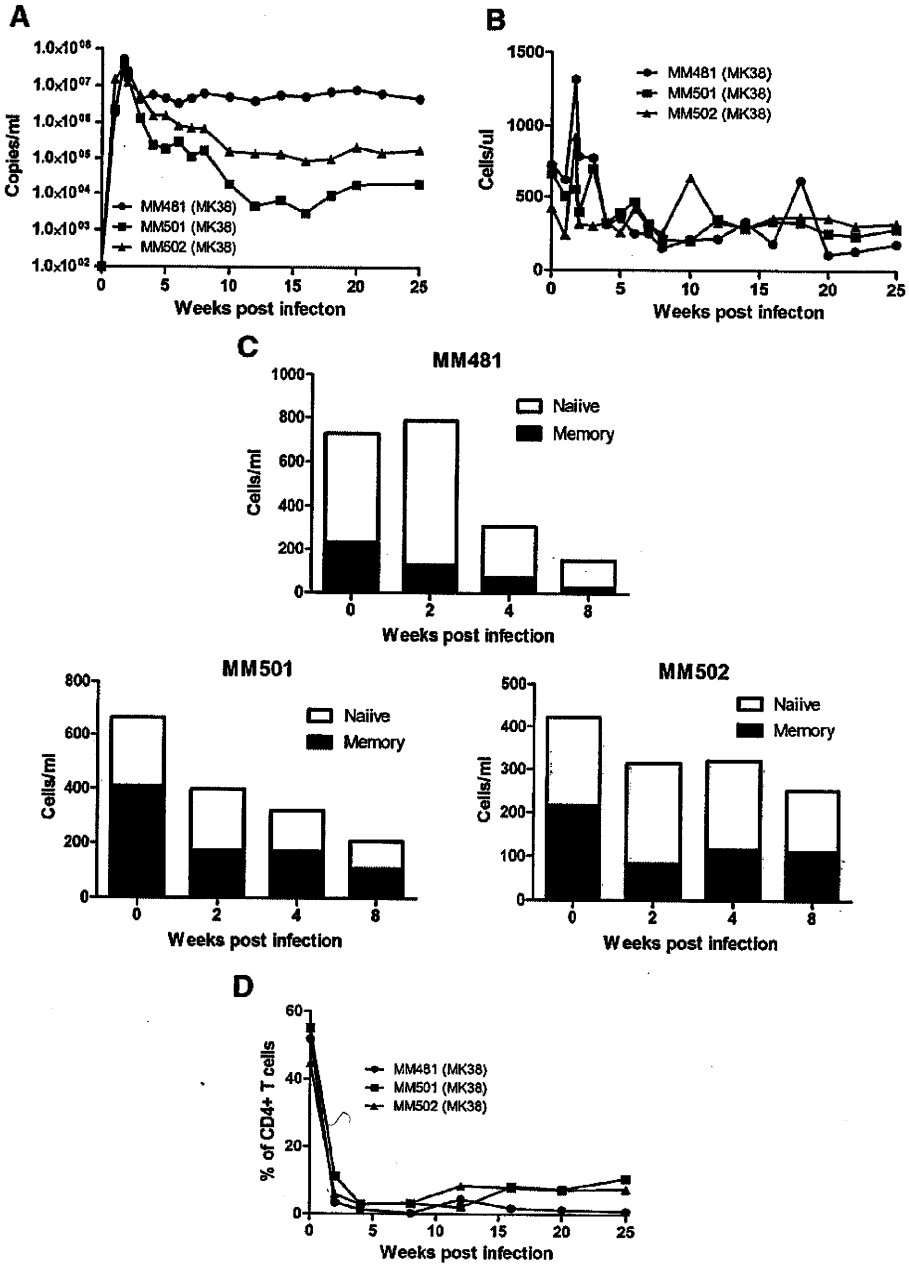

Fig. 4. *In vivo* replication of SHIV-MK38. (A) Plasma viral RNA loads in SHIV-infected rhesus monkeys were measured at the indicated times. A total of 20,000 TCID50 SHIV-MK38 were inoculated into MM481, MM501, and MM502. (B) CD4+ T lymphocytes were enumerated using FACS analysis in SHIV-MK38-infected 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-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 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 $1200\times g$ 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′ 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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