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In vivo inactivation of Nef ITAM motif of chimeric simian/human immunodeficiency virus SHIVsbg-YE correlates with absence of increased virulence in Chinese rhesus macaques

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Abstract

SHIVsbg, expressing Vpu, Tat, Rev, and Env proteins of HIV-1 Lai, was shown to be infectious for rhesus macaques. In this study, we mutated SHIVsbg Nef amino acids 17–18 from RQ to YE, conferring to SHIVsbg-YE the ability to replicate in vitro in unstimulated macaque PBMC. Juvenile macaques inoculated intravenously or orally with SHIVsbg-YE developed persistent infection. All macaques lost weight during the first 17 weeks but recovered afterward. All animals developed a strong HIV-specific humoral immune response. Viruses isolated 2 years postinoculation lost the ability to replicate in unstimulated macaque PBMC. Point mutations or 33-bp-wide deletions in the *nef* ITAM motif were responsible for this phenotype and correlated with clinical improvement of the infected macaques. These data demonstrate that the ITAM domain is inactivated in animals developing an acute antiviral immune response and may be detrimental to viral replication, perhaps by interfering with other well-conserved functions of SIV Nef protein.

Keywords: Nef; ITAM; SHIV; Macaques

Introduction

Since the isolation of human immunodeficiency virus type 1 (HIV-1) and HIV-2 (Barre- Sinoussi et al., 1983; Clavel et al., 1986), progress has been made in the understanding of in vivo viral dynamics (Chun et al., 1997; Ho et al., 1995; Mellors et al., 1996; Wei et al., 1995), but further comprehension of HIV-induced pathology and development of a vaccine require the use of an animal model. As HIV-1 does not induce disease in experimentally infected nonhuman primates, infection of macaques with simian immunodeficiency virus (SIV) has been used as surrogate to study pathogenesis induced by primate lentiviruses (Kestler et al., 1990). However this model has limited utility for developing a vaccine based on induction of an envelope-specific immune response due to the differences between HIV and SIV envelope glycoproteins (Burns and Desrosiers, 1991; Javaherian et al., 1992; Overbaugh et al., 1991).

To overcome this problem, chimeric simian–human immunodeficiency viruses (SHIV) have been constructed by replacing *tat*, *rev*, and *env* genes of the pathogenic SIVmac239 molecular clone by *tat*, *rev*, *vpu*, and *env* genes of HIV-1 strains (Bogers et al., 1997; Igarashi et al., 1994; Kuwata et al., 1996; Li et al., 1992, 1995; Luciw et al., 1995; Reimann et al., 1996a; Sakuragi et al., 1992; Shibata et al., 1991, 1997). We previously described the infection of rhesus and cynomolgus macaques with SHIVsbg, a virus carrying *tat*, *rev*, *vpu*, and *env* genes of HIV-1 Lai (Dunn et al., 1996). Although SHIVsbg replicated to high levels in macaques during primary infection and caused $CD4^+$ lymphocyte depletion, macaques remained asymptomatic for years and few animals developed simian AIDS (Dunn et al.,

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1996; and our unpublished data). Several groups have obtained SHIVs with increased virulence after serial passages in macaques (Joag et al., 1996; Reimann et al., 1996b; Stephens et al., 1996). This process of adaptation results in multiple mutations and the emergence of viruses with increased replication capacity in macaques (Cayabyab et al., 1999; Harouse et al., 1999; Igarashi et al., 1996; Karlsson et al., 1997). Introduction of specific sequences from pathogenic SIV strains is another approach. For example, SIVsmmPBj14 is a highly virulent strain, which naturally evolved in a pig-tailed macaque (*Macaca nemestrina*) infected with SIVsmm9 (Fultz et al., 1989; Tao and Fultz, 1995). The viral strain, recovered 14 months after inoculation, was extremely pathogenic for pig-tailed macaques. Indeed, intravenously infected monkeys developed acute disease (diarrhea, anorexia, depression, and rash) and died within 1 or 2 weeks postinoculation. SIVsmmPBj14 replicated to a high level and induced extensive hyperplasia of T -cell zones, especially in the gut-associated lymphoid tissues (Fultz, 1994). The in vivo phenotype of SIVsmmPBj14 was associated with the ability of the virus to replicate in vitro in unstimulated macaque peripheral blood mononuclear cells (PBMC) and to induce cell proliferation (Fultz, 1991; Fultz and Zack, 1994). Using molecular clones having varying pathogenicity, Novembre et al. demonstrated that multiple genes are involved in SIVsmmPBj14 acute virulence but deletion of *nef* gene abrogated the lethal phenotype in macaques, indicating a major contribution of this gene product (Novembre et al., 1993, 1996). Furthermore the SIVsmmPBj14 *nef* gene has a sequence variant YE at amino acids 17–18 that creates an immunoreceptor tyrosine base motif (ITAM, $TyrX_2Leu/IleX_7TyrX_2Leu/Ile$, where X is any amino acid). Conversion of sequence of SIVmac239 *nef* gene at these positions from RQ to YE conferred the mutated virus the capacity to replicate in resting PBMC in vitro and induces the SIVsmmPBj14 phenotype in vivo (Du et al., 1995, 1996; Sasseville et al., 1996).

In this study, we introduced the YE sequence variant into SHIVsbg *nef* gene. This molecular clone is an X4 virus and therefore its cell tropism differs from that of YE virus mutants generated earlier as SIVmac239 uses CCR5 as coreceptor while SHIVMD14YE has an envelope derived from a dual tropic HIV-1 isolate (Du et al., 1995; Shibata et al., 1997). Nevertheless, the mutation RQ to YE conferred to SHIVsbg the ability to replicate in unstimulated macaque PBMC. Juvenile macaques inoculated intravenously and orally with SHIVsbg-YE developed persistent infection and lost weight during the first 17 weeks. However they did not manifest other symptoms of acute SIVsmmPBj14 infection. Viruses isolated 2 years postinoculation did conserve the CXCR4 coreceptor usage as the inoculated virus but had lost the ability to replicate in unstimulated macaque PBMC. Different alterations in the *nef* gene ITAM motif were responsible for this phenotype. This data demonstrate that ITAM domain is inactivated in animals developing an acute antiviral immune response and may be detrimental to viral replication, perhaps by interfering with other well-conserved function of SIV Nef protein.

Results

In vitro characterization of SHIVsbg-YE

The goal of our study was to evaluate the impact of the Nef $_{17}$YE sequence variant on SHIVsbg virulence. The *nef* mutation was introduced by targeted mutagenesis in SHIVsbg genome to generate SHIVsbg-YE. We also replaced the *nef* gene of SIVmac239 by the mutated allele to serve as positive control in in vitro infection of unstimulated PBMC. SHIVsbg-YE virus was first evaluated by infecting CEMx174 cells and human or macaque PHA-activated PBMC in vitro.

SHIVsbg-YE replicated in CEMx174 cells to the same levels as the parental virus SHIVsbg (data not shown). It also replicated efficiently as SHIVsbg in activated human PBMC (Fig. 1A). In contrast to SHIVsbg, which replicated better on human than rhesus PBMC, SHIVsbg- YE replicated equally well on activated PBMC from both species (Fig. 1B). In macaque PBMC, SIVmac239-YE replicated slightly better than the parental virus (data not shown).

SHIVsbg-YE multiplication in unstimulated macaque PBMC

We next evaluated the replication capacity of SHIVsbg-YE in unstimulated macaque PBMC. Preliminary experiments demonstrated that the presence of fetal calf serum was sufficient to activate macaque PBMC and allow replication of all viruses (data not shown). Moreover, IL-2 addition results in proliferation of in vivo activated cells and replication of all viruses. To avoid this activation and proliferation, PBMC were infected directly after isolation and cultured in serum-free medium AIM grade V in the absence of IL-2. In these culture conditions, cell viability was somewhat reduced and few fusiform cells were observed in cultures infected with unmutated RQ viruses or in uninfected cultures (Fig. 2A). However, in cultures infected with YE viruses, cell viability improved and more adherent fusiform cells were observed (Figs. 2B and C). Only viruses bearing the YE mutations were able to replicate in these conditions (Fig. 1C). SIVmac239-YE generally replicated faster than SHIVsbg-YE but in some experiments similar replication kinetics were observed. Progeny viruses were infectious and replicated efficiently in CEMx174 cells (data not shown). Therefore, the Nef YE protein allows the replication of SHIVsbg in unactivated macaque PBMC, a property that could enhance its capacity to replicate in vivo.

Infection of rhesus macaques with SHIVsbg-YE

To evaluate the impact of the Nef YE sequence on SHIVsbg in vivo, we inoculated five Chinese rhesus ma-

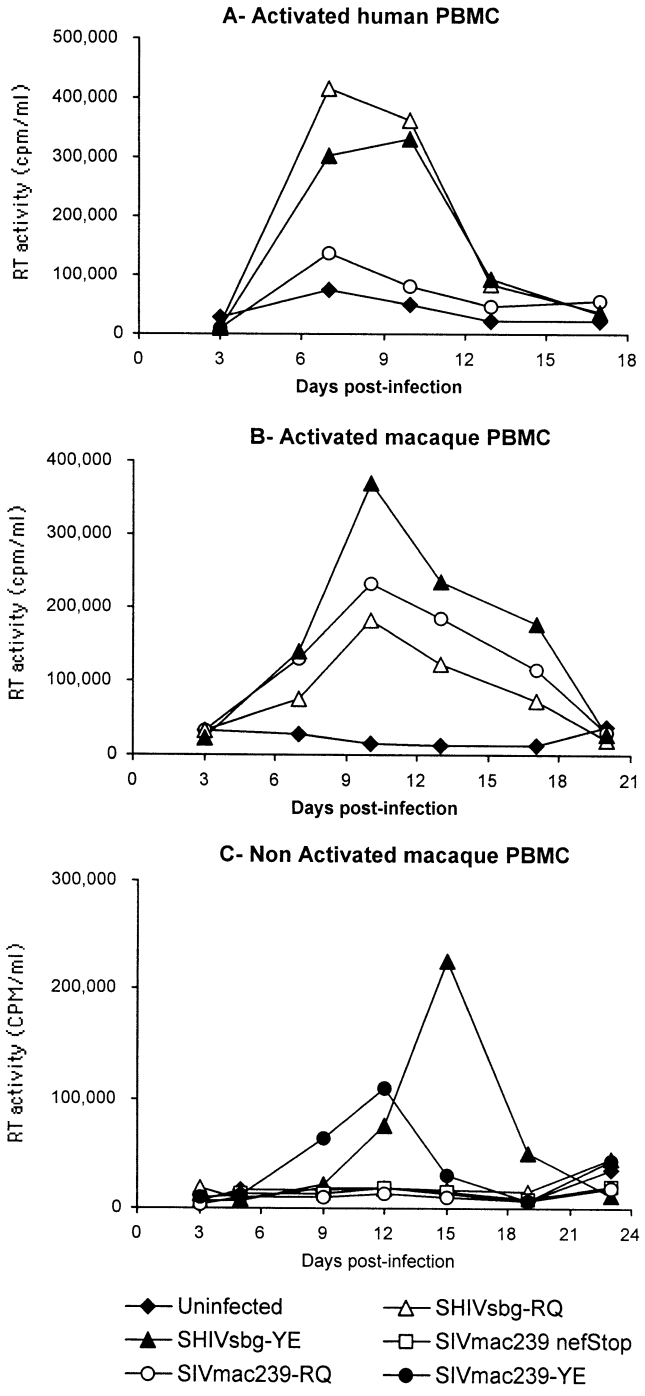

Fig. 1. Replication of SHIVsbg-YE and its parental viruses in human and rhesus macaque PBMC. PBMC were infected with cell-free stock of SHIVsbg, SHIVsbg-YE, SIVmac239, SIVmac- YE, or mock infected. Virus replication was monitored by measuring RT activity in the culture supernatant. (A) PHA-activated human PBMC; (B) PHA-activated rhesus macaque PBMC; (C) unstimulated macaque PBMC.

caques with SHIVsbg-YE. Three monkeys were inoculated intravenously (iv) with 440 $TCID_{50}$ and two rhesus macaques were inoculated with 7×10^3 $TCID_{50}$ by the oral route. Monkeys were followed by clinical examination, measurement of the cell-associated viral load, serum RNA viral load, lymphocyte subsets in peripheral blood, and HIV-specific humoral immune response.

All five macaques became infected. Clinical examination revealed persistent lymphadenopathy detectable as soon as 1 week postinoculation for the axillary lymph nodes. Macaques did not develop the severe disease induced by SIVsmmPBj14 infection but weight loss was observed during the first 17 weeks of infection (Fig. 3). At the nadir, mean weight loss was 9% global weight (range 3.1–14.6%) and was more pronounced for orally infected monkeys (11.7%) than for iv inoculated animals (7.2%). Such weight loss was not observed during primary infection of six rhesus macaques of Chinese and Indian origin inoculated intrave-

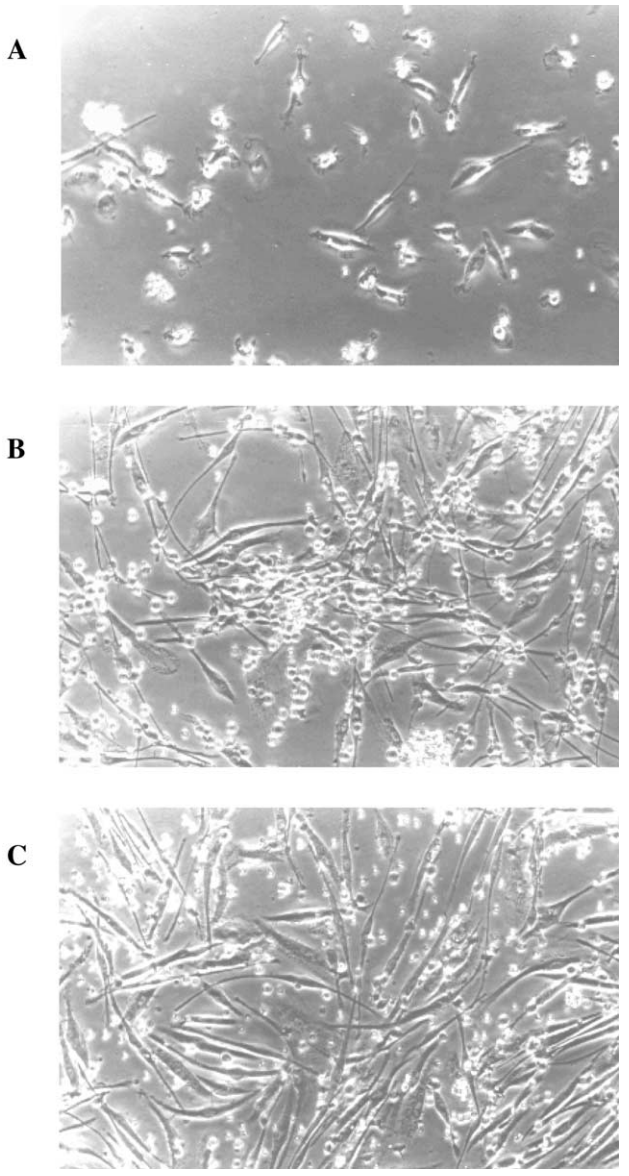

Fig. 2. Adherent cells after infection of unstimulated macaque PBMC. The adherent cell fraction observed 9 days after infection with SHIVsbg (A), SHIVsbg-YE (B), and SIVmac239-YE (C) are shown. Mock-infected cultures and SIVmac239*nef* stop or SIVmac239*nef* open infected cultures were similar to (A).

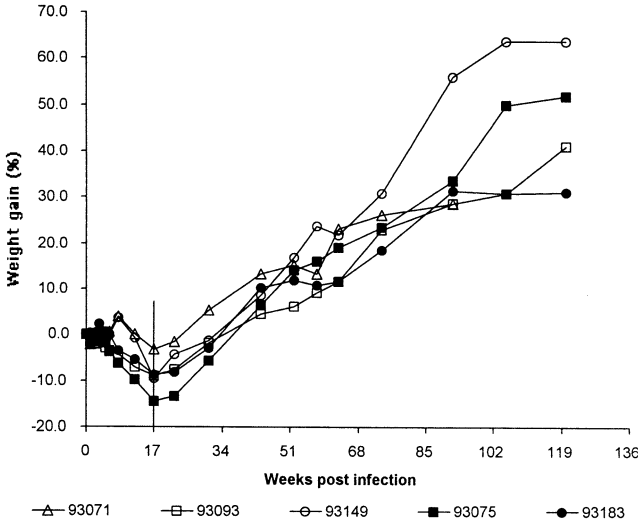

Fig. 3. Evolution of macaques weight. Macaques were weighed after anesthesia at each blood collection. The weight is expressed as the percentage of body weight increase compare to the day of virus inoculation. Macaque 93071 was euthanized 94 weeks p.i.

nously with the same amount of SHIVsbg (Dunn et al., 1996). All SHIVsbg-YE infected monkeys had recovered preinfection weight by 38 weeks postinoculation.

Viral RNA was detected in serum of intravenously infected macaques as early as 4 days postinoculation and later in orally infected animals. The peak of viremia was reached after 1.5 or 2 weeks for four of five macaques and was comprised between 3.9×10^5 and 2.0×10^6 RNA copies per milliliter (Fig. 4). In the case of macaque 93183, the number of RNA copies remained quasiconstant (4.2×10^5–6.6×10^5 copies per ml) between week 2 and 5 postinfection (Fig. 4B). Viral loads decreased afterward in all monkeys and after 2 years, viral set point was comprised between 7.7×10^2 and 3.6×10^4 copies per milliliter. Similarly, PBMC-associated viral load peaked at week 2 or 3 postinfection (50 to 6250 infected cells per 10^6 PBMC) and decreased thereafter in all monkeys but less rapidly in orally infected animals. Low levels of cell-associated virus were detected for up to 2.4 years (data not shown).

Analysis of lymphocyte subsets in peripheral blood demonstrated that iv inoculated monkeys had a rapid depletion during the primary infection (2–3 weeks) and for two of them $CD4^+$ lymphocytes increased thereafter to preinfection level (Fig. 5A). For macaque 93149, the restoration of $CD4^+$ lymphocyte subset was slower and took 1 year to return to baseline value. The orally inoculated macaques had a partial depletion of $CD4^+$ lymphocytes persisting for 17 weeks, but later they recovered to preinfection values (Fig. 5B). After 2 years, macaque 93075 developed AIDS characterized by a $CD4^+$ lymphocyte count below 200 cells/μl and thrombocytopenia (data not shown).

All monkeys developed HIV-specific antibodies in the serum (Fig. 6) and seroconversion was concomitant with the rapid increase of $CD20^+$ lymphocyte population in the peripheral blood (data not shown). Seroconversion occurred after 2 weeks of infection in iv inoculated macaques and at 3 weeks for orally infected animals. HIV-specific antibody titers increased sharply for the two groups during the first month. A continuous increase during the following 2 years led to sustained levels of HIV-specific antibodies, slightly higher for the orally infected monkeys.

Growth in unstimulated macaque PBMC and nef sequences

To evaluate the biological properties of viruses still present in the infected monkeys, we isolated viruses by cocultivation of infected macaque PBMC with healthy macaque and tested their capacity to replicate in unstimulated macaque PBMC. SHIVsbg, SHIVsbg-YE, and SIVmac239-YE were used as controls. Whereas virus replication was observed in cultures infected with YE viruses, no virus replication was detected in cultures infected with the viral isolates or the parental SHIVsbg (Fig. 7).

To evaluate if *nef* gene alterations could be responsible for the failure to replicate in nonstimulated PBMC, *nef*

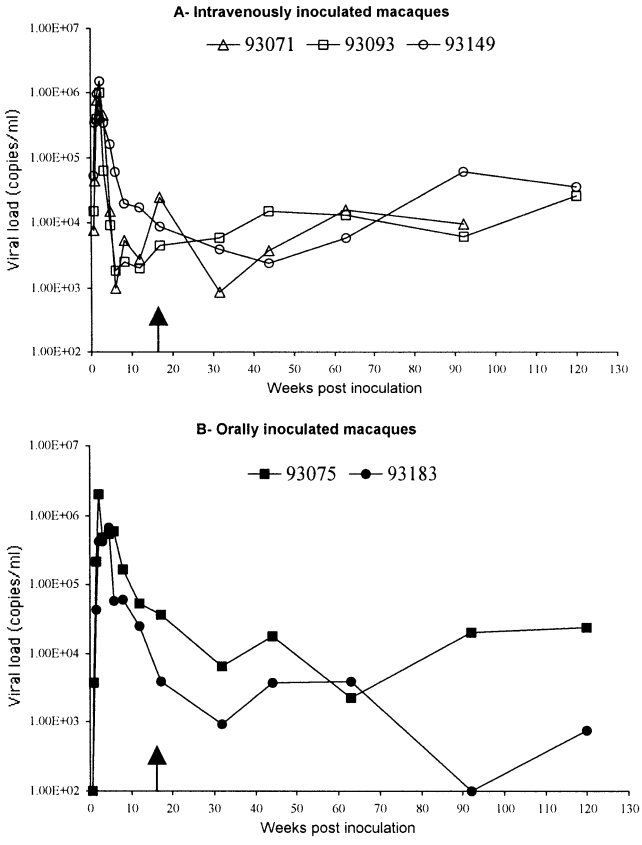

Fig. 4. Viral RNA copies in the serum of SHIVsbg-YE-infected macaques. The number of RNA copies, expressed per ml of serum, was determined by real-time RT-PCR for (A) iv infected monkeys (93071, 93093, and 93149) and (B) orally infected macaques (93075 and 93183). Black arrow indicates the time postinfection at which nadir of body weight loss was observed.

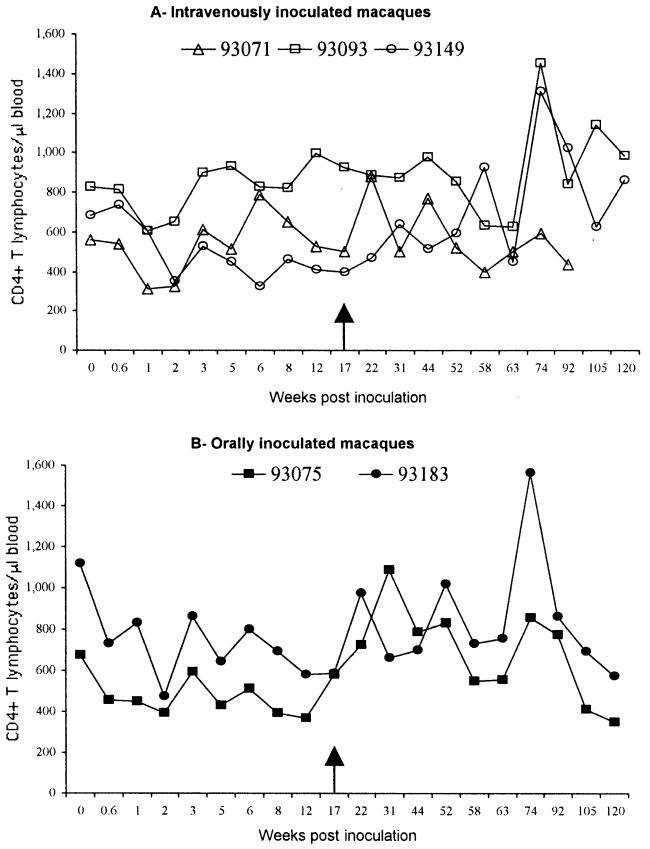

Fig. 5. Variation of $CD4^+$ lymphocytes in rhesus macaques infected with SHIVsbg-YE. Absolute number of $CD4^+$ lymphocytes are represented for (A) iv infected monkeys (93071, 93093, and 93149) and (B) orally infected macaques (93075 and 93183). Black arrow indicates the time postinfection at which nadir of body weight loss was observed.

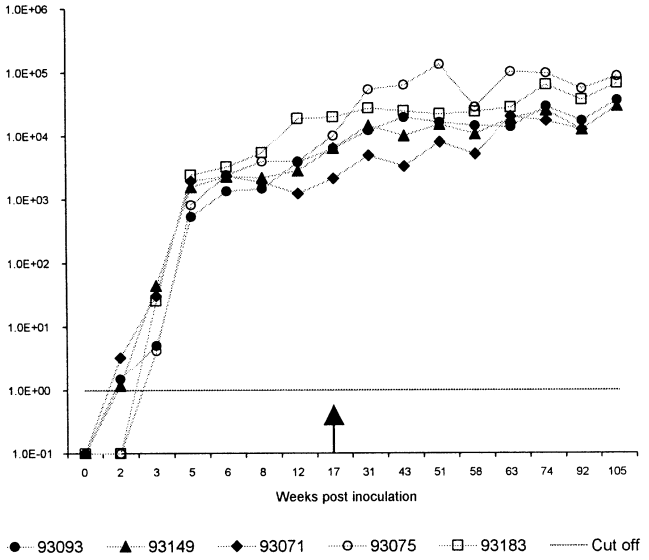

Fig. 6. HIV-specific antibody titers in SHIVsbg-YE-infected macaques. Antibody response was determined on serum of the infected animals with a commercial HIV ELISA kit (Murex HIV 1.2.O). Antibody titers are the reciprocals of the highest serum dilution giving an optical density of at least 0.1 above the cutoff value. Cutoff value (1) is depicted by a line and antibody titer below are considered as negative. Orally infected macaques (93075 and 93183) are depicted by open symbols, iv inoculated monkeys (93071, 93093, and 93149) are depicted by filled symbols. Black arrow indicates the time postinfection at which nadir of body weight loss was observed.

genes were amplified and sequenced (Fig. 8). Extensive alterations were observed in the *nef* genes of viruses isolated from two iv inoculated macaque viruses. Although the YE site was retained in most of these viruses, 33-bp in-frame deletions were detected in multiple isolates from macaques 93071 and 93149. The deletions observed in viruses from these two monkeys differed from each other by a two codon shift but both impaired the $TyrX_2Leu$ site located seven amino acids downstream of the YE site. In contrast, no deletion was observed in the *nef* gene of viral isolates obtained from orally infected macaques. However codon 17 reverted back from tyrosine to arginine, inactivating also the ITAM motif.

Retrospectively, we analyzed *nef* genes of viruses isolated at earlier time points to determine if *nef* alteration could be implicated in the clinical improvement. The *nef* deletion was detected as soon as 8 weeks postinoculation in viruses isolated from macaque 93071 but full-length *nef* (YE) was still present (representing approximately 20%). At 12 weeks, leucine 31 was substituted by a proline and after 17 weeks postinoculation only deleted *nef* genes were revealed in isolates originating from PBMC or different lymph nodes collected at time of necropsy. For macaque

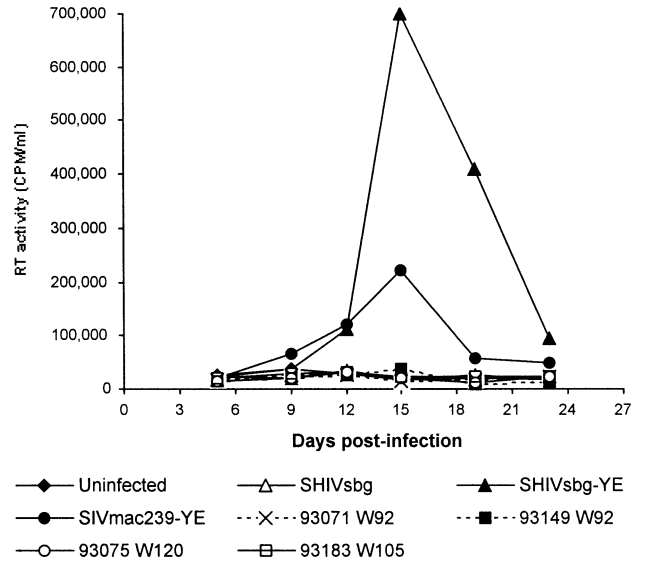

Fig. 7. In vitro phenotype in unstimulated macaque PBMC of viral isolates recovered from SHIVsbg-YE-infected monkeys. Replication of viruses isolated from macaque 93071 (92 weeks p.i.), 93149 (92 weeks p.i.), 93075 (120 weeks p.i.), and 93183 (105 weeks p.i.) was evaluated in unstimulated macaque PBMC as described under Materials and methods. Infection with SHIVsbg-YE and SIVmac239- YE served as positive controls and cultures uninfected or infected with SHIVsbg as negative controls.

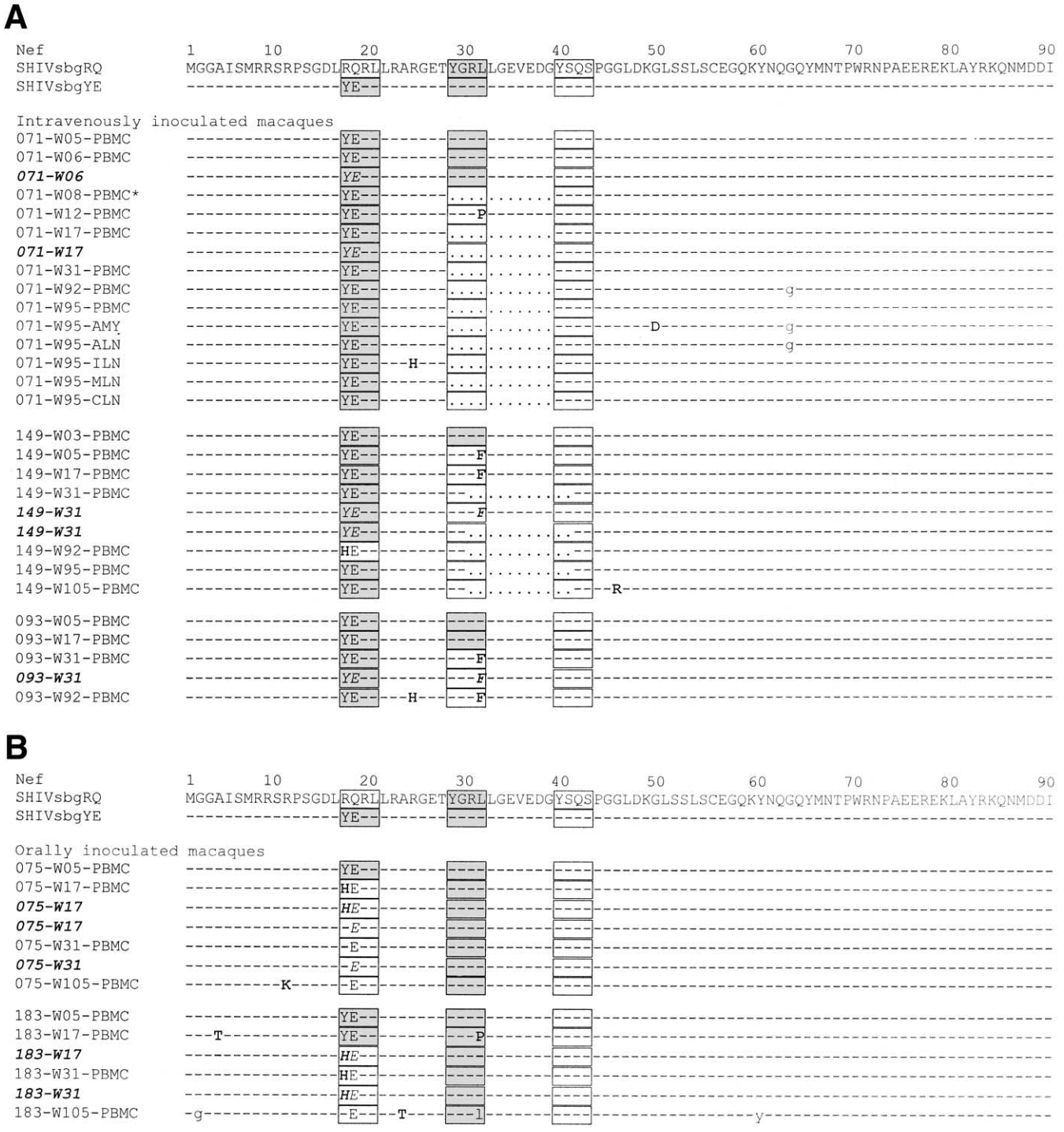

Fig. 8. Sequence of the N-terminal extremity of Nef protein from virus isolated from SHIVsbg-YE infected macaques. Virus were isolated from several tissues: PBMC, tonsil (TON), mesentheric lymph nodes (MLN), inguinal lymph nodes (ILN), axillary lymph nodes (ALN), and colonic lymph nodes (CLN). The *nef* gene of virus isolates was sequenced and amino acid sequences of the N-terminal extremity are depicted. Dashes indicate amino acids identical to SHIVsbg Nef protein. Dots indicate deleted amino acids. An asterisk marks isolate containing parental and deleted genes. Mutations are indicated in bold capitals for nonsynonymous mutations and in lowercase letters for synonymous mutations. Localization of the tyrosine potentially involved in TyrXXLeu motifs is highlight by a box. The two consensus TyrXXLeu motifs, constituting an ITAM domain, are indicated as gray boxes, whereas mutated sequences are in white boxes. Sequences of seric viruses collected at different time points after infection were determined by direct RT-PCR sequencing and are indicated in italic. For some samples, two populations of viruses were detected concomitantly; both sequences are indicated. Note that the nadir of body weight loss was observed 17 weeks postinfection.

93149, the deletion was established 31 weeks postinoculation. It was preceded by the mutation of leucine 31 in phenylalanine as early as 5 weeks postinoculation. For the third animal (93093) the same punctual mutation of leucine 31 was detected after 31 weeks postinoculation, suggesting that this mutagenesis event may be necessary for the following deletion. In the orally infected group, viruses isolated from both monkeys carried an altered ITAM motif from 17 weeks postinoculation. The reversion of tyrosine 17 (TAC) to arginine (CGC) uses an intermediate transition in histidine (CAC). Therefore all viruses carried a mutated ITAM motif. As SIVsmmPBJ14 replication capacity is associated with the presence in Nef protein of two $TyrX_2Leu$ motifs forming an ITAM motif, the two types of Nef alterations could be responsible for the absence of replication of the viral isolates in unstimulated macaque PBMC. However the Nef protein of all mutated viruses had maintained a motif of two tyrosines spaced by 11 amino acids, using in the case of deleted *nef* genes either Tyr 17 and 39 (93071) or Tyr 17 and 28 (93149). For orally infected macaques, Tyr 28 and 39 were maintained after Tyr 17 reverted back to Arg (Fig. 8).

Sequence determination on genome of viruses collected in the serums at different times postinfection indicates that mutations detected in viruses produced in vitro by activated PBMC are also present in viruses produced in vivo.

Discussion

In this study we describe the effect of $_{17}$YE Nef protein mutation on multiplication of SHIVsbg in vitro and its virulence in Chinese rhesus macaques. Introduction of the $_{17}$YE mutation allowed SHIVsbg to replicate in unstimulated rhesus macaque PBMC. This observation is consistent with previously published characteristics of SIVmac239-YE and the acute pathogenic SIVsmmPBj14, although these viruses use a different coreceptor in vitro (Du et al., 1995; Schwiebert et al., 1997). Ability to replicate in resting PBMC was also observed with a chimeric virus ($SHIV_{PPc}$P-Bjnef) bearing the *nef*/LTR region from SIVsmmPBj14, but in this case, coreceptor usage was not established (its envelope gene was cloned from a virus stock of sequentially passaged SHIV-4 and compared to this, later, predicted amino acids substitutions in the V3 loop were reported, Stephens et al., 1998). We hypothesized that the capacity to replicate in nonstimulated PBMC would allow SHIVsbg to infect productively a larger number of cells in vivo and therefore confer an increased virulence. However, after intravenous and oral inoculation of juvenile rhesus macaques, no acute phenotype was observed in the infected animals. SHIVsbg-YE replicated efficiently during primary infection but was not highly pathogenic.

Macaques species, route of inoculation, and viral dose could be factors involved in this weak virulence (Shibata et al., 1997). Indeed after iv inoculation with SIVsmmPBj14, only 30– 50% of rhesus macaques suffered from severe symptoms and died, in contrast to a 100% mortality observed in pig-tailed macaques (Lewis et al., 1992). Moreover the route of inoculation can play an important role. For example, pig-tailed macaques infected by rectal or vaginal exposure to SIVsmmPBj14 generally developed an acute disease syndrome (diarrhea, anorexia, depression, and rash) but less than 50% died (Fultz et al., 1995). Previous study has shown that day of onset of disease and death was a function of the inoculated dose (Fultz and Zack, 1994). Similarly, Endo and collaborators reported recently that rhesus monkey iv inoculated with 650 $TCID_{50}$ of pathogenic $SHIV_{DH12R}$ or more suffered irreversible $CD4^+$ loss in contrast to animals inoculated with lower doses (Endo et al., 2000). Coreceptor utilization may also play a role as animals infected with the R5 and X4 SHIV, expressing in these cases the SIVmac239nef gene, have distinct pathogenic outcomes despite comparable levels of viral replication (Harouse et al., 1999). Clinical examination of SHIVsbg-YE-infected macaques revealed only a persistent lymphadenopathy and a weight loss up to 14.6% at the nadir (17 weeks postinoculation), which was not observed in the course of SHIVsbg infection. It could be associated with viral replication in lymphoid tissues such as lymph node and gut-associated lymphoid tissues (GALT). Indeed, GALT is a major site for lentiviral replication, especially for SIVsmmPBj14 and SIVmac239-YE that induce hyperplasia of Peyer's patches (Fultz and Zack, 1994; Sasseville et al., 1996; Veazey et al., 1998; Veazey and Lackner, 1998). The extremely intense lymphoproliferation observed in GALT of SIVsmmPBj14-infected monkeys was associated with the expression on infected cells of $\alpha^E\beta_7$ integrin (CD103), an adhesion molecule involved in GALT homing of lymphocytes (Gummuluru et al., 1996). Moreover, acute replication was associated with elevated levels of inflammatory cytokines (IL-6 and TNFα), which could be responsible for maladsorption and weight loss observed during acute infection.

Depletion of the $CD4^+$ lymphocytes was more prolonged in orally exposed monkeys than in two of the three iv infected macaques. For the latter monkeys (93071, 93093), serum viral load was also 10 times lower from 5 to 12 weeks p.i. After 17 weeks of infection, clinical parameters started to return to their preinfection level. Concomitantly $CD4^+$ lymphocytes increased in the blood and cell-associated viral load decreased, demonstrating that a better control of viral multiplication was achieved by the immune system of the infected macaques. The infection was persistent as, after 2 years postinoculation, viral set point was comprised between 7.7×10^2 and 2.4×10^4 viral RNA copies per milliliter, even when virus could not be isolated from 10^6 PBMC. Comparison with historical SHIVsbg infection can be made as animals inoculated intravenously received the same dose of virus (Dunn et al., 1996). In the case of SHIVsbg infection, following the peak of viremia (mean value 0.9×10^6 RNA copies/ml serum) viral set

point measured was between 10^2 and 10^3 RNA copies/ml serum (L. Gloeckler, unpublished data).

To evaluate virus variation after 2 years of replication in vivo, analyses were done on isolated virus rather than directly amplified proviral DNA. Indeed studies have shown that during the asymptomatic phase of HIV infection, most of the viral sequences are found in quiescent $CD4^+$ cells, in an unintegrated form that does not represent replication-competent virus (Chun et al., 1997). Moreover defective proviruses accumulated in asymptomatic patients (Sanchez et al, 1997). We could recover viruses from PBMC as well as lymphoid organs, confirming that replication-competent viruses were indeed present in the infected animals after 2 years of infection. Phenotypic tests and envelope gene sequencing demonstrated that these viral isolates had not changed their CXCR4 usage (data not shown), indicating that oral infection can take place with an X4 virus.

The viruses isolated 2 years postinoculation no longer replicated in unstimulated macaques PBMC. Sequence analysis of *nef* genes demonstrated that the ITAM motif was inactivated by either a point mutation of tyrosine 17 or a 33-bp downstream deletion, altering the second $TyrX_2Leu$ site. These modifications were detected not only in the genome of viruses produced in vitro by activated PBMC but also in the genome of viruses present in the plasma. The two different mechanisms of inactivation were observed depending on the route of inoculation. In pig-tailed macaques persistently infected by SIVsmmPBj14 after mucosal exposure, previous sequences analyses of *nef* gene have also shown that the ITAM motif was inactivated by point mutations of the first or the second tyrosine (Schwiebert et al., 1997). We have shown that ITAM alteration correlates with clinical improvement of the infected monkeys.

Why is this motif inactivated? On one hand, the ITAM motif in Nef may confer a replication advantage to the virus in persistently infected animals. SIV possessing the Nef YE allele were isolated in several studies in animals enduring an increase of viral replication and a progression to death (Dewhurst et al., 1990; Kirschhoff et al., 1999). It would contribute to cell activation after phosphorylation of the two tyrosines by Lck and interaction with ZAP-70, a T cell specific tyrosine kinase, when lymphocytes are in an anergic state during long-term infection (Luo and Peterlin, 1997). Presence of macrophages is also necessary to trigger lymphoproliferation and costimulatory molecules such as B7-1, B7-2, and CD40 are involved in the process (Du et al., 1995; Whetter et al., 1998). On the other hand the ITAM motif appeared to be counterselected in animals that did not succumb to the acute infection after SIVsmmPBj14 mucosal exposure or in our case iv and oral SHIVsbg-YE infections. These contrasting observations indicate that after primary infection and the development of an immune response, presence of an ITAM motif could be detrimental for viral replication as recovered viruses bear mutations altering this motif. Macaques infected with SIVsmmPBj14 generally died between 6 and 14 days postinoculation when immune responses were minimal or undetectable (Fultz and Zack, 1994; Lewis et al., 1992). This strong selection may be due to an interference of ITAM with other well-conserved functions of Nef protein important for evading immune control. The well-conserved tyrosines in position 28 and 39 of SIV Nef protein have been shown to be involved in the interaction and cellular colocalization of Nef with the $\mu 2$ subunit of AP-2 (Lock et al., 1999; Piguet et al., 1998). Moreover studies indicate that these tyrosine-based motifs play a role in CD4 down regulation, which is best revealed in the context of composite mutations with the leucine-based motif at position 194–195 (Bresnahan et al., 1999). The ITAM motif partially overlaps the tyrosine domains and may interfere, by steric obstruction or tyrosine phosphorylation, with functions associated with these two tyrosines in vivo. In persistently infected animals with a strong immune response, the function associated with these tyrosines could be more beneficial to virus replication than cell activation that is generally high. At later times, in animals having a weaker immune system and anergic lymphocytes, Nef protein with an ITAM motif could stimulate viral replication, thereby accelerating progression to death. Further analyses will be needed to clarify the precise function of Nef–$\mu 2$ interaction in infected animals.

Materials and methods

Construction of SHIVsbg-YE and SIVmac239-YE

Classical techniques of molecular biology used were similar to those described by Sambook et al (1989). The 17th and 18th codons of SIVmac239 *nef* gene were mutated from CGACAG to TACGAG by targeted mutagenesis using the Scultor in vitro mutagenesis system (Amersham). Briefly, the pTG5043 plasmid, encoding the 3′ half genome of SHIVsbg, was digested with *Sma*I and *Pvu*II. The 1311-bp fragment encompassing the *nef* gene was cloned into the *lac*Zα gene of the M13TG131 phage vector linearized with *Sma*I and dephosphorylated with calf intestine phosphatase. XL-1 Blue *Escherichia coli* were transformed with the ligation products and white plaques were picked. Double-stranded DNA was prepared and screened for the insertion by *Bam*HI and *Hin*dIII restriction analysis. Single-stranded DNA was prepared and used with a phosphorylated primer NEFOMYE (5′- CGCCCGCAAGAGTCTCT**CGTA**-CAGATCTCCAGACGG-3′) in a targeted mutagenesis reaction according to the manufacturer's instructions. XL-1 Blue *E. coli* were transformed with the mutagenesis reaction product and phages were screened for the presence of the mutations by sequencing single-stranded DNA using the Sequenase kit (Amersham). The 201-bp mutated *nef* fragment was purified after digestion of double-stranded DNA with *Rsr*II and *Nco*I and cloned into pTG5043 digested by *Rsr*II and *Nco*I and dephosphorylated. The *nef* gene in the

resulting recombinant plasmid p3′SHIV-YE was sequenced using the Sequenase kit.

The mutagenized *nef* gene was also introduced into the SIVmac239 genome. The 1694-bp *Bgl*II-*Eco*RI fragment of p3′SHIV-YE, encompassing the mutated *nef* gene and the LTR, was ligated to the p239SpE3′ plasmid, encoding the 3′ half genome of SIVmac239, previously digested by *Bgl*II and *Eco*RI. The resulting plasmid p3′SIV-YE was verified by restriction analysis and *nef* gene sequencing using Sequenase kit.

Cells and viruses

CEMx174, a $CD4^+$ T/B cell hybrid cell line, was maintained at 37°C and 5% CO_2 in RPMI complete medium [RPMI 1640 (Gibco) supplemented with 10% decomplemented fetal calf serum (FCS), 2 mM L-alanyl-L-glutamine (Glutamax I, Gibco), 100 μg of streptomycin per ml, and 100 IU of penicillin per ml].

Human and macaque PBMC were isolated from whole blood of healthy donors by density gradient centrifugation on a Ficoll–Hypaque cushion (Eurobio, France), washed with phosphate-buffered saline (PBS), and counted. They were stimulated for 3 days with 4 μg phytohemagglutinin A (PHA, Sigma) per milliliter in RPMI complete medium supplemented with 20 IU interleukin-2 (IL-2) per milliliter. Unstimulated macaque PBMC were cultured in AIM grade V medium (Gibco) with 2 mM L-alanyl-L- glutamine without any other supplementation.

SHIVsbg, SHIVsbg-YE, SIVmac239nefstop, and SIVmac239-YE were produced in CEMx174 cells. Viral genomes were reconstructed in vitro by ligation of *Sph*I linearized plasmids encoding the 5′ and 3′ half genomes: pTG4036 and pTG5043 for SHIVsbg (Dunn et al., 1996), pTG4036 and p3′SHIV-YE for SHIVsbg-YE, p239SpSp5′, and p239SpE3′ (kindly provided by Dr. Desrosiers through the NIH AIDS reagent program) for SIVmac239nef stop (Naidu et al., 1988; Regier and Desrosiers, 1990), and p239SpSp5′ and p3′SIV-YE for SIVmac239-YE. Ten million CEMx174 cells were electroporated with 10 μg of ligated DNA in 800 μl RPMI complete medium under 1500 μF and 280 V in an Easyject Plus apparatus (Eurogentech, Seraing, Belgium) and then cultured at 1 million cells per milliliter in RPMI complete medium. Cultures were observed for cytopathic effects and virus production was evaluated by measuring reverse transcriptase (RT) activity in culture supernatant (Moog et al., 1994). Once increasing RT activity was detected, 24 h supernatants were harvested for virus stocks and filtered through 0.45-μm filters.

For macaque inoculation, we used a SHIVsbg-YE stock grown in macaque PBMC, having a titer of 2330 $TCID_{50}$ per milliliter on CEMx174 cells and 39 ng of p27 antigen per milliliter as determined by ELISA (Innotest HIV antigen mAb screening, Innogenetics, Zwijndrecht, Belgium).

Infection of cell line and PBMC

Five million CEMx174 cells were infected with identical quantities of viruses determined on the basis of RT activity (10^5 cpm). Cells were incubated with the virus for 1 h at 37°C and then washed with PBS and cultured at 1 million cells per milliliter in RPMI complete medium. Culture medium was replaced twice weekly and cell concentrations were adjusted at these occasions.

A similar protocol was used to infect activated human and rhesus macaque PBMC cultured in RPMI complete medium supplemented with 20 IU IL-2 per milliliter.

Unstimulated macaque PBMC were infected immediately following isolation with the same protocol used for stimulated PBMC. After 1-h incubation at 37°C, cells were washed with PBS and cultured at 1–2 million cells per milliliter in AIM grade V medium with 2 mM L- alanyl-L-glutamine. Half of the culture medium was replaced with fresh medium twice weekly without perturbing the cells.

Animals

Chinese rhesus macaques (*Macaca mulatta*), 3- to 4-year-old juvenile monkeys, were maintained according to the conditions stipulated in the European guidelines. Animals were sedated with ketamine HCl (10 mg/kg; Imalgene, Mérieux) for clinical examination, blood sampling, lymph node biopsies, and virus inoculation. Blood samples were collected by saphenous venipuncture. Three monkeys (93071, 93093, and 93149) were inoculated in the saphenous vein with 440 $TCID_{50}$ of the cell-free SHIVsbg-YE stock produced in macaque PBMC. After examination of the oral cavity to verify the absence of lesions and inflammation, two other rhesus monkeys (93075 and 93183) were inoculated orally with 7.0×10^3 $TCID_{50}$ in 3 ml. During the primary infection, clinical examination of monkeys was performed twice weekly and less frequently thereafter.

Immunophenotyping

Lymphocyte subsets were evaluated by flow cytometry. Whole-blood samples were stained with either anti-CD4 PE (OKT4, Ortho, Roissy, France) or anti-CD8 FITC (Leu2a, Becton–Dickinson, Le Pont de Claix, France) monoclonal antibodies or with anti-CD20 FITC monoclonal antibody (B9H9, Immunotech, Marseille, France). Red blood cells were lysed with the Facs lysing solution (Becton–Dickinson) according to the manufacturer's instructions. Cells were washed twice in FACSflow and resuspended in 1.5% formaldehyde in PBS. Samples were analyzed on a FACScan flow cytometer (Becton–Dickinson) and data were collected in list mode using the CellQuest software with at least 3000 events in a lymphocyte gate defined by the FSC-SSC parameters.

Serology

HIV-specific antibody titers in the sera of the infected macaque were quantified with a commercial HIV ELISA kit (Murex HIV-1.2.O, Murex Diagnostics S.A., Chatillon, France) following the manufacturer's instructions. Antibody titers were determined as the reciprocal of the highest dilution giving an optical density of a least 0.1 above the cutoff values.

Determination of cell-associated viral load

Macaque PBMC were purified from heparinized blood by density gradient centrifugation on a Ficoll–Hypaque cushion (Eurobio). Quantitative determination of the cell-associated viral load was performed by cocultivating 10^6, 5×10^5, and five-fold serial dilutions of the PBMC with 1.5×10^5 CEM $\times$ 174 cells in 24-well plates. The cocultures were maintained for 4 weeks and treated twice weekly, once to change the culture medium (RPMI complete medium) and once to divide the cells. The RT activity in culture supernatant was determined at each time and its increase was taken as evidence of viral replication. The cell-associated viral load was expressed as the number of infected cells per 10^6 cells. After 12 weeks of infection, cocultures of $CD8^+$ depleted PBMC were also performed in parallel.

Quantification of viral RNA in serum

Viral RNA was extracted from 250 μl of macaque serums collected at different times postinfection as described in the paragraph RT-PCR amplification and concentrated in 25 μl of water. Determination of the number of viral RNA was made in duplicate by real-time RT-PCR (5 μl of RNA per assay), with the TaqMan EZ RT-PCR kit (PE Applied Biosystems), according to the protocol described (Hofmann-Lehmann *et al.*, 2000) using an i-cycler (Bio-Rad). Under these conditions, a concentration of 400 copies of viral RNA per milliliter of serum was measurable.

Virus isolation from macaque PBMC and lymphoid organs

PBMC were purified from infected macaque blood using Ficoll–Hypaque cushion and $CD8^+$ cells were removed using magnetic beads coupled to antihuman CD8 monoclonal antibody (Dynal). Two million $CD8^+$-depleted PBMC were cultivated in complete medium [RPMI 1640 + 10% FBS + 20 IU IL-2 per ml] supplemented with 4 μg PHA per milliliter. After 3 days, the cells were washed with PBS and cocultivated in complete medium with 2 million $CD8^+$-depleted, PHA-activated PBMC from a healthy macaque. Virus replication was checked twice weekly by quantifying RT activity in culture supernatant or by quantifying p27 antigen with a p24 ELISA (Innotest HIV antigen mAb screening; Innogenetics).

For virus isolation from the organ cells, lymph node samples were dissociated with scissors and vigorous pipetting followed by filtration through a 70-μm nylon cell strainer (Becton–Dickinson) to obtain a single-cell suspension. Cells were depleted of $CD8^+$ cells and then cultivated in the same conditions used for PBMC.

Determination of co-receptor usage

Evaluation of chemokine receptor usage for cell entry was performed using the GHOST cell lines, obtained from the AIDS Research Project (Program EVA centralized Facility–NIBSC, Potters Bar, UK). GHOST cells are derived from the human osteoscarcoma (HOS) cell line that expresses CD4 alone or with one of the chemokine receptors (CCR5, CXCR4, Bob, Bonzo) known to be used as coreceptor by primate lentiviruses. These cells also carry the gene for green fluorescent protein (GFP) under the control of the HIV-2 promoter, which is *trans*-activated by Tat following virus entry. Infected cells expressing GFP were enumerated by flow cytometry. GHOST cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, 500 μg Geneticin sulfate (Gibco-BRL) per milliliters and 100 μg hygromycin (Sigma) per milliliter. For cell lines expressing one of the chemokine receptors, culture medium was supplemented with 1 μg puromycin per milliliter. Cells were distributed in 12-well plates at 10^5 cells per well and cultured overnight. They were infected the following day for 1 h with SHIVsbg, SHIVsbg-YE, SIVmac239-YE, HIV-1 B$\times$08, and viruses isolated from SHIVsbg- YE-infected macaques. After 2 days of culture in DMEM + 10% FCS, cells were detached from the well by incubation with trypsin–EDTA, washed in PBS, resuspended in 500 μl 1.5% formaldehyde in PBS, and analyzed for GFP expression on a FACScan flow cytometer (Becton–Dickinson).

RT-PCR amplification

The sequences encoding Nef protein were amplified by RT-PCR from genomic RNA of SHIVsbg, SHIVsbg-YE, or viral isolates. Viral RNA was extracted by mixing 250 μl of serum with 750 μl Tri Reagent (Molecular Research Center Inc., Cincinati, OH). After 5 min, 200 μl of trichloroethane was added and 5 min later the aqueous phase was recovered after centrifugation. The viral RNA was isopropanol-precipitated, pelleted by centrifugation, and washed with 70% ethanol. The RNA pellet was solubilized in 25 μl water. Reverse transcription was carried out in 20 μl containing 35 pmol reverse primer NEF2 (5′- AGAACCTCCCAGGGCTCAATCT-3′), 2 μl of 10$\times$ PCR Buffer II (Perkin–Elmer Applied Biosystem Division, Foster City, CA), 5 mM $MgCl_2$, 1 mM dNTP, 20 U RNasin (Pharmacia Biotech, Uppsala, Sweden), and 50 U MuLV reverse transcriptase (Perkin–Elmer) and incubated for 30 min at 42°C.

The PCR amplifications were carried out in a DNA

thermal cycler (Perkin–Elmer) in 100 μl reaction volume consisting of 20 μl reverse-transcribed RNA, 75 mM Tris–HCl pH 9.0, 20 mM $(NH_4)_2SO_4$, 0.01% (w/v) Tween 20, 2.5 mM $MgCl_2$, 200 μM dNTP, 35 pmol OPEL sense primer (5′-CTTGTAGAGCTATTCGCCACATAC-3′), and 1.5 U of Gold Star DNA polymerase (Eurogentec). The reverse primer, already present in the sample, was not added again in the PCR mix. The amplification program comprised 5 min denaturation at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, and finally 8 min at 72°C.

The PCR products were separated on a 2% agarose gel and purified with the Wizard PCR preps DNA purification System (Promega Corp., Madison, WI) according to the manufacturer's specifications. The sequencing reactions were performed directly on the PCR products by Genome Express Co. (Grenoble, France) using the fluorescent dideoxynucleotide terminator protocol and analyzed on an automated 377 ABI Prism sequencer.

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References

Barré-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyre, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vézinet-Brun, F., Rouzioux, C., Rozenbaum, W., Montagnier, L., 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220, 868–871.

Bogers, W.M.J.M., Dubbes, R., ten Haaft, P., Niphuis, H., Cheng-Mayer, C., Stahl- Hennig, C., Hunsmann, G., Kuwata, T., Hayami, M., Jones, S., Ranjbar, S., Almond, N., Stott, J., Rosenwirth, B., Heeney, J.L., 1997. Comparison of in vitro and in vivo infectivity of different clade B HIV-1 envelope chimeric simian/human immunodeficiency viruses in *Macaca mulatta*. Virology 236, 110–117, doi:10.1006/viro.1997.8744.

Bresnahan, P.A., Yonemoto, W., Greene, W.C., 1999. Cutting edge: SIV Nef protein utilizes both leucine- and tyrosine-based protein sorting pathways for down-regulation of CD4. J. Immunol. 163, 2977–2981.

Burns, D.P.W., Desrosiers, R.C., 1991. Selection of genetic variants of simian immunodeficiency virus in persistently infected rhesus monkeys. J. Virol. 65, 1843–1854.

Cayabyab, M., Karlsson, G.B., Etemad-Moghadam, B.A., Hofmann, W., Steenbeke, T., Halloran, M., Fanton, J.W., Axthelm, M., Letvin, N.L., Sodroski, J., 1999. Changes in human immunodeficiency virus type 1 envelope glycoproteins responsible for the pathogenicity of a multiply passaged simian-human immunodeficiency virus (SHIV-HXBc2). J. Virol. 73, 976–984.

Chun, T.W., Carruth, L., Finzi, D., Shen, K., DiGiuseppe, J.A., Taylor, H., Hermankova, M., Chadwick, K., Margolick, J., Quinn, T.C., Kuo, Y.H., Brookmeyer, R., Zeiger, M.A., Barditch-Crovo, P., Siliciano, R.F.R., 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature 387, 183–188.

Clavel, F., Guétard, D., Brun-Vézinet, F., Chamaret, S., Rey, M.A., Santos-Ferreira, M.O., Laurent, A.G., Dauguet, C., Katlama, C., Rouzioux, C., Klatzmann, D., Champalimaud, J.L., Montagnier, L., 1986. Isolation of a new human retrovirus from west african patients with AIDS. Science 233, 343–346.

Dewhurst, S., Embretson, J.E., Anderson, D.C., Mullins, J.I., Fultz, P.N., 1990. Sequence analysis and acute pathogenicity of molecularly cloned $SIV_{SMM\text{-}PBj14}$. Nature 345, 636–640.

Du, Z., Ilyinskii, P.O., Sasseville, V.G., Newstein, M., Lackner, A.A., Desrosiers, R.C., 1996. Requirements for lymphocyte activation by unusual strains of simian immunodeficiency virus. J. Virol. 70, 4157–4161.

Du, Z., Lang, S.M., Sasseville, V.G., Lackner, A.A., Ilyinskii, P.O., Daniel, M.D., Jung, J.U., Desrosiers, R.C., 1995. Identification of a *nef* allele that causes lymphocyte activation and acute disease in macaque monkeys. Cell 82, 665–674.

Dunn, C.S., Beyer, C., Kieny, M.P., Gloeckler, L., Schmitt, D., Gut, J.P., Kirn, A., Aubertin, A.M., 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.

Endo, Y., Igarashi, T., Nishimura, Y., Buckler, C., Buckler-White, A., Plishka, R., Dimitrov, D.S., Martin, M.A., 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.

Fultz, P.N., 1991. Replication of an acutely lethal simian immunodeficiency virus activates and induces proliferation of lymphocytes. J. Virol. 65, 4902–4909.

Fultz, P.N., 1994. SIVsmmPBj14: an atypical lentivirus. Curr. Top. Microbiol. Immunol. 188, 65–76.

Fultz, P.N., McClure, H.M., Anderson, D.C., Switzer, W.M., 1989. Identification and biologic characterization of an acutely lethal variant of simian immunodeficiency virus from sooty mangabeys (SIV/SMM). AIDS Res. Hum. Retroviruses 5, 397–409.

Fultz, P.N., Schwiebert, R., Stallworth, J., 1995. AIDS-like disease following mucosal infection of pig-tailed macaques with SIVsmmPBj14. J. Med. Primatol. 24, 102–107.

Fultz, P.N., Zack, P.M., 1994. Unique lentivirus-host interactions: SIVsmmPBj14 infection of macaques. Virus Res. 32, 205–225.

Gummuluru, S., Novembre, F.J., Seshi, B., Dewhurst, S., 1996. $SIV_{smmPBj14}$ induces expression of a mucosal integrin on macaque lymphocytes. Virology 215, 97–100, doi:10.1006/viro.1996.0010.

Harouse, J.M., Gettie, A., How Tan, R.C., Blanchard, J., Cheng-Mayer, C., 1999. Distinct pathogenic sequela in rhesus macaques infected with CCR5 or CXCR4 utilizing SHIVs. Science 284, 816–819.

Ho, D.D., Neumann, A.U., Perelson, A.S., Chen, W., Leonard, J.M., Markowitz, M., 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373, 123–126.

Hofmann-Lehmann, R., Swenerton, R.K., Liska, V., Leutenegger, C.M., Lutz, H., McClure, H.M., Ruprecht, R., 2000. Sensitive and robust one-tube real-time reverse transcriptase-polymerase chain reaction to quantify SIV RNA load: comparison of one- versus two-enzyme systems. AIDS Res. Hum. Retroviruses 16, 1247–1257.

Igarashi, T., Kuwata, T., Takehisa, J., Ibuki, K., Shibata, R., Mukai, R., Komatsu, T., Adachi, A., Ido, E., Hayami, M., 1996. Genomic and biological alteration of a human immunodeficiency virus type 1 (HIV-1)-simian immunodeficiency virus strain mac chimera, with HIV-1 Env, recovered from a long-term carrier monkey. J. Gen. Virol. 77, 1649–1658.

Igarashi, T., Shibata, R., Hasebe, F., Ami, Y., Shinohara, K., Komatsu, T., Stahl-Hennig, C., Petry, H., Hunsmann, G., Kuwata, T., Jin, M., Adachi, A., Kurimura, T., Okada, M., Miura, T., Hayami, M., 1994. Persistent infection with SIVmac chimeric virus having *tat*, *rev*, *vpu*,

env and *nef* of HIV type 1 in macaque monkeys. AIDS Res. Hum. Retroviruses 10, 1021–1029.

Javaherian, K., Langlois, A.J., Schmidt, S., Kaufmann, M., Cates, N., Langedijk, J.P.M., Meloen, R.H., Desrosiers, R.C., Burns, D.P.W., Bolognesi, D.P., LaRosa, G.J., Putney, S.D., 1992. The principal neutralization determinant of simian immunodeficiency virus differs from that of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA 89, 1418–1422.

Joag, S.V., Li, Z., Foresman, L., Stephens, E.B., Zhao, L.-J., Adany, I., Pinson, D.M., McClure, H.M., 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.

Karlsson, G.B., Halloran, M., Li, J., Park, I.W., Gomila, R., Reimann, K.A., Axthelm, M.K., Iliff, S.A., Letvin, N.L., Sodroski, J., 1997. Characterization of molecularly cloned simian-human immunodeficiency viruses causing rapid CD4+ lymphocyte depletion in rhesus macaques. J. Virol. 71, 4218–4225.

Kestler, H., Kodama, T., Ringler, D., Marthas, M., Pedersen, N., Lackner, A., Regier, D., Sehgal, P., Daniel, M., King, N., Desrosiers, R., 1990. Induction of AIDS in rhesus monkeys by molecularly cloned simian immunodeficiency virus. Science 248, 1109–1112.

Kirchhoff, F., Carl, S., Sopper, S., Sauermann, U., Mätz-Rensing, K., Stahl-Hennig, C., 1999. Selection of the R17Y substitution in SIVmac239 Nef coincided with a dramatic increase in plasma viremia and rapid progression to death. Virology 254, 61–70, doi:10.1006/viro.1998.9522.

Kuwata, T., Shioda, T., Igarashi, T., Ido, E., Ibuki, K., Enose, Y., Stahl-Hennig, C., Hunsmann, G., Miura, T., Hayami, M., 1996. Chimeric viruses between SIVmac and various HIV-1 isolates have biological properties that are similar to those of the parental HIV- 1. AIDS 10, 331–1337.

Lewis, M.G., Zack, P.M., Elkins, W.R., Jahrling, P.B., 1992. Infection of rhesus and cynomolgus macaques with a rapidly fatal SIV ($SIV_{SMM/PBj}$) isolate from sooty mangabeys. AIDS Res. Hum. Retroviruses 8, 1631–1639.

Li, J.T., Halloran, M., Lord, C.I., Watson, A., Ranchalis, J., Fung, M., Letvin, N.L., Sodroski, J.G., 1995. Persistent infection of macaques with simian-human immunodeficiency viruses. J. Virol. 69, 7061–7071.

Li, J.T., Lord, C.I., Haseltine, W., Letvin, N.L., Sodroski, J.G., 1992. Infection of cynomolgus monkeys with a chimeric HIV-1/SIV_{mac} virus that expresses the HIV-1 envelope glycoproteins. J. Acquired Immune Defic. Syndr. 5, 639–646.

Lock, M., Greenberg, M.E., Iafrate, A.J., Swigut, T., Muench, J., Kirchhoff, F., Shohdy, N., Skowronski, J., 1999. Two elements target SIV Nef to the AP-2 clathrin adaptor complex, but only one is required for the induction of CD4 endocytosis. EMBO J. 18, 2722–2733.

Luciw, P.A., Pratt-Lowe, E., Shaw, K.E., Levy, J.A., Cheng-Mayer, C., 1995. Persistent infection of rhesus macaques with T-cell-line-tropic and macrophage-tropic clones of simian/human immunodeficiency viruses (SHIV). Proc. Natl. Acad. Sci. USA 92, 7490–7494.

Luo, W., Peterlin, B.M., 1997. Activation of the T-cell receptor signalling pathway by Nef from an aggressive strain of Simian Immunodeficiency Virus. J. Virol. 71, 9531–9537.

Mellors, J.W., Rinaldo Jr., C.R., Gupta, P., White, R.M., Todd, J.A., Kingsley, L.A., 1996. Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science 272, 1167–1170.

Moog, C., Wick, A., Le Ber, P., Kirn, A., Aubertin, A.M., 1994. Bicyclic imidazo derivatives, a new class of highly selective inhibitors for the human immunodeficiency virus type 1. Antiviral Res. 24, 275–288.

Naidu, Y.M., Kestler III, H.K., Li, Y., Butler, C.V., Silva, D.P., Schmidt, D.K., Troup, C.D., Sehgal, P.K., Sonigo, P., Daniel, M.D., Desrosiers, R.C., 1988. Characterisation of infectious molecular clones of simian immunodeficiency virus (SIV_{mac}) and human immunodeficiency virus type 2: persistent infection of rhesus monkeys with molecularly cloned SIV_{mac}. J. Virol. 62, 4691–4696.

Novembre, F.J., Johnson, P.R., Lewis, M.G., Anderson, D.C., Klumpp, S., McClure, H.M., Hirsch, V., 1993. Multiple viral determinants contribute to pathogenicity of the acutely lethal simian immunodeficiency virus SIV_{smmPBj} variant. J. Virol. 67, 2466–2474.

Novembre, F.J., Lewis, M.G., Saucier, M.M., Yalley-Ogunro, J., Brennan, T., McKinnon, K., Bellah, S., McClure, H.M., 1996. Deletion of the *nef* gene abrogates the ability of SIVsmmPBj to induce acutely lethal disease in pigtail macaques. AIDS Res. Hum. Retroviruses 12, 727–736.

Overbaugh, J., Rudensey, L.M., Papenhausen, M.D., Benveniste, R.E., Morton, W.R., 1991. Variation in simian immunodeficiency virus env is confined to V1 and V4 during progression to simian AIDS. J. Virol. 65, 7025–7031.

Piguet, V., Chen, Y.-L., Mangasarian, A., Foti, M., Carpentier, J.L., Trono, D., 1998. Mechanism of Nef-induced CD4 endocytosis: nef connects CD4 with the μ chain of adaptor complexes. EMBO J. 17, 2472–2481.

Regier, D.A., Desrosiers, R.C., 1990. The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. AIDS Res. Hum. Retroviruses 6, 1221–1231.

Reimann, K.A., Li, J.T., Veazey, R., Park, I.W., Karlsson, G.B., Sodroski, J., Letvin, N.L., 1996b. 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.

Reimann, K.A., Li, J.T., Voss, G., Lekutis, C., Tenner-Racz, K., Racz, P., Lin, W., Montefiori, D.C., Lee-Parritz, D.E., Lu, Y., Collman, R.G., Sodroski, J., Letvin, N.L., 1996a. An *env* gene derived from a primary human immunodeficiency virus type 1 isolate confers high in vivo replicative capacity to a chimeric simian/human immunodeficiency virus in rhesus monkeys. J. Virol. 70, 3198–3206.

Sakuragi, S., Shibata, R., Murai, R., Kotmatsu, T., Fukasawa, M., Sakai, H., Sakuragi, J.I., Kawamura, M., Ibuki, K., Hayami, M., Adachi, A., 1992. Infection of macaque monkey with a chimeric human and simian immunodeficiency virus. J. Gen. Virol. 73, 2983–2987.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning—A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sanchez, G., Xu, X., Chermann, J.C., Hirsch, I., 1997. Accumulation of defective viral genomes in peripheral blood mononuclear cells of human immunodeficiency virus type-1 infected individuals. J. Virol. 71, 2233–2240.

Sasseville, V.G., Du, Z., Chalifoux, L.V., Pauley, D.R., Young, H.L., Sehgal, P.K., Desrosiers, R.C., Lackner, A.A., 1996. Induction of lymphocyte proliferation and severe gastrointestinal disease in macaques by a nef gene variant of SIVmac239. Am. J. Pathol. 149, 163–176.

Schwiebert, R.S., Tao, B., Fultz, P.N., 1997. Loss of the $SIV_{smmPBj14}$ phenotype and *nef* genotype during long-term survival of macaques infected by mucosal routes. Virology 230, 82–92, doi:10.1006/viro.1997.8469.

Shibata, R., Kawamura, M., Sakai, H., Hayami, M., Ishimoto, A., Adachi, A., 1991. Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells. J. Virol. 65, 3514–3520.

Shibata, R., Maldarelli, F., Siemon, C., Matano, T., Parta, M., Miller, G., Fredrickson, T., Martin, M.A., 1997. Infection and pathogenicity of chimeric simian human immunodeficiency viruses in macaques: determinant of high virus loads and CD4 cell killing. J. Infect. Dis. 176, 362–376.

Stephens, E.B., Joag, S.V., Sheffer, D., Liu, Z.Q., Mukherjee, S., Foresman, L., Adany, I., Li, Z., Pinson, D., Narayan, O., 1996. Initial characterization of viral sequences from a SHIV-inoculated pig-tailed macaque that developed AIDS. J. Med. Primatol. 25, 175–185.

Stephens, E.B., Mukherjee, S., Liu, Z.Q., Sheffer, D., Lam-Warton, R., Leung, K., Zhuge, W., Joag, S.V., Li, Z., Foresman, L., Adany, I., Narayan, O., 1998. Simian-human immunodeficiency virus (SHIV)

containing the *nef*/long terminal repeat region of the highly virulent SIV_{smm}PBj14 causes PBj-like activation of cultured resting peripheral blood mononuclear cells, but the chimera showed no increase in virulence. J. Virol. 72, 5207–5214.

Tao, B., Fultz, P.N., 1995. Molecular and biological analyses of quasispecies during evolution of a virulent simian immunodeficiency virus, SIVsmmPBj14. J. Virol. 69, 2031–2037.

Veazey, R.S., De Maria, M.A., Chalifoux, L.V., Shvetz, D.E., Pauley, D.R., Knight, H.L., Rosenzweig, M., Johnson, R.P., Desrosiers, R.C., Lackner, A.A., 1998. Gastrointestinal tract as a major site of CD4+ T cell depletion and viral replication in SIV infection. Science 280, 427–431.

Veazey, R.S., Lackner, A.A., 1998. The gastrointestinal tract and the pathogenesis of AIDS. AIDS 12, S35–S42.

Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., Alizon, M., 1985. Nucleotide sequence of the AIDS virus, LAV. Cell 40, 9–17.

Wei, X., Ghosh, S.K., Taylor, M.E., Johnson, V.A., Emini, E.A., Deutsch, P., Lifson, J.D., Bonhoeffer, S., Nowak, M.A., Hahn, B.H., Saag, M.S., Shaw, G.M., 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature 373, 117–122.

Whetter, L., Novembre, F.J., Saucier, M., Gummuluru, S., Dewhurst, S., 1998. Co- stimulation pathways in lymphocyte proliferation induced by the simian immunodeficiency virus SIVsmmPBj14. J. Virol. 72, 6155–6158.