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ORIGINAL ARTICLE

Viral outcome of simian–human immunodeficiency virus SHIV-89.6P adapted to cynomolgus monkeys

A. Borsetti · S. Baroncelli · M. T. Maggiorella · S. Bellino · S. Moretti · L. Sernicola · R. Belli · B. Ridolfi · S. Farcomeni · D. R. M. Negri · A. Cafaro · B. Ensoli · F. Titti

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Abstract Simian–human immunodeficiency virus (SHIV) 89.6P is considered to be one of the most pathogenic chimeric viruses in rhesus macaques. However, when crossing from one to another species of monkeys the pathogenicity of this virus may be affected. By using SHIV-89.6P$_{cy243}$, a virus obtained by passaging SHIV-89.6P in cynomolgus macaques, we investigated the dynamics of viral replication and the impact of the inoculum size (from 10 up to 50 monkey infectious dose) on the progression of the infection in 22 cynomolgus macaques. SHIV-89.6P$_{cy243}$ caused massive depletion of CD4+ T-cells within 4 weeks of the inoculum, followed by an irreversible immune deficiency in a high proportion of the infected monkeys. This study demonstrates that SHIV-89.6P$_{cy243}$ is pathogenic in cynomolgus macaques and that the dynamics of the viral replication and the rate of clinical progression depend on the size of the inoculum. Our findings provide unique and relevant data, particularly with regard to the value of the in vivo titration used to select the most appropriate infectious dose to study the "virus-host" interplay.

Nucleotide sequences data reported are available in the GenBank databases under the accession numbers U89134; AF217181; EF672090.

A. Borsetti · S. Baroncelli · M. T. Maggiorella · S. Bellino · S. Moretti · L. Sernicola · R. Belli · B. Ridolfi · S. Farcomeni · D. R. M. Negri · A. Cafaro · B. Ensoli · F. Titti (✉)
National AIDS Center, Istituto Superiore di Sanità,
V.le Regina Elena 299, 00161 Rome, Italy
e-mail: fausto.titti@iss.it

Present Address:
S. Baroncelli
Department of Drugs and Evaluation,
Istituto Superiore di Sanità, 00161 Rome, Italy

Present Address:
D. R. M. Negri
Department of Infectious, Parasitic and Immune-mediated Diseases-Istituto Superiore di Sanità, 00161 Rome, Italy

Introduction

Simian–human immunodeficiency virus (SHIV) infection of macaques provides an excellent model for studying human immunodeficiency virus-1 (HIV-1) pathogenesis and for HIV-1/acquired immunodeficiency syndrome (AIDS) vaccine development. Although many of the SHIVs are infectious in macaques, only a few have been found to be pathogenic. In fact, SHIVs do not readily induce disease in macaques and require adaptation via serial in vivo passages to generate variants able to replicate at high titre, deplete CD4+ T-cells and to induce AIDS [6, 12, 15, 27]. The induction of immunodeficiency by pathogenic SHIVs appears to require the elimination of virtually all CD4+ lymphocytes [8, 25]. In contrast, when the loss of CD4+ cells is only partial, the resulting infection is rarely associated with clinical symptoms, and animals usually remain disease-free for several years [11, 26].

SHIV-89.6P, a pathogenic CXCR4-tropic virus obtained after serial in vivo passages in rhesus macaques, has been extensively used as a model for the preclinical evaluation of HIV/AIDS vaccine candidates [1, 2]. However, the use of SHIV-89.6P as a challenge virus is currently controversial because in HIV-1-infected humans, a pattern of disease progression like that following SHIV-89.6P infection of macaques is extremely rare [9]. The pathogenicity of SHIV-89.6P is characterized by a profound and irreversible loss of CD4+ circulating T-cells, high levels of viremia and AIDS-like disease [13, 14, 25]. However, it

has been recently shown that infection with this virus in Chinese rhesus macaques and cynomolgus macaques results in lower levels of plasma viremia, CD4+ T depletion, and a more attenuated pathogenicity as compared to Indian rhesus macaques [26]. The different clinical outcome observed in these monkey species has been related to genetic factors or to differences in the host immune response to the virus [26].

In the present study, we report results obtained by infecting 22 cynomolgus macaques with different doses of SHIV-89.6P$_{cy243}$, a virus obtained by in vivo passage of the rhesus-derived SHIV-89.6P in cynomolgus macaques [3, 4, 18]. SHIV-89.6P$_{cy243}$ was highly pathogenic in this monkey species, as evidenced by the dose-dependent levels of plasma viremia, the rapid decline of CD4+ T-cells and by the survival rates of infected monkeys.

To ensure infection success in the experimental infection of macaques, the doses of the challenge virus are usually much higher than those estimated to be transmitted in the course of natural HIV infection, or, inappropriately, tissue culture infectious doses (TCID$_{50}$) are used, which do not reflect the situation in vivo [24]. Here, we provide clear evidence that the infectious dose, obtained by performing a rigorous in vivo virus titration, affects the clinical progression to AIDS in cynomolgus monkeys.

Materials and methods

Animals

Twenty-two adult cynomolgus macaques (Macaca fascicularis) imported from Mauritius and tested seronegative for STLV-1, SIV, simian Type D retrovirus and simian herpes B virus infections, were housed in single cages within level 3 biosafety facilities according to the European guidelines for nonhuman primate care (EEC, Directive No. 86-609, November 24, 1986). Before the start of the experimentation, the animal protocol received approval from the ethics committee of the Istituto Superiore di Sanità. Animals were clinically examined, and weight and rectal temperature were measured while they were under ketamine hydrochloride anaesthesia (10 mg/kg). Macaques used in this study were part of different experimental protocols as naïve or control groups. Control animals were treated with different adjuvants. Adjuvants included: aluminum phosphate (ALUM), 250 μL subcutaneous (s.c.); RIBI, 250 μL s.c. or microspheres (H1D), 60 μg intramuscular (i.m.) [5]. Animals were inoculated intravenously with different doses of the same SHIV-89.6P$_{cy243}$ virus stock [range:10–50 monkey infectious dose (MID$_{50}$)]. All animals used in these studies were males except for four females (monkeys 9406, 9610, 9503, 9104).

Origin of SHIV-89.6P$_{cy243}$

The virus was derived as previously described [3]. Briefly, SHIV-89.6P$_{cy243}$ was obtained by infecting a cynomolgus macaque (monkey 55111) with 50 MID$_{50}$ of the SHIV-89.6P obtained from Letvin's laboratory [3,25]. Blood, lymph nodes and spleen mononuclear cells were collected and CD8+-depleted cells were stimulated with PHA and interleukin-2 (IL-2) to induce massive virus replication. At the peak of RT activity (95,000 cpm/mL), determined as previously described [30], the cell-free supernatant was stocked and frozen. The new viral stock was termed SHIV-89.6P$_{cy243.}$ This viral stock was titred in vitro on human cell lines (CEMX174, C8166) resulting in titres of 5.6×10^3 and 1.6×10^4 TCID$_{50}$/mL, respectively, and on PBMCs from four naïve monkeys, its mean titre value was 3.23×10^3 TCID50/mL. To titre the virus in vivo, eight cynomolgus monkeys were exposed to serial dilutions of SHIV-89.6P$_{cy243}$ (dilution range-1:50 one monkey, 1:500 one monkey, 1:5,000 two monkeys, 1:50,000 two monkeys, 1:500,000 one monkey, 1:5,000,000 one monkey). The SHIV-89.6P$_{cy243}$ viral stock contained 4.9×10^5 MID$_{50}$/mL.

Plasma viral RNA measurements and proviral DNA detection

Plasma levels of SHIV-89.6P$_{cy243}$ were quantified using a highly sensitive quantitative competitive RNA-polymerase chain reaction assay (QT-RNA-PCR) [29]. Both methodologies were comparable in terms of sensitivity (50 RNA eq/mL). To determine the cell-associated viral load, DNA was extracted from 400 μL of whole citrated blood by using the QIAamp DNA Blood Mini Kit (QIAGEN, Milan, Italy) according to the manufacturer's instructions. SHIV proviral copy number was determined by a semi-quantitative DNA PCR utilizing 1 μg of DNA and amplifying a 496-bp region of the *gag* gene of SIVmac239, as already described [3].

Lymphocyte subset determination

Citrated peripheral blood cells were stained with FITC-conjugated anti-CD3, phicoerythrin PE-conjugated anti-CD4 and peridin chlorophyll protein (PerCP)-conjugated anti-CD8 mAb (Becton-Dickinson, Mountain View, CA, USA), and analyzed with a FACScan cytometer and software (Becton-Dickinson) as described previously [7]. Absolute cell numbers were calculated from the blood cell counts, performed by an automatic cell counter (Coulter Onyx, Beckman Coulter, Milan, Italy).

RT-PCR and sequencing analysis

Viral RNA was isolated from the SHIV-89.6P$_{cy243}$ viral stock by using the QIAamp viral RNA kit (QIAGEN, GmbH, Hilden, Germany). RT-PCR was performed using SuperScript III (Invitrogen, Carlsband, CA, USA) and Platinum Taq High Fidelity (Invitrogen) according to the manufacturer's instructions. Primers were designed based on the SHIV-89.6P KB9 sequence present in GenBank under accession number U89134. Primers (+)5′-CAGTC GCTCTGCGGAGAGGC-3′[nt1-20]), (-)5′-CATCTCCCA CTCTATCTTA-3′[nt520-538]), (+)5′-GCTGTCTTTTAT CCAGGAAGG-3′[nt494-514]), (-)5′-TGCTTGCCCAACT GCATGTAG-3′[nt1951-1971]), (+)5′-GCTGTGGATCTG CTAAAGAA-3′[nt1931-1970]), (-)5′-GTCTCACTCCATT GGTATGTG-3′[nt3410-3430]), (+)5′-GAGTCAGGACAA TCAGTGGTC-3′[nt3327-3347]), (-)5′-GTCCACCATGCC CATCCGAC-3′[nt4961-4980]), (+)5′-AGAGGCTAGAGA GGTGGCATAG-3′[nt4872-4893]), (-)5′-GGCTGAAAGG ATACCTTTGGAC-3′[nt6961-6982]), (+)5′-GTAACACC TCAGTCATTACACAG-3′[nt6934-6956]), (-)5′-AGGAA CACAGCTCCTATTCC-3′[nt7872-7891]), (+)5′-CCATC ACAGGACAAATTAGATG-3′[nt7642-7663]), (-)5′-AAGT GCCAAGGATCCGTTCA-3′[nt8578-8597]), and (+)5′-CC TGGATGGAGTGGGAAAGA-3′[nt8212-8231]), (-)5′-GT CTAAGATTCTATGTCTTC-3′[nt9191-9210]), (+)5′-TGG AAGGGATTTATTACAGTG-3′[nt9167-9187]), (-)5′-TAC TTCTAAAATGGCAGCTTT-3′[nt9840-9860]) were used for reverse transcription of the entire viral genome. These PCR products were directly sequenced.

Statistical analysis

Plasma virus levels were expressed as $\log_{10}$ copies/mL before all analysis. Plasma virus and CD4 T-cell count data were reduced to eight measurements for each macaque. To compare the different dose groups, three measurements of viral load and CD4+ T-cells were determined: acute period (median of 2–4 weeks p.i.), post-acute period (median of weeks 8–21) and chronic phase of infection (median of weeks 25–35). The Kruskall–Wallis test was used to assess overall differences among the four dose groups, and the Wilcoxon rank sum test was performed to compare each group to the others.

The smoothed curves of viral load and CD4 T-cells were estimated using a smooth local regression (LOESS), with a quadratic fit and a span of 0.5. Log-rank test was used to compare the survivor functions estimated by the Kaplan–Meier method. Statistical tests were performed at a two-sided 5% significance level. All statistical analysis and data processing were performed using SAS® software-version 8.1 for Windows (SAS Institute, Cary, NC, USA).

Results

Origin of SHIV-89.6P$_{cy243}$

The original SHIV-89.6P viral stock was inoculated intravenously at a dose of 50 MID_{50} in 3 cynomolgus macaques. The course of infection was quite different in the three animals (Fig. 1). In fact, in monkey 9, plasma virus reached 1.6×10^7 copies/mL 2 weeks post-infection (p.i.) and was maintained at 10^5 copies/mL thereafter. Peripheral CD4+ T-cells slightly decreased at 2 weeks p.i. and dropped at 4 weeks, remaining below 1% until week 16. At this time, when plasma viral RNA was 6.8×10^5 copies/mL, this monkey was euthanized because of AIDS-related syndrome. In contrast, monkey 27 displayed high viremia at 2 weeks p.i. (4.4×10^6 copies/mL), which declined sharply at 4 weeks p.i. (2.1×10^3 copies/mL), remaining below the detection limit (<50 copies/mL) from week 8 to week 57 p.i., and subsequently rebounded to 10^2–10^3 copies/mL by the end of the follow-up (week 77). This monkey experienced a profound CD4+ T-cell depletion starting 2 weeks p.i., reaching a nadir of 6% at week 4 p.i. After the acute phase of infection, this animal showed a partial CD4+ T-cell recovery which, however, remained below 10% up to week 77. The third animal (monkey 55111) exhibited high primary viremia at 2 weeks p.i. (1.5×10^6 copies/mL), which was coincident with a decline in CD4+ T-cells, that, however, was less pronounced (33% at 2 week p.i.) than in monkeys 9 and 27. According to our experience, it is difficult to produce a large-scale stock of virus from a monkey that exhibits a significant depletion of CD4+ T cells, since CD4+ T cells are the primary target in which virus actively replicates. Thus, as a source of virus, we selected monkey 55111 because it presented a less pronounced decline of CD4+ T-cells compared to the other two monkeys. Monkey 55111 was sacrificed at 3 weeks after infection, and the new stock of virus, termed SHIV-89.6P$_{cy243}$, was titrated in cynomolgus macaques.

Plasma viral load and CD4+ T-cell levels in cynomolgus macaques inoculated with SHIV-89.6P$_{cy243}$

Naive or control macaques that were part of different experimental protocols were used in this study. The identification number, the viral dose and the treatment for each of the animal are listed in Table 1. Viral RNA levels were analyzed and compared between the different groups at three different time points. These included: peak of plasma viremia (2–4 weeks p.i.) as the acute phase of infection, post-acute period (median of weeks 8–21), and the chronic

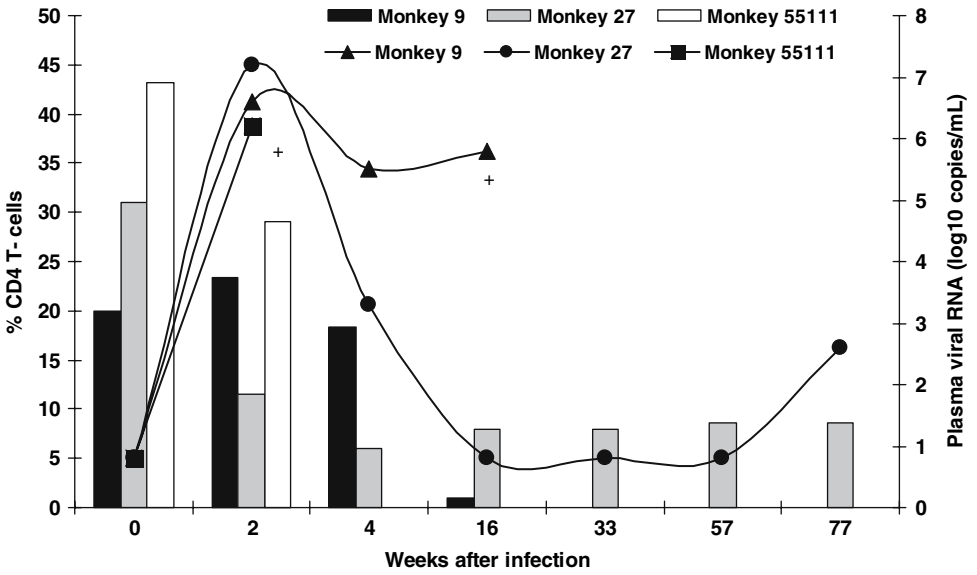

Fig. 1 Profiles of CD4+ T-cells (*bars*) and plasma viral loads (*lines*) of cynomolgus macaques infected with 50 MID_{50} of SHIV-89.6P. Monkey 55111 was sacrificed at 3 weeks p.i. to obtain a new stock of virus (SHIV-89.6P_{cy243})

phase of infection (median of weeks 25–35). In all animals, high levels of viral RNA were rapidly detected and peaked at 2 weeks p.i. (Fig. 2). The peak of viral RNA was significantly higher in the 50-MID_{50} group than in the 15-MID_{50} group ($P < 0.05$), although for the 10-MID_{50} group the P value was near the significance level ($P = 0.0526$). In the post-acute phase (weeks 8–21), a 50% reduction in the plasma RNA levels was observed in the 10-MID_{50} groups, while the other dose-groups maintained a higher level of viral replication (>10^4 RNA copies/mL) (Fig. 2). During this time, no significant differences in viral RNA levels among the groups were observed. In the chronic phase of infection (weeks 25–35), the median levels of plasma viremia ranged from 1×10^2 in the lowest dose group to 1.1×10^5 RNA copies/mL in the highest dose group. During this observation period, the levels of plasma viremia fell and remained below detection (<50 RNA copies/mL for at least two measurements) in 2/3 (66%) and in 3/6 (50%) of the monkeys inoculated with 10 and 15 MID_{50}, respectively. In contrast, only one out of three and one out of four animals in the 20-MID_{50} and 50-MID_{50} dose groups, respectively, displayed undetectable plasma viremia levels in the same time period. Although a trend emerged suggesting an association between the amounts of virus administered and viral load, this correlation was not statistically significant. Nevertheless, the comparison between the 15 and the 50 MID_{50} dose group was near the significance level ($P = 0.0599$).

According to the smooth regression line for the plasma virus level (Fig. 3a), it was clear that although viral RNA was detectable in a few animals in the 15-MID_{50} and 50-MID_{50} dose groups one week p.i. (average 1.7×10^2 RNA copies/mL at 15 MID_{50} and 2.0×10^4 RNA copies/mL at 50 MID_{50}), the data consistently indicated that the peak of plasma viremia was at week 2 p.i. Moreover, the viral long-term set point level was reduced in the lower dose groups compared to the higher ones. Monkeys in the 20-MID_{50} and 50-MID_{50} groups displayed a higher long-term set point viremia as compared to monkeys in the 10-MID_{50} and 15-MID_{50} groups, suggesting that viral

Table 1 Monkeys used in the study

Animal ID	Treatment	Inoculum size
61754	None	10 MID_{50}
61784	None	
55123	ALUM	
55129	RIBI	
AC032	H1D	15 MID_{50}
AC739	H1D	
AC921	H1D	
AC734	None	
AC756	None	
BD585F	None	
AC225	ALUM	
AC277	ALUM	
AC637	ALUM	
AF838	None	
AF710	None	
61768	None	20 MID_{50}
61776	None	
61763	None	
9406	None	50 MID_{50}
9610	None	
9503	None	
9104	None	

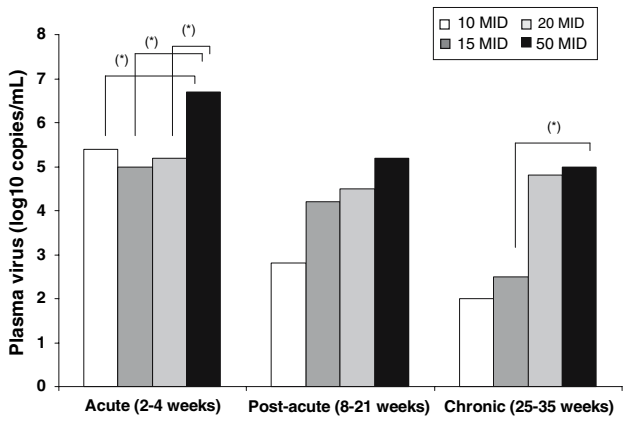

Fig. 2 Dynamics of viral infection relative to different inoculum size. Viral RNA levels during the acute, post-acute and chronic phase post-infection of cynomolgus monkeys inoculated with 10, 15, 20 and 50 MID_{50} of the SHIV-89.6P_{cy243}, respectively. The *bars* represent the median values of RNA copies/mL in the different phases of infection. *Asterisk* indicates a significant difference between 10–50 MID_{50} dose group ($P = 0.05$), 15–50 MID_{50} dose group ($P = 0.0055$) or 15–50 MID_{50} dose group ($P = 0.0259$)

dynamic was dependent on the inoculum size. The CD4+ T-cell levels in the different dose groups of monkeys were also compared during the acute, post-acute and long-term set point period. The trend line of the smooth local regression (loess) of the absolute CD4+ T-cell number for each group of monkeys is shown in Fig. 3b. All animals suffered a progressive and profound CD4+ T-cell decline within 4 weeks p.i. Monkeys that in the acute phase of infection developed a rapid and almost complete loss of CD4+ T-cells (i.e., below 50 cells/mm^3) showed an irreversible CD4+ T-cell depletion during the follow-up period. In contrast, a partial recovery of CD4+ T-cells was observed in the remaining monkeys from all groups starting at week 4 p.i., although they never returned to pre-infection values (Fig. 3b). However, the trend line of CD4+ T counts in the 10- and 15-MID_{50} groups during all three post-inoculation time periods analyzed was higher than in the 20- and 50-MID_{50} groups. Thus, as for plasma viremia, these results indicate that the CD4 T lymphopenia is related to the amounts of the administered virus.

Clinical outcome and survival analysis in cynomolgus macaques inoculated with SHIV-89.6P_{cy243}

Monkeys were monitored for evidence of immunodeficiency and AIDS-related symptoms. Twelve animals with high levels of viral replication and profound CD4+ T-cell depletion in peripheral blood were sacrificed at the first onset of AIDS as required by regulatory authorities for animal welfare. One animal belonging to the 50-MID_{50} group was lost for causes not related to the infection. Cumulative survival rates of the four dose groups through 80 weeks after inoculation with SHIV-89.6P_{cy243} are shown in the Kaplan–Meier curve (Fig. 4). Although statistical analysis using a log-rank test did not reveal significant differences among the groups ($P = 0.4843$), a clear correlation between the inoculum dose and the rate of disease progression was observed. Conversely, nine monkeys remained asymptomatic for 80 weeks p.i., during which they were monitored for clinical and virological parameters. After a marked loss of CD4+ T-cells in all monkeys at 4 weeks p.i., the three monkeys in the 10-MID_{50} group completely recovered CD4+ T-cells, whereas one out of the five animals in the 15-MID_{50} group and one in the 20-MID_{50} group nearly returned to preinoculation levels at 80 weeks p.i. (Table 2). All surviving animals became aviremic 8–76 weeks p.i., during which proviral DNA was detected in peripheral blood at different time points (Table 2). Interestingly, five out of eight monkeys that received adjuvants survived up to week 80, suggesting a potential beneficial effect of the adjuvants on the dynamics of viral replication. However, their virological pattern, such as the absence of plasmaviremia and the presence of provirus, did not differ from that of monkeys that were not treated before infection. In addition, monkeys AC225 and AC637 showed a significant reduction of CD4+ T cells at 80 weeks p.i. as compared to the value of CD4+ T cells of the other monkeys. Altogether, our data indicate that the adjuvants do not have a significant effect on the pathogenicity of the cynos-grown SHIV89.6P nor on the clinical progression of the infection.

Genomic sequence of SHIV-89.6P_{cy243}

Since SHIV-89.6P_{cy243} consistently induced high levels of viral load and rapid disease progression, investigations were done to verify whether the virus had acquired mutations during the in vivo passage that might have changed its replication efficiency and virulence. In order to determine the molecular changes upon transmission to the new host, the entire genome of the SHIV-89.6P_{cy243} was sequenced (GenBank accession number EF672090) and compared to the published SHIV-89.6P sequence (GenBank accession number U89134). The viral gene sequences were determined directly from PCR products, and they therefore represent the predominant genotype of the viral population. This analysis revealed that SHIV-89.6P_{cy243} and SHIV-89.6P differed by eight amino acids. One mutation in the gag region (457 M/I) and three mutations in pol region (65 A/T, 412 S/L and 770 K/R) of the SHIV-89.6P_{cy243} were detected. Three amino acids changes were observed in the gp41 cytoplasmic domain of the envelope protein (756 D/A, 779 I/T and 842 L/V). One change (109 R/K) was found

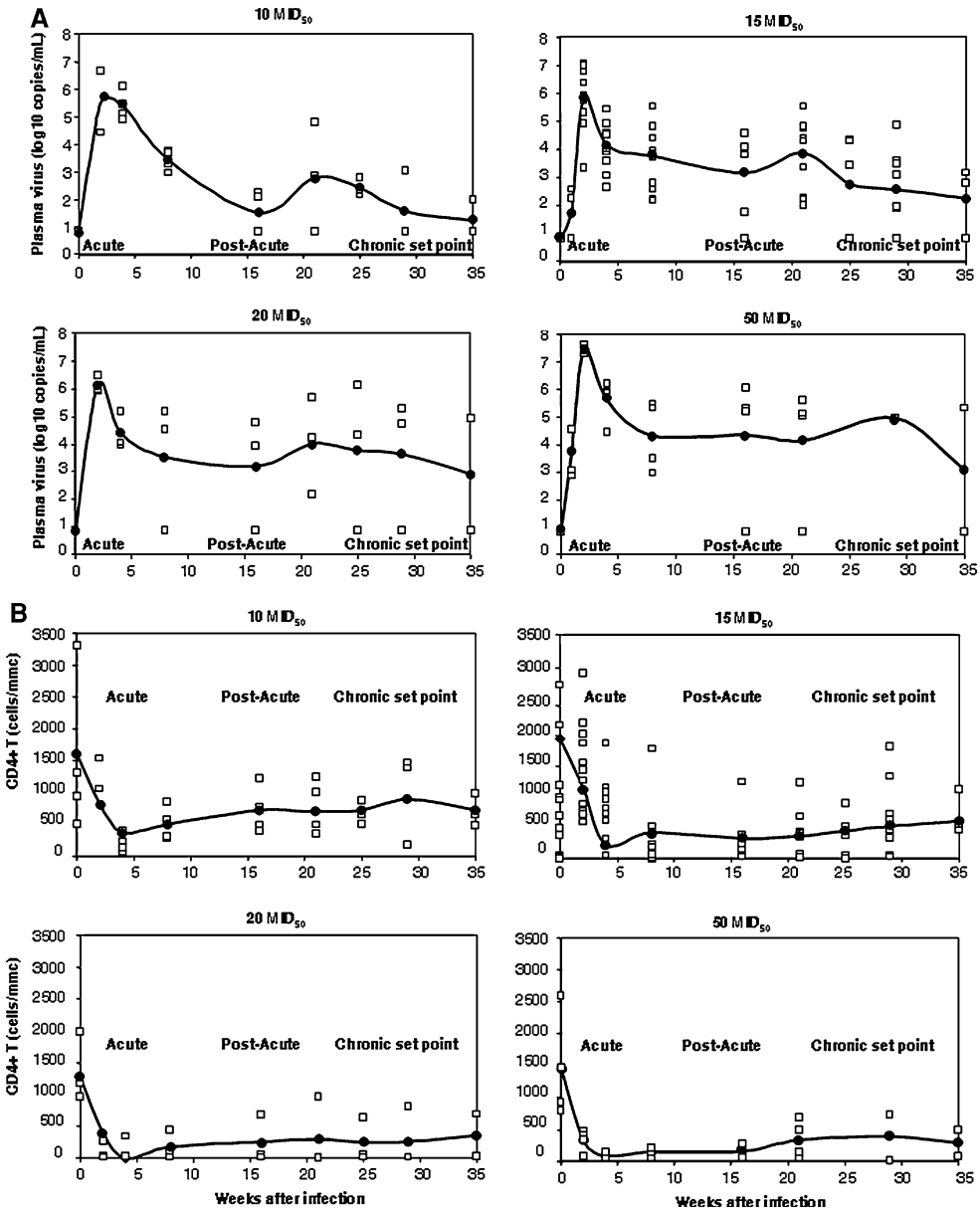

Fig. 3 Changes in plasma virus levels and CD4+ T-cell number after infection with SHIV-89.6P$_{cy243}$. The trend line for each panel is a LOESS smoothed average analysis **a,** of plasmaviremia and **b,** of absolute CD4+ T-cell counts, calculated for the acute, post-acute phase and for the chronic phase set points. On the top of each panel is reported the dose of SHIV89.6P $_{cy243}$ used to infect the cynomolgus monkeys. From week 13 up to week 35, nine animals that developed simian AIDS were euthanized

in the nef protein. A single nucleotide difference at position 312 in the primer binding site region of the 5′ long terminal repeat (LTR) was also identified (Table 3). Of note, these amino acid changes were found in the SHIV-C2/1 virus (GenBank accession number AF217181), a SHIV-89.6 variant isolated by in vivo passage in cynomolgus macaques [27]. In fact, SHIV-89.6P$_{cy243}$ showed 100% identity to the sequence of the highly pathogenic SHIV-C2/1 [4, 24, 25, 26].

Discussion

SHIV-89.6P is noted for its ability to cause an unusually and typically irreversible CD4 T-cell depletion accompanied by rapid progression to simian AIDS and death, and it has been used as a challenge virus for the evaluation of vaccine efficacy in pre-clinical models [13, 14, 25]. However, the infection of monkeys with SHIV-89.6P does not mimic the natural course of HIV infection observed in humans, and therefore it cannot represent the ideal challenge model [9]. In this study, the pathogenicity of SHIV-89.6P$_{cy243,}$ a virus obtained by passaging SHIV-89.6P in cynomolgus macaques, was determined by evaluating levels of plasma viremia, CD4+ T-cell number and disease progression [16, 20, 23]. Accordingly, SHIV-89.6P$_{cy243}$ was highly pathogenic in cynomolgus macaques, indicating that this virus is suitable for assessing vaccine efficacy and that the SHIV-cynomolgus system is a relevant nonhuman primate model for studying lentivirus pathogenesis. In fact,

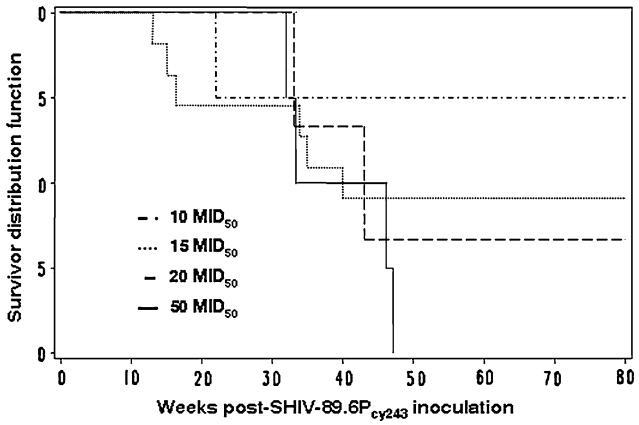

Fig. 4 A Kaplan–Meier plot of survival rates of monkeys up to 80 weeks post-challenge with the pathogenic SHIV-89.6P_{cy243} is shown. One animal inoculated with 50 MID_{50} was lost for reasons not related to the infection. Statistical analysis showed that the result is not significant ($P = 0.4843$), but a correlation between the inoculum size and the rate of disease progression was observed

Table 2 Virological markers in monkeys that survived simian–human virus (SHIV)-89.6P_{cy243} infection up to 80 weeks

Inoculum size	Animal ID	Week of first undetectable plasma viremia[a]	CD4+ T-cells[b]	Proviral DNA[c]
10 MID_{50}	61754	29	3332/1578	14 (1–140)
	55129	46	505/556	<1 (<1–6)
	55123	23	947/1180	28 (3–80)
15 MID_{50}	AC921	21	1438/770	48 (33–63)
	BD585F	13	1944/1450	14 (9–36)
	AC225	28	2006/636	5 (2–10)
	AC637	76	2829/311	46
	AF838	46	1200/787	2 (2–3)
20 MID_{50}	61776	8	1169/827	60 (2–1000)

Monkeys were grouped according to the virus dose they received. Animals were constantly monitored for virological status until week 80

[a] Absence of plasma viremia from the week shown in the table

[b] CD4+ T-cell number $(mm)^3$ at the day of challenge and at 80 weeks p.i.

[c] Median and range of proviral copies/μg DNA determined in peripheral blood mononuclear cells (PBMC) of infected animals during the aviremic period

SHIV-89.6P_{cy243} caused a massive depletion of circulating CD4+ T-cells early after infection and replicated at high rates, as shown by the high levels of plasma viremia observed in all infected animals. When CD4+ T-cell counts dropped below 50 cells/μL in the first few weeks after infection, monkeys did not recover and progressed to AIDS. This rapid and dramatic depletion of CD4+ T-cells has been extensively described not only for SHIV-89.6P but also for other SHIVs [6, 11, 26]. In our studies, 8 out of 22 animals belonging to the different dose groups, experienced almost a total CD4+ T-cell depletion in the acute phase of infection that persisted up to the time of death, which occurred by week 35 post-infection. Of note, these animals remained viremic until death. Conversely, when a partial recovery of CD4+ T-cells occurred, the majority of animals contained the viral load and remained asymptomatic for more than 1 year and a half. However, in most of these animals the recovery of circulating CD4+ T-cells was partial since their level remained below 50% of the values observed before infection. Moreover, in 10 out of the 22 infected animals, the plasma viremia remained detectable over 35 weeks of follow-up. Lastly, even if the number of animals that died of AIDS did not statistically correlate with the inoculum viral dose, a trend toward a positive correlation between the infection and the rate of disease progression was observed in the different dose groups. Thus, according to our data, the cynomolgus-derived SHIV-89.6P_{cy243} is highly pathogenic in cynomolgus macaques.

However, Reimann et al. [26] have reported attenuated pathogenicity of SHIV-89.6P in cynomolgus macaques. In particular, upon infection with 100 MID_{50} of SHIV-89.6P grown in rhesus monkey, they found lower levels of viremia, better preservation of CD4+ T-cell counts and longer survival of cynomolgus macaques than of rhesus macaques. According to these authors, many factors, such as the origin of the virus and of the animals, could have contributed to determining the diminished susceptibility of cynomolgus macaques to infection. In fact, propagation of virus in an heterologous species may have favoured the selection of viral variants adapted to a more efficient replication and/or enhanced pathogenicity in that species but not necessarily in others. On the other hand, they ascribed the different pathogenicity in the two monkey species to a stronger antiviral immune response occurring in cynomolgus than in rhesus macaques. Moreover, they suggested that changes of viral tropism, such as a lower replication rate in cynomolgus target T-cells, could explain the longer survival observed in this monkey species. In contrast, our results are consistent with studies performed with SHIV-C2/1, a variant of SHIV-89.6 isolated by in vivo passage in cynomolgus macaques, which is able to infect cynomolgus macaques [7, 21, 32]. Similar to SHIV-89.6P_{cy243}, SHIV-C2/1 was shown to infect cynomolgus macaques, inducing high levels of viremia and marked CD4+ T-cell depletion within 2 weeks of the inoculation [7, 21, 32].

With regard to the association of genetic changes with pathogenicity, a limited number of amino acid substitutions in the genome of SHIV-89.6P were found after passage in a cynomolgus macaque. In fact, the SHIV-89.6P_{cy243} sequence differed from that of SHIV-89.6P KB9 by eight amino acids substitutions, whereas it was identical to the

Table 3 Amino acid changes in the simian–human immunodeficiency virus (SHIV)-89.6P$_{cy243}$ genome

	Gag (aa 457)	Pol (aa 65, 412,770)	Env (aa 756, 779, 842)	Nef (aa 109)
SHIV-89.6P KB9	M	A S K	D I L	R
SHIVC2/1	I	T L R	A T V	K
SHIV-89.6P$_{cy243}$	I	T L R	A T V	K

Amino acid sequence alignments of SHIV-89.6P KB9, SHIVC2/1, SHIV-89.6P$_{cy243}$ genomes showed differences in the gag, pol, env, and nef genes, which are indicated in the table (GenBank accession number U89134; AF217181)

sequence of SHIV-C2/1. Three out of eight specific mutations were detected at the junction between the HIV-1 and SIVmac gp41 cytoplasmic tail, and one of these was associated with a charge change. Since it has been shown that the cytoplasmic domain of Env may play an important role in virus infection by modulating its immunogenicity, it is possible that serial in vivo passage of the SHIV-89.6P in a different monkey species may have induced genetic changes responsible for the acquisition of the high virulence of SHIV-89.6P$_{cy243}$. A particular aspect that deserves discussion is the dose of the challenge virus used to infect macaques in vaccine protocols. In fact, experimental infections in preclinical studies are usually performed with very high doses of challenge virus to ensure that all monkeys become infected. These high infectious doses exceed the amounts of virus that humans are naturally exposed to by many fold [24]. Moreover, many preclinical trials in macaques have been conducted using an estimated infectious dose exclusively based on in vitro titration (TCID$_{50}$), which usually does not correspond to the infectious dose determined by in vivo titration. Similarly, the infectious dose obtained from an in vivo virus titration by intravenous exposure could be not directly translated to that necessary to infect monkey via the mucosal route since the mechanisms of infection are different. The mucosa, and in particular, the gastrointestinal tract, represent the major site of HIV replication and amplification, the reservoir, and the initial site of CD4+ T-cell depletion [31]. The primary events of cell-virus interactions at the epithelial barrier are multifaceted, involving a complex virus-host interplay that is not present at the peripheral level [10, 19, 31]. Thus, an infectious dose able to infect all monkeys when given intravenously is likely to not infect all monkeys when given via the mucosal route. Yet, among the mucosal surfaces (oral, rectal, and vaginal) the architecture of the tissue is different and therefore even the infectious doses might be different. In a vaccine efficacy trial, the exposure of macaques to a low dose will impede the understanding of the protective efficacy due the increased risk of having some control monkeys remain aviremic. On the other hand, the exposure of macaques to limited doses of SIV or SHIV generates mucosal immune responses that are able to contain virus replication to some extent though the induction of different protective humoral and cell-mediated responses [17, 22]. Furthermore, as we concluded in a previous work on SIV- and SHIV-infected macaques, once infection has occurred, there is no influence of either intravenous, rectal or vaginal administration on plasma viremia [28].

We think that a good compromise between the too-stringent high infectious dose and the more realistic low doses would be an accurate estimate of the MID$_{50}$ based on data generated from in vivo titration.

In conclusion, SHIV-89.6P$_{cy243}$ is pathogenic in cynomolgus macaques, and the clinical outcome seems to be related to the size of the viral inoculum and to individual host determinants. Ongoing studies on the viral genotype will define more clearly the contribution of molecular changes on disease development and progression. Our data provide clear and systematic information on the importance of the infectious dose on disease progression in monkeys. This finding is not strictly related to the usage of SHIV-89.6P but could be easily extended also to SIV and other SHIV infections.

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References

1. Amara RR, Villinger F, Altman JD, Lydy SL, O'Neil SP, Staprans SI, Montefiori DC, Xu Y, Herndon JG, Wyatt LS, Candido MA, Kozyr NL, Earl PL, Smith JM, Ma HL, Grimm BD, Hulsey ML, Miller J, McClure HM, McNicholl JM, Moss B, Robinson HL (2001) Control of a mucosal challenge and prevention of AIDS by a multiprotein DNA/MVA vaccine. Science 292:69–74
2. Barouch DH, Santra S, Schmitz JE, Kuroda MJ, Fu TM, Wagner W, Bilska M, Craiu A, Zheng XX, Krivulka GR, Beaudry K, Lifton MA, Nickerson CE, Trigona WL, Punt K, Freed DC, Guan L, Dubey S, Casimiro D, Simon A, Davies ME, Chastain M, Strom TB, Gelman RS, Montefiori DC, Lewis MG, Emini EA,

Shiver JW, Letvin NL (2000) Control of viremia and prevention of clinical AIDS in rhesus monkeys by cytokine-augmented DNA vaccination. Science 290:486–492
3. Cafaro A, Caputo A, Maggiorella MT, Baroncelli S, Fracasso C, Pace M, Borsetti A, Sernicola L, Negri DR, ten HP, Betti M, Michelini Z, Macchia I, Fanales-Belasio E, Belli R, Corrias F, Buttò S, Verani P, Titti F, Ensoli B (2000) SHIV89.6P pathogenicity in cynomolgus monkeys and control of viral replication and disease onset by human immunodeficiency virus type 1 Tat vaccine. J Med Primatol 29:193–208
4. Cafaro A, Titti F, Fracasso C, Maggiorella MT, Baroncelli S, Caputo A, Goletti D, Borsetti A, Pace M, Fanales-Belasio E, Ridolfi B, Negri DR, Sernicola L, Belli R, Corrias F, Macchia I, Leone P, Michelini Z, ten Haaft P, Buttò S, Verani P, Ensoli B (2001) Vaccination with DNA containing tat coding sequences and unmethylated CpG motifs protects cynomolgus monkeys upon infection with simian/human immunodeficiency virus (SHIV89.6P). Vaccine 19:2862–2877
5. Caputo A, Brocca-Cofano E, Castaldello A, De MR, Altavilla G, Marchisio M, Gavioli R, Rolen U, Chiarantini L, Cerasi A, Dominici S, Magnani M, Cafaro A, Sparnacci K, Laus M, Tondelli L, Ensoli B (2004) Novel biocompatible anionic polymeric microspheres for the delivery of the HIV-1 Tat protein for vaccine application. Vaccine 22:2910–2924
6. Dunn CS, Beyer C, Kieny MP, Gloeckler L, Schmitt D, Gut JP, Kirn A, Aubertin AM (1996) High viral load and CD4 lymphopenia in rhesus and cynomolgus macaques infected by a chimeric primate lentivirus constructed using the env, rev, tat, and vpu genes from HIV-1 Lai. Virology 223:351–361
7. Eda Y, Murakami T, Ami Y, Nakasone T, Takizawa M, Someya K, Kaizu M, Izumi Y, Yoshino N, Matsushita S, Higuchi H, Matsui H, Shinohara K, Takeuchi H, Koyanagi Y, Yamamoto N, Honda M (2006) Anti-V3 humanized antibody KD-247 effectively suppresses ex vivo generation of human immunodeficiency virus type 1 and affords sterile protection of monkeys against a heterologous simian/human immunodeficiency virus infection. J Virol 80:5563–5570
8. Endo Y, Igarashi T, Nishimura Y, Buckler C, Buckler-White A, Plishka R, Dimitrov DS, Martin MA (2000) Short- and long-term clinical outcomes in rhesus monkeys inoculated with a highly pathogenic chimeric simian/human immunodeficiency virus. J Virol 74:6935–6945
9. Feinberg MB, Moore JP (2002) AIDS vaccine models: challenging challenge viruses. Nat Med 8:207–210
10. Haase AT (2005) Perils at mucosal front lines for HIV and SIV and their hosts. Nat Rev Immunol 5:783–792
11. Igarashi T, Endo Y, Nishimura Y, Buckler C, Sadjadpour R, Donau OK, Dumaurier MJ, Plishka RJ, Buckler-White A, Martin MA (2003) Early control of highly pathogenic simian immunodeficiency virus/human immunodeficiency virus chimeric virus infections in rhesus monkeys usually results in long-lasting asymptomatic clinical outcomes. J Virol 77:10829–10840
12. Joag SV, Li Z, Foresman L, Stephens EB, Zhao LJ, Adany I, Pinson DM, McClure HM, Narayan O (1996) Chimeric simian/human immunodeficiency virus that causes progressive loss of CD4+ T cells and AIDS in pig-tailed macaques. J Virol 70:3189–3197
13. Karlsson GB, Halloran M, Li J, Park IW, Gomila R, Reimann KA, Axthelm MK, Iliff SA, Letvin NL, Sodroski J (1997) Characterization of molecularly cloned simian–human immunodeficiency viruses causing rapid CD4+ lymphocyte depletion in rhesus monkeys. J Virol 71:4218–4225
14. Karlsson GB, Halloran M, Schenten D, Lee J, Racz P, Tenner-Racz K, Manola J, Gelman R, Etemad-Moghadam B, Desjardins E, Wyatt R, Gerard NP, Marcon L, Margolin D, Fanton J, Axthelm MK, Letvin NL, Sodroski J (1998) The envelope glycoprotein ectodomains determine the efficiency of CD4+ T lymphocyte depletion in simian–human immunodeficiency virus-infected macaques. J Exp Med 188:1159–1171
15. Li JT, Halloran M, Lord CI, Watson A, Ranchalis J, Fung M, Letvin NL, Sodroski JG (1995) Persistent infection of macaques with simian–human immunodeficiency viruses. J Virol 69:7061–7067
16. Lifson JD, Nowak MA, Goldstein S, Rossio JL, Kinter A, Vasquez G, Wiltrout TA, Brown C, Schneider D, Wahl L, Lloyd AL, Williams J, Elkins WR, Fauci AS, Hirsch VM (1997) The extent of early viral replication is a critical determinant of the natural history of simian immunodeficiency virus infection. J Virol 71:9508–9514
17. Ma ZM, Abel K, Rourke T, Wang Y, Miller CJ (2004) A period of transient viremia and occult infection precedes persistent viremia and antiviral immune responses during multiple low-dose intravaginal simian immunodeficiency virus inoculations. J Virol 78:14048–14052
18. Maggiorella MT, Baroncelli S, Michelini Z, Fanales-Belasio E, Moretti S, Sernicola L, Cara A, Negri DR, Butto S, Fiorelli V, Tripiciano A, Scoglio A, Caputo A, Borsetti A, Ridolfi B, Bona R, ten HP, Macchia I, Leone P, Pavone-Cossut MR, Nappi F, Ciccozzi M, Heeney J, Titti F, Cafaro A, Ensoli B (2004) Long-term protection against SHIV89.6P replication in HIV-1 Tat vaccinated cynomolgus monkeys. Vaccine 22:3258–3269
19. Mattapallil JJ, Douek DC, Hill B, Nishimura Y, Martin M, Roederer M (2005) Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature 434:1093–1097
20. Mellors JW, Munoz A, Giorgi JV, Margolick JB, Tassoni CJ, Gupta P, Kingsley LA, Todd JA, Saah AJ, Detels R, Phair JP, Rinaldo CR Jr (1997) Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. Ann Intern Med 126:946–954
21. Miyake A, Ibuki K, Suzuki H, Horiuchi R, Saito N, Motohara M, Hayami M, Miura T (2005) Early virological events in various tissues of newborn monkeys after intrarectal infection with pathogenic simian–human immunodeficiency virus. J Med Primatol 34:294–302
22. Murphey-Corb M, Wilson LA, Trichel AM, Roberts DE, Xu K, Ohkawa S, Woodson B, Bohm R, Blanchard J (1999) Selective induction of protective MHC class I-restricted CTL in the intestinal lamina propria of rhesus monkeys by transient SIV infection of the colonic mucosa. J Immunol 162:540–549
23. Parker RA, Regan MM, Reimann KA (2001) Variability of viral load in plasma of rhesus monkeys inoculated with simian immunodeficiency virus or simian–human immunodeficiency virus: implications for using nonhuman primate AIDS models to test vaccines and therapeutics. J Virol 75:11234–11238
24. Regoes RR, Longini IM, Feinberg MB, Staprans SI (2005) Preclinical assessment of HIV vaccines and microbicides by repeated low-dose virus challenges. PLoS Med 2:e249
25. Reimann KA, Li JT, Veazey R, Halloran M, Park IW, Karlsson GB, Sodroski J, Letvin NL (1996) A chimeric simian/human immunodeficiency virus expressing a primary patient human immunodeficiency virus type 1 isolate env causes an AIDS-like disease after in vivo passage in rhesus monkeys. J Virol 70:6922–6928
26. Reimann KA, Parker RA, Seaman MS, Beaudry K, Beddall M, Peterson L, Williams KC, Veazey RS, Montefiori DC, Mascola JR, Nabel GJ, Letvin NL (2005) Pathogenicity of simian–human immunodeficiency virus SHIV-89.6P and SIVmac is attenuated in cynomolgus macaques and associated with early T-lymphocyte responses. J Virol 79:8878–8885
27. Shinohara K, Sakai K, Ando S, Ami Y, Yoshino N, Takahashi E, Someya K, Suzaki Y, Nakasone T, Sasaki Y, Kaizu M, Lu Y,

Honda M (1999) A highly pathogenic simian/human immunodeficiency virus with genetic changes in cynomolgus monkey. J Gen Virol 80(Pt 5):1231–1240
28. ten Haaft P, Almond N, Biberfeld G, Cafaro A, Cranage M, Ensoli B, Hunsmann G, Polyanskaya N, Stahl-Hennig C, Thortensson R, Titti F, Heeney J (2001) Comparison of early plasma RNA loads in different macaque species and the impact of different routes of exposure on SIV/SHIV infection. J Med Primatol 30:207–214
29. ten Haaft P, Verstrepen B, Uberla K, Rosenwirth B, Heeney J (1998) A pathogenic threshold of virus load defined in simian immunodeficiency virus- or simian–human immunodeficiency virus-infected macaques. J Virol 72:10281–10285
30. Titti F, Zamarchi R, Maggiorella MT, Sernicola L, Geraci A, Negri DR, Borsetti A, Menin C, D'Andrea E, Modesti A, Masuelli L, Verani P, Chieco-Bianchi L, Amadori A (2002) Infection of simian B lymphoblastoid cells with simian immunodeficiency virus is associated with upregulation of CD23 and CD40 cell surface markers. J Med Virol 68:129–140
31. Veazey RS, DeMaria M, Chalifoux LV, Shvetz DE, Pauley DR, Knight HL, Rosenzweig M, Johnson RP, Desrosiers RC, Lackner AA (1998) Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science 280:427–431
32. Yamakami K, Honda M, Takei M, Ami Y, Kitamura N, Nishinarita S, Sawada S, Horie T (2004) Early bone marrow hematopoietic defect in simian/human immunodeficiency virus C2/1-infected macaques and relevance to advance of disease. J Virol 78:10906–10910