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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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 diarrhea (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
maque 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
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).

Naive 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

Table 1. Anti-HIV antibody titres in infected monkeys

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Intrarectal inoculation</th>
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<tr>
<td></td>
<td>MM243</td>
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<td>1</td>
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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...
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 (Kauffman 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 $2 \times 10^7$ 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 anticoagulant 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.

**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 naive and memory CD4$^+$ cells were analysed by a combination of FITC-conjugated anti-human CD45 (clone DX2; BD Pharmingen) and allophycocyanin-conjugated anti-human CD4 (clone L200; BD Pharmingen). CD95$^+$ CD4$^+$ CD4$^+$ cells were defined as naive 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; Novacasta Laboratories), anti-SIV Nef (diluted 1:500; FIT Biotech), anti-human CD68 (diluted 1:50; clone KP-1; Dako) and anti-human Ki67 (Ki-55; 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 (VassarWave).

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**REFERENCES**


(2003). Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS* 17, 1881–1888.


