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SHIV89.6P pathogenicity in cynomolgus monkeys and control of viral replication and disease onset by human immunodeficiency virus type 1 Tat vaccine

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Abstract: The Tat protein of human immunodeficiency virus (HIV) is produced very early after infection, plays a key role in the virus life cycle and in acquired immunodeficiency syndrome (AIDS) pathogenesis, is immunogenic and well conserved among all virus clades. Notably, a Tat-specific immune response correlates with non-progression to AIDS. Here, we show that a vaccine based on the Tat protein of HIV blocks primary infection with the simian/human immunodeficiency virus (SHIV)89.6P and prevents the CD4 T cell decline and disease onset in cynomolgus monkeys. No signs of virus replication were found in five out of seven vaccinated macaques for almost 1 year of follow-up. Since the inoculated virus (derived from rhesus or from cynomolgus macaques) is shown to be highly pathogenic in cynomolgus macaques, the results indicate efficacy of Tat vaccination in protection against highly pathogenic virus challenge. Finally, the studies of the Tat-specific immunological responses indicate a correlation of protection with a cytotoxic T cell response. Thus, a Tat-based vaccine is a promising candidate for preventive and therapeutic vaccination in humans.

Key words: correlation – CTL – macaque – preventive – therapeutic

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Introduction

Most acquired immunodeficiency syndrome (AIDS) vaccine strategies are aimed at inducing sterilizing immunity [1, 11] by blocking virus entry with neutralizing antibodies directed against the human immunodeficiency virus (HIV)-1 envelope (*env*) protein. However, such strategies have failed to protect against heterologous viruses because of the Env strain variability [32, 37, 54]. Vaccines with live attenuated viruses have provided the best protection data against heterologous virus challenge in non-human primate models [1, 19, 37, 57]. However, very recent data indicating limited pro-

tection against some of the heterologous challenges [40, 64] and the appearance of reverent pathogenic viruses [4, 25, 62] hamper their use in humans, at least in their current form. This has led to the concept that the control of viral infection and the block of disease onset may be a more achievable goal to develop a vaccine against AIDS. Such a vaccine should use a viral component that plays a key role in the virus life cycle, is immunogenic and is conserved at least in its immunodominant epitopes.

We chose to target the Tat protein of HIV since it possesses all these characteristics.

Tat is produced early after infection and is essential for virus replication and infectivity [3, 17, 29]. During acute infection of T cells by HIV, Tat is released into the extracellular milieu and is taken up by infected cells where it trans-activates virus gene expression and replication [5, 16, 22, 23, 30], and by uninfected cells where it favours the transmission of both macrophage-tropic and T lymphocyte-tropic HIV-1 strains by inducing the expression of the chemokine receptors, CCR5 and CXCR4 [34, 42]. Tat is also involved in the pathogenesis of AIDS and AIDS-associated malignancies such as Kaposi's sarcoma (KS) which is the most frequent tumor in AIDS patients [5–8, 22, 24, 41, 55, 60, 61, 68].

In the natural infection, the presence of a humoral immune response against Tat correlates with the control of disease progression [48, 50, 52, 67]. Anti-Tat antibodies can, in fact, inhibit the uptake and, therefore, the effect of extracellular Tat on HIV replication and virus transmission [23, 48] and its immunosuppressive effects on T cells [65]. Similarly, an early anti-Tat cytotoxic T cell response (CTLs) is present in HIV-1 infected long-term non-progressors and inversely correlates with the progression to the symptomatic stage of the infection [31, 58, 59]. Of note, Tat is a rare example of a soluble protein that can be very efficiently taken up by antigen-presenting cells (Barillari et al., in preparation) and can induce $CD8^+$ T cell-mediated CTL-responses by entering the major histocompatibility complex (MHC) class I pathway [38]. Finally, Tat is a well-conserved protein, particularly in its immunogenic epitopes, among the different HIV-1 clades with the exception of the O subtype [43] (S. Buttò, unpublished data). Altogether, these data suggest that a Tat-based vaccine may control virus replication and transmission and can block disease onset and/or progression.

We have recently shown that a subunit vaccine consisting of a biologically active HIV-1 Tat protein can control replication of the highly pathogenic simian/human immunodeficiency virus (SHIV) 89.6P to undetectable levels in most (five out of seven) of the vaccinated macaques, preventing the $CD4^+$ T cell decline and disease onset [12]. Similarly, a *tat-rev* vaccine has been shown to protect rhesus macaques from pathogenic simian immunodeficiency virus (SIV) challenge [44].

Protection correlated with the anti-Tat CD8-mediated cytotoxic T cell response and Th-1 cytokine production that were present in all protected animals [12]. These data are consistent with results in humans indicating that an early immune response to Tat is present in long-term non-progressors and correlates with non-progression to AIDS [31, 48, 50, 52, 58, 66] (our unpublished data).

Here, we extended the analysis of the vaccinated macaques up to about 1 year post-challenge, and confirmed the control of infection in five out of seven monkeys. In addition, detailed data are presented on the pathogenicity of the SHIV89.6P utilized for the challenge, a critical issue for the relevance of the protection observed.

Materials and methods

Monkeys

Adult cynomolgus monkeys (*Macaca fascicularis*) were housed in single cages and were clinically examined. Weight and rectal temperature was measured while they were under ketamine hydrochloride anaesthesia (10 mg/kg). Blood samples for hematological, immunological and virological analysis were obtained in the morning prior to food administration.

HIV-1 Tat protein expression, purification and inoculation

HIV-1 Tat from the human T lymphotropic virus type IIIB (HTLV-IIIB) isolate (subtype B) was expressed in *Escherichia coli* and purified to homogeneity by heparin-affinity chromatography and high pressure liquid chromatography (HPLC) as described previously [16]. The purified Tat protein was $>95\%$ pure as tested by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and had full biological activity as tested by the rescue assays [16, 23] and by the proliferation of KS cells [22]. The Tat protein was stored lyophilized at $-80°C$. Tat used for *in vitro* studies was resuspended in degassed buffer before use as described [16, 23]. The plasticware was previously rinsed in phosphate buffered saline-bovine serum albumine (PBS-BSA) or in RPMI-1640 supplemented with antibiotics, 2 mM L-glutamine and containing 10% fetal calf serum. In addition, since Tat is also photo- and thermo-sensitive [23], the handling of the protein was performed in the dark

and on ice. For the injection in vaccinees, lyophilized Tat was resuspended in 100 μl of saline containing 20% of autologous serum at the concentration of 4 mg/ml (stock). Additional dilutions were made in the same buffer. For skin tests, 10 μg of Tat were diluted in 200 μl of saline. Before use, the plasticware, syringes and needles were rinsed in saline containing 20% of autologous serum to prevent Tat from sticking to their surfaces.

Adjuvants

RIBI adjuvant [containing monophosphoryl lipid A, trehalose dimycolate, cell wall skeleton in oil (squalene) and Tween 80], was obtained through the European Concerted Action on Macaque models for AIDS Research from the AIDS Reagent Project, National Institute for Biological Standards and Control (Potters Bar, UK). Aluminium phosphate (alum) was a gift from P. Frezza (Hardis Ltd., Naples, Italy). ISCOMs containing the Tat protein (80 μg/ml of ISCOMs) were prepared with a previously described procedure [20]. ELISA and western blot analysis were performed to confirm the co-localization of the ISCOMs with the Tat protein (data not shown).

Detection of anti-Tat antibodies

Polyvinyl chloride microtiter plates were coated with Tat (100 ng in 200 μl/well of 0.05 M carbonate buffer, pH 9.6) for 12 hours at 4°C. Plates were then extensively washed with PBS without Ca^{2+} and Mg^{2+} containing 0.05% Tween 20 (PBS/Tween) and 200 μl of plasma, diluted (in the buffer) as indicated, were added to each well in duplicate. Plates were incubated for 90 minutes at 37°C, washed five times with PBS/Tween and 100 μl of the horseradish peroxidase-conjugated secondary antibody (Sigma, St Louis, MO) diluted 1:1000 in PBS/Tween (containing 1% BSA) was added for an additional 90 minutes at 37°C. After extensive washing, 100 μl of peroxidase substrate (ABTS 1 mM, Amersham Pharmacia Biotech, Milan, Italy) was added to each well and the optical density (OD, 405 nm) was read with a spectrophotometer. A rabbit polyclonal anti-Tat serum was used at serial 2-fold dilutions (1:200–1:6,400) as the positive control, whereas the pre-immune plasma (diluted 1:50 and 1:100) from each monkey constituted the negative controls. The mean of the negative controls + 3 standard deviations (SD) represented the cut-off value in each test. The minimal plasma dilution used was 1:50.

Neutralization of Tat activity on HIV replication by the rescue assay

Tat activity was measured by the rescue assay in which the replication of *tat*-defective HIV-1 proviruses is induced by serial concentrations of exogenous Tat protein added to HLM-1 cells (HeLa $CD4^+$ cells containing a *tat*-defective HIV-1 provirus) as described previously [5, 16, 23]. Three hundred microlitres of growth medium containing either pre-immune or immune plasma were added to each well and plates were incubated for 48 hours. Supernatants were then collected and p24 Gag content determined by an antigen capture assay (NEN Life Science Inc., Boston, MA). All samples were tested in duplicate.

Inhibition of *in vitro* SHIV89.6P acute infection by plasma

CEMx174 cells (obtained from AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) (3×10^4/well) were infected (2 hours at 37°C) with the SHIV89.6P [5×10^{-5} median tissue culture infectious doses (TCID50)/cell]. After two washings in growth medium, cells were resuspended in 150 μl of medium containing 5% of heat-inactivated pre-immune or immune plasma (in duplicate). Supernatants were collected at day 7 after infection. Inhibition of virus replication was measured by p27 SIV-Gag antigen capture ELISA (Coulter International, Miami, FL) and results were expressed as the mean of the p27 SIV-Gag values (pg/ml). A 90% reduction of p27 production, as compared to the pre-immune plasma, was considered indicative of a blocking activity.

Lymphoproliferative responses

Two $\times 10^5$ peripheral blood mononuclear cells (PBMCs), resuspended in 100 μl of growth medium, were plated in triplicate in flat-bottomed 96-well plates. One hundred microlitres of each stimulus [medium control; phytohemagglutinin (PHA), 2 μg/ml; tetanus toxoid (TT), 5 μg/ml; Tat, 5 μg/ml] were added to triplicate wells. After 5 days of culture, 1 μg of ^{3}[H]-thymidine was added to each well and samples were harvested 16 hours later for scintillation counting with a Betaplate (Wallac, Turku, Finland). A stimulation index greater than three was considered positive. All monkeys responded to PHA.

CTL assay

CTL assays were performed as described [12]. PBMCs were seeded (5×10^6/well in 0.5 ml of

complete medium) in a 24-well plate with Tat (1 μg). One day later, 5×10^6 PBMCs were incubated for 3 hours with Tat (1 μg), washed twice and added to the wells containing the PBMCs stimulated previously. On day 2, 2 IU of recombinant human interleukin (rhIL)-2 were added to the wells. Half of the supernatant was replaced with medium containing rhIL-2 twice per week. On day 14, cells were harvested, counted, resuspended in growth medium containing 1 mM sulfinpyrazone (Sigma) and seeded (96-well round-bottom plate) at serial 2-fold dilutions (in duplicate) (Effectors). The day before the assay, herpesvirus papio-transformed autologous B-lymphocytes (BLCL) [18] were pulsed overnight with or without Tat (4 μg/10^6 cells) (Targets), labeled with the Fluorescence Enhancing Ligand (BATDA) according to the manufacturer's instructions (Delfia, Wallac) [26], and 5×10^3 cells per well added to the Effectors. After 2 hours, 20 μl of supernatants was mixed with 200 μl of the Europium solution and fluorescence measured after 20 minutes with a time-resolved fluorescence reader (Victor, Wallac). The percent specific lysis was calculated for each E:T ratio as follows: (test release − spontaneous release)/(maximum release − spontaneous release) × 100. The % specific lysis against unpulsed autologous BLCL was calculated and subtracted from the % specific lysis against the Tat-pulsed targets. The assay was considered positive for values exceeding 5%. This cut-off was determined by experiments in which the cytolytic activity of $CD8^+$ T cells was measured against targets labeled in parallel with Europium or ^{51}Chromium. The results demonstrated that the time-resolved fluorescence method has a 50% lower sensitivity than the radioactive assay (R. Gavioli and F. Micheletti, Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm, Sweden and Department of Biochemistry and Molecular Biology, University of Ferrara, Italy, unpublished data). Accordingly, the cut-off of 10% lysis commonly used for ^{51}Chromium release corresponded to a 5% value with the Europium method.

Determination of tumor necrosis factor (TNF)-α production from PBMCs

TNF-α production was measured by seeding in duplicate 10^6 PBMCs in a 24-well plate in 1 ml of complete medium. Cells were then stimulated with PHA (2 μg/ml) (PHA-P, Sigma-Aldrich, Srl) or Tat (5 μg/ml). After 2 days, 100 μl of the cell supernatants were harvested and TNF-α production was measured by ELISA (Cytoscreen Monkey for TNF-α, Biosource International, Camarillo, CA) as indicated by the manufacturer, and as described previously [12].

Skin tests to TT and Tat

For both TT and Tat skin tests, 150 μl of PBS containing 0.1% BSA plus either TT (7 μg) or Tat (5 or 1 μg) were injected intradermally (ID) on a previously thricotomized area of the upper back. Buffer alone was used as the negative control. Monkeys were monitored at 24, 48 and 72 hours for the appearance of erythema at the site of inoculation. The skin test was considered strongly positive (++) or positive (+) when an induration was appreciated and the diameter of the erythematous area was ≥ 5 or 1–4 mm, respectively. Erythema without induration was considered a weakly positive (±) response, whereas an erythematous area < 1 mm was considered negative (−).

Generation of the SHIV89.6P virus stock, and *in vivo* and *in vitro* titration

The parental SHIV89.6P [36, 49], obtained from Dr N. Letvin (Harvard Medical School, Boston, MA), was expanded *in vivo* in a *M. fascicularis* [12]. Virus pathogenicity in cynomolgus and the 50% monkey infectious dose (MID_{50}) were determined in a previous study [12]. Briefly, the original virus stock obtained from the rhesus monkey and the virus stock obtained from the cynomolgus macaque were inoculated into six (range of 50–5 MID_{50}) and eight (dilutions range 1:50–1:5,000,000 corresponding, retrospectively, to a range of 28,520–0.285 MID_{50}) monkeys, respectively.

p27 determination in plasma

Levels of p27 SIV-Gag protein were measured in plasma by using an antigen capture ELISA assay (Innotest, Innogenetics, Wwijndrecht, Belgium), as described [12].

Quantitation of the SHIV RNA copies in plasma

Quantitation of SHIV89.6P RNA copies was performed in the Bayer-Chiron Diagnostics Reference Testing Laboratory (Amsterdam, The Netherlands) by a branched DNA signal amplification assay (bDNA) recognizing the *pol* region of the SIVmac strains, as described [12], with a cut-off of 1,500 RNA copies/ml. Results below 10,000 RNA copies/ml were confirmed by a quantitative competitive RNA-polymerase chain reaction (RNA-PCR) as described previously [56].

Proviral DNA detection

SIV proviral copy number was determined in DNA extracted from whole blood (QIAamp Blood Kit, Qiagen GmbH, Hilden, Germany) by a semi-quantitative DNA-PCR amplifying a 496-bp region of the *gag* gene of SIVmac251, as described previously [12]. The lower limit of detection was one SIV proviral copy/μg of DNA. Samples containing ten SIV proviral copies were always positive.

Virus isolation and cell associated viral load (cytoviremia)

For virus isolation, $CD8^+$ T cell depleted PBMCs (3×10^6) were cocultured with 1×10^6 human CEMx174 cells after PHA and rhIL-2 (10 IU/ml) stimulation, as described previously [12]. Virus isolation was also performed by culturing CD8-depleted PBMCs for 3 weeks in 96-well plates previously coated with anti-CD3 monoclonal antibodies (2.5 μg/ml, clone FN-18, Biosource International) in the presence of rhIL-2 (10 IU/ml). Culture supernatants from both methods were tested for p27 production twice per week.

Cytoviremia was determined in CD8-depleted PBMCs by a limiting dilution microtiter assay. Serial 2-fold dilutions of the cell suspension (from 10^6 to 4.8×10^2 cells/well in duplicate, standard in quadruplicate), were cocultured with CEMx174 cells (10^4 cells/well) and scored for the presence of p27 antigen production. The 50% endpoints were calculated using the method of Reed and Muench and the results expressed as the number of infected cells per 10^6 PBMCs.

Anti-SIV and anti HIV-Env Ab titers and *in vitro* antibody production (IVAP)

Since there is a high sequence homology between HIV-2 and SIV, antibody titers to SIV were determined by end-point dilution using an HIV-2 ELISA assay (Elavia Ac-Ab-Ak II kit, Diagnostic Pasteur, Paris, France). Ab titers against the HIV-1 Env were by an HIV-1 ELISA assay (HIV-1/HIV-2 Third Generation Plus, Abbott, Chicago, IL). The mean of the negative controls + 3 SD represented the cut-off value.

For IVAP, 2×10^6 PBMCs/well were seeded onto a 24-well culture plate, stimulated with 2 μg/ml of pokeweed mitogen (Sigma) [26] and incubated for 7 days at 37°C, 5% CO_2 and 100% humidity. On day 7, the culture supernatants were collected, centrifuged (5,000 rpm for 10 minutes) and used for anti-HIV antibody detection by ELISA (Abbott, HIV-1/HIV-2 EIA Third Generation Plus).

Determination of the $CD4^+$ and $CD8^+$ T cell counts

Phycoerythrin (PE)-conjugated anti-CD4 (Biosource International) and peridinin chlorophyll protein (PerCP)-conjugated anti-CD8 monoclonal antibodies (Becton-Dickinson, Mountain View, CA) were used for staining citrated peripheral blood. Stained cells were then analyzed with a FACScan cytometer and software (Becton-Dickinson) as described previously [12]. Isotype matched PE- and PerCP-labelled mouse monoclonal antibodies were used to determine background fluorescence. Absolute cell numbers were calculated from the blood cell counts.

Results

Safety and immunogenicity of Tat vaccine

Six cynomolgus monkeys (*M. fascicularis*) were immunized subcutaneously (SC) with 10 μg of Tat and the adjuvant RIBI (three monkeys) or alum (three monkeys), and one animal with Tat (6 μg) ID, in the absence of adjuvants. Boosts were given at 2, 6, 11, 15, 21, 28, 32 weeks after the first immunization and the last boost was given intramuscularly at week 36 with Tat in immune-stimulating complexes (16 μg/200 μl) [20], except for the monkey vaccinated with Tat ID that did not receive this last boost. Two monkeys served as controls and were injected with RIBI or alum alone, respectively. Moreover, at the time of the viral challenge an additional monkey (naive) was added to the protocol as an additional control for the infectivity of the virus inoculum.

No toxicity (acute or chronic; local or systemic) was ever detected in the vaccinated animals throughout the immunization period. Vaccination with Tat induced both humoral and cellular anti-Tat specific responses (summarized in Table 1).

The six monkeys inoculated with Tat and RIBI or alum seroconverted by week 6 after the first immunization and the antibody titres increased up to 1:25,600 in the monkeys immunized with Tat and alum, and up to 1:12,800 in the monkeys immunized with Tat and RIBI, respectively, and remained stable over time. In contrast, the animal given Tat ID developed low and transient titres of anti-Tat antibodies (1:100), that were detected only up to 32 weeks after immunization. In addition, plasma from the monkeys inoculated with Tat and RIBI or alum were capable of neutralizing the activity of increasing concentrations of exogenous Tat protein (60–500 ng/ml), as compared to the pre-immune plasma, in a test of rescue of Tat-defective proviruses induced by the addition of exogenous Tat and measured by determining p24

antigen production in the cell supernatants [5, 16, 23]. Furthermore, plasma from three vaccinated monkeys (given RIBI or alum and Tat) were capable of blocking the replication of the SHIV89.6P after *in vitro* acute infection of CEMx174 cells. In both assays, inhibition correlated with the anti-Tat antibody titres (Table 1).

Tat vaccination also induced a specific cellular immune response. In particular, all the monkeys vaccinated with Tat and RIBI or alum showed a Tat-specific proliferation, whereas no response was detected in the macaque vaccinated ID with Tat alone and in the control animals (Table 1). The lymphoproliferative response to TT, used as a control since all animals had been vaccinated with TT, was always present (data not shown).

In the vaccinated monkeys, a specific anti-Tat CTL activity began to be detectable at week 28 after immunization and increased to higher levels at week 36. This occurred in one of the two macaques tested that were vaccinated with Tat and RIBI, in two of the three monkeys that were vaccinated with Tat and alum and in the monkey vaccinated with Tat alone ID (Table 1). Since anti-Tat CTLs generally do not show high levels of killing, as compared to CTLs directed against structural proteins, these data were confirmed by the production of TNF-α after stimulation of PBMCs with Tat. Only the animals that showed an anti-Tat CTL activity also produced TNF-α upon Tat stimulation, whereas monkeys that were negative for CTL activity produced TNF-α upon PHA but not Tat stimulation (Table 1). In addition, cell fractionation studies indicated that the main source of TNF-α (about 90%) was the $CD8^+$ T cell fraction, confirming the presence of $CD8^+$ anti-Tat CTLs [12].

SHIV89.6P pathogenicity in cynomolgus monkeys

The SHIV89.6P was chosen because it is highly pathogenic in macaques and because it contains the tat gene of HIV-1 in the SIV genome backbone [49]. Since the virus was previously prepared and titrated by Dr Letvin in rhesus macaques, prior to challenge of our animals, the original viral stock (obtained from Dr Letvin's laboratory) was inoculated into six cynomolgus macaques to assess its pathogenicity in that species. A viral stock was then prepared from another cynomolgus monkey and used to infect eight additional cynomolgus monkeys, to titrate it and to determine the MID_{50} to be used for the challenge. High levels of viral replication indicated by p27 antigenemia, plasma viremia, proviral DNA, anti-SIV antibody titers, and a profound and persistent decrease in CD4 T cell counts were observed in all monkeys (except for the animal inoculated with 1:5,000,000 dilution of the virus stock corresponding to 0.285 MID_{50}) independently from the virus stock utilized, and no differences were found when these data were com-

Table 1. Summary of the immunological responses to Tat in vaccinated monkeys

		Pre-challenge						
Mk	Vaccination	Ab titers[1]	Neutralization[2]	SHIV inhibition[3]	Prolif. response[4]	DTH[5]	CTL[6]	TNF-α induction[7]
54844	RIBI+Tat (10 μg, SC)	12,800	++	NT	+	−	+	+
54879	RIBI+Tat (10 μg, SC)	12,800	+++	NT	+	+	NT	+
54963	RIBI+Tat (10 μg, SC)	12,800	++++	+	+++	±	−	NT
54899	Alum+Tat (10 μg, SC)	25,600	++++	+	+++	++	+	+
55396	Alum+Tat (10 μg, SC)	25,600	++++	+	+	++	−	−
55240	Alum+Tat (10 μg, SC)	25,600	++++	NT	+	±	+	+
54222	Tat (6 μg, ID)	100	NT	NT	−	−	+	+
55123	Control RIBI	0	NT	NT	−	NT	−	−
55129	Control alum	0	NT	NT	−	NT	−	−

[1] Reciprocal of the last positive dilution by ELISA (cut-off: mean of pre-immune sera +3 SD).
[2] Neutralizing index measured as the capability of sera to block (>50%) the rescue of *tat*-defective provirus by the addition of serial concentrations of Tat protein: +, 60 μg/ml; ++, 120 μg/ml; +++, 240 μg/ml; ++++, 500 μg/ml.
[3] Inhibition of replication, (measured as p27-Gag content in the culture supernatant) of the SHIV89.6P after *in vitro* acute infection of CEMx174 cells, by plasma (final dilution 1:20) from vaccinated animals.
[4] Stimulation index (ratio between Tat-specific and the control proliferative response): −, <3; +, 3–10; ++ , 11–30; +++, >30.
[5] Tat-skin test, ∅ measured at 48 hrs: −, <1 mm , +/−, erythema without induration; +, 1–4 mm; ++, ≥5 mm.
[6] Specific killing (cut-off: 5%).
[7] TNF-α production from PBMCs; values represent the average of duplicate wells. Values below cut-off (15.6 pg/ml) were given a value of 0.
NT, not tested; SC, subcutaneously; ID, intradermally.

Table 2. Detection of proviral DNA (copies/μg of DNA) after intravenous infection of cynomolgus monkeys with SHIV89.6P grown in rhesus or cynomolgus macaques

Monkey		Inoculum (MID_{50})	Week post-infection								
			2	4–5	7–8	10–12	14–16	18–19	23–24	28–35	>35
SHIV89.6P (cynomolgus → cynomolgus)											
193	1:50[1]	28,520[2]	NT	78	3	27 (died)[3]					
8112	1:500	2,852	NT	782	68	52	41	57 (died)[3]			
3V	1:5,000	285.2	NT	160	61	48	71	93	NT	NT	NT
8106	1:5,000	285.2	NT	967	NT	52	78	114	NT	NT	Died[3] (81 WPI)
5	1:50,000	28.52	NT	118	32	25	95	69 (died)[3]			
2	1:50,000	28.52	NT	1,516	744	1,099	135	160	NT	88	Died[3] (46 WPI)
12	1:500,000	2.852	NT	136	43	55	24	56	43	11	18

[1] Virus dilution.
[2] Retrospective calculation of the infectious doses.
[3] Death or euthanasia because of severe worsening of clinical conditions.
NT, not tested. WPI, week post-infection.
Shown are the number of proviral DNA copies (copies/μg of DNA) present in PBMCs from cynomolgus monkeys infected with inocula of different sizes (range 50–5 MID_{50}) of the SHIV89.6P virus stock obtained from the same laboratory (rhesus → cynomolgus), and from cynomolgus macaques infected with inocula of different sizes (range 28,520–2.8 MID_{50}) of the same SHIV89.6P virus stock but after passage in a cynomolgus monkey (cynomolgus → cynomolgus).

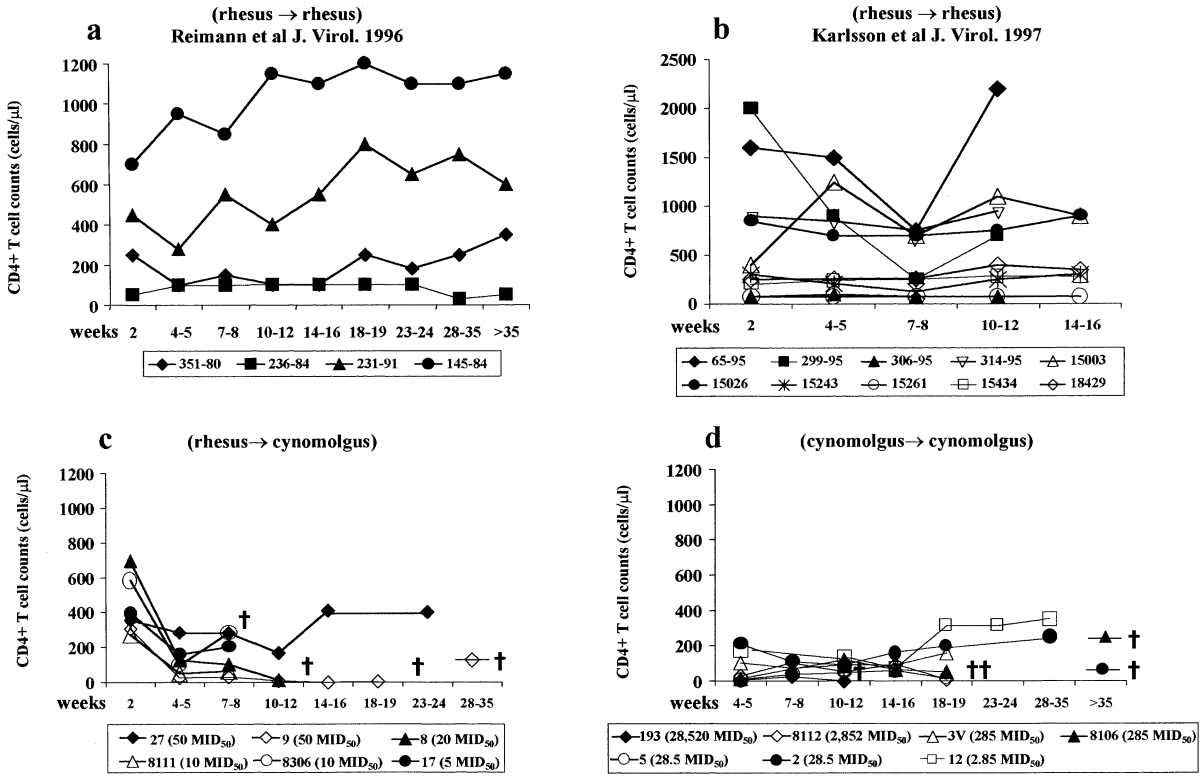

Fig. 1. CD4 T cell counts per μl in rhesus or cynomolgus macaques inoculated intravenously with SHIV89.6P virus stocks derived from a rhesus or a cynomolgus monkey. Shown are the CD4 T cell counts in (a) rhesus monkeys infected (inoculum size unknown) with a SHIV89.6P grown in a rhesus macaque (rhesus → rhesus) as reported in Reimann et al. [49]; (b) rhesus monkeys infected (inoculum size unknown) with SHIV89.6P molecular clones (rhesus → rhesus) as reported in Karlsson et al. [36]; (c) cynomolgus monkeys infected with inocula of different sizes (range 50–5 MID_{50}) of the SHIV89.6P virus stock from a rhesus macaque obtained from the same laboratory, and (d) cynomolgus macaques infected with inocula of different sizes (range 28,520–2.8 MID_{50}) of the same SHIV89.6P virus stock but after passage in a cynomolgus monkey (cynomolgus → cynomolgus). The † indicates the time when a monkey died or was euthanized due to worsening of the clinical conditions.

pared to those already published, including the rate of animal death [12, 36, 49] (Table 2, Figs. 1–3).

Specifically, as shown in Fig. 1, rhesus macaques inoculated with the original viral stock derived from a rhesus monkey (Fig. 1a) [49] or with the SHIV89.6P molecular clones also derived from rhesus (Fig. 1b) [36] showed a decrease in the number of CD4 T cells over time that was similar to that observed after the inoculation of cynomolgus monkeys with either the original SHIV89.6P virus stock from rhesus (Fig. 1c) or the virus stock that we prepared after passage in a cynomolgus monkey (Fig. 1d). Of note, in both the latter groups of animals CD4 T cells showed a decline even more profound and prolonged than that observed in the previous studies (compare Fig. 1c and d to a and b), suggesting a higher pathogenicity of the original virus stock in cynomolgus monkeys.

Similarly, as shown in Fig. 2, the levels of plasma viremia found in rhesus monkeys inoculated with the original virus stock [49] (Fig. 2a) were in the same range or lower than the values detected in the cynomolgus macaques inoculated with either the original SHIV89.6P stock from rhesus (Fig. 2b) or with the virus stock derived from a cynomolgus monkey (Fig. 2c), which were often higher than five logs, a limit that some authors consider characteristic of very pathogenic viruses.

Moreover, by using the molecular clones from the SHIV89.6P [36], a peak of p27 in plasma (plasma viremia data were not shown) was observed within the first 2 weeks (Fig. 3a) as observed in our infected monkeys (Fig. 3b and c).

In addition to these data, the pathogenicity in cynomolgus macaques of the SHIV89.6P stock derived from rhesus or the virus stock derived from cynomolgus was also confirmed by quantitative proviral DNA-PCR (Table 2) and repeated virus isolation (data not shown).

The most convincing evidence of the high pathogenicity in cynomolgus monkeys of both the SHIV89.6P stock from rhesus and the virus stock

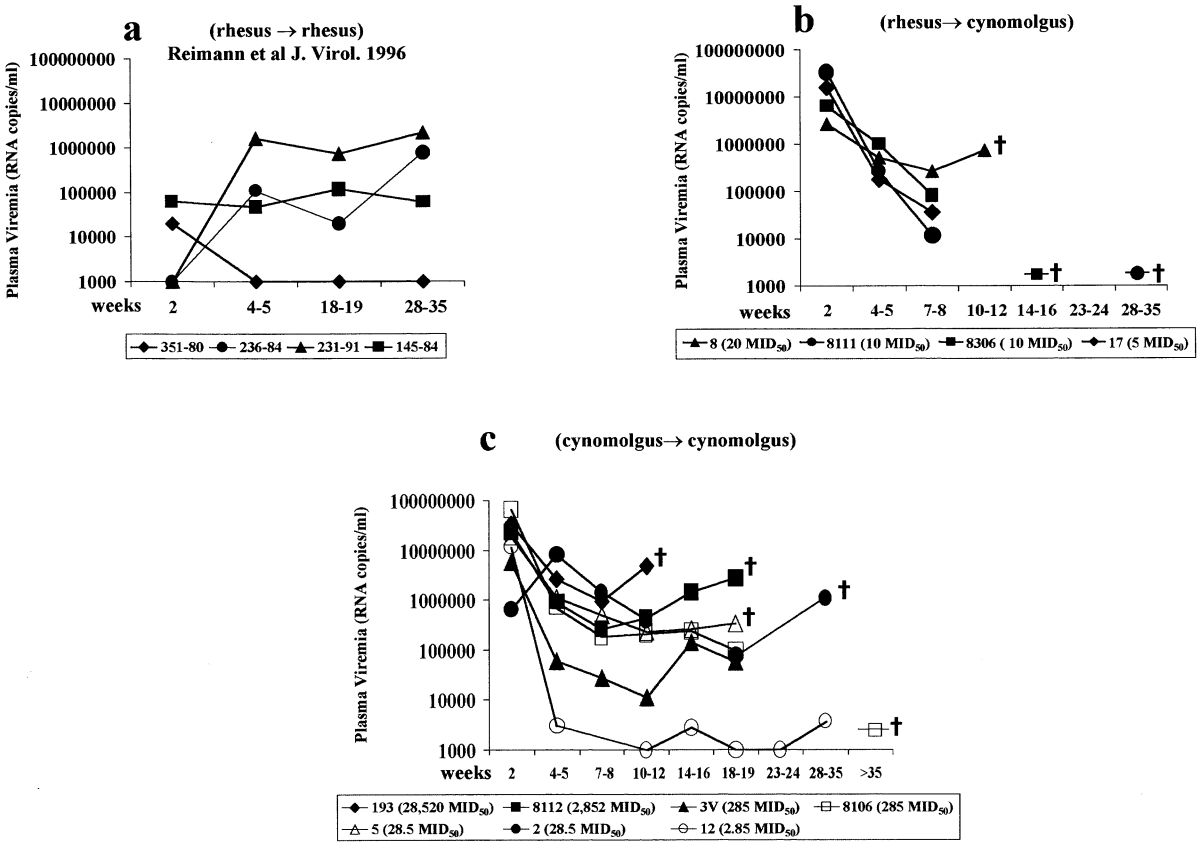

Fig. 2. Plasma viremia (RNA copies/ml) in rhesus or cynomolgus macaques inoculated intravenously with SHIV89.6P virus stock derived from a rhesus or cynomolgus monkey. Plasma viremia is shown for (a) rhesus monkeys infected (inoculum size unknown) with a SHIV89.6P grown in a rhesus macaque (rhesus → rhesus) as reported in Reimann et al [49]; (b) cynomolgus monkeys infected with inocula of different sizes (range 50–5 MID_{50}) of the original SHIV89.6P virus stock grown in rhesus macaques and provided by the same laboratory (rhesus → cynomolgus); (c) cynomolgus macaques infected with inocula of different sizes (range 28,520–2.8 MID_{50}) of the same SHIV89.6P virus stock but after passage in a cynomolgus monkey. The † indicates the time when a monkey died or was euthanized due to worsening of the clinical conditions.

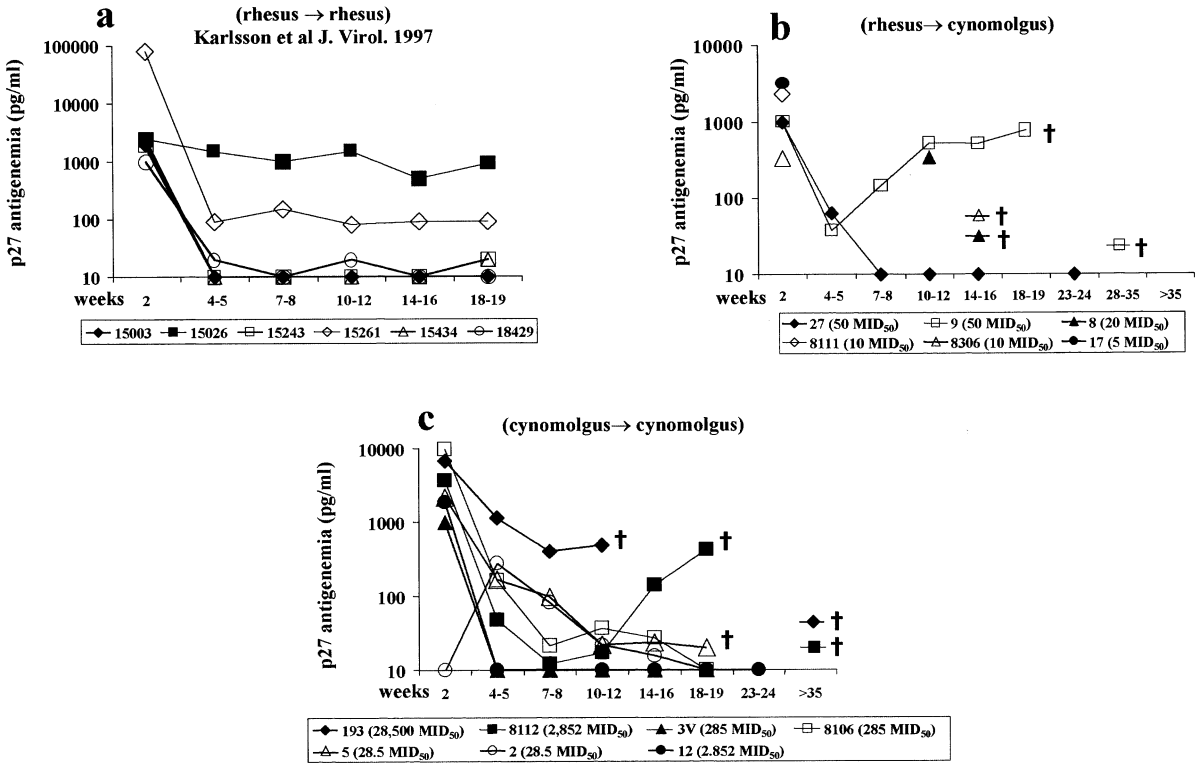

Fig. 3. Measurement of p27-Gag antigenemia (pg/ml, log scale) in rhesus or cynomolgus macaques inoculated intravenously with SHIV89.6P virus stock derived from a rhesus or cynomolgus monkey. Shown are the amounts of p27 antigen present in the plasma of (a) rhesus monkeys infected (inoculum size unknown) with SHIV89.6P molecular clones from rhesus macaques (rhesus → rhesus) as reported in Karlsson et al. [36]; (b) cynomolgus monkeys infected with inocula of different sizes (range 50–5 MID_{50}) of the original SHIV89.6P virus stock grown in rhesus macaques and obtained from the same laboratory (rhesus → cynomolgus); (c) cynomolgus macaques infected with inocula of different sizes (range 28,520–2.8 MID_{50}) of the same SHIV89.6P virus stock but after passage in a cynomolgus monkey. The † indicates the time when a monkey died or was euthanized due to worsening of the clinical conditions.

from cynomolgus monkeys is provided by the rapid progression of the infection toward disease and death that has already occurred in nine of the infected cynomolgus monkeys (Table 2).

By passaging the original SHIV89.6P in cynomolgus macaque, the pathogenicity increases rather than diminishes, which is confirmed by other studies in which, upon inoculation of a cynomolgus monkey with the SHIV89.6 (from which SHIV89.6P was derived), a virus more pathogenic than the parental stock was obtained [53]. Notably, a second *in vivo* passage in another cynomolgus monkey further increased its pathogenicity, as indicated by a rapid $CD4^+$ T cell decline and the high p27 values within the first 3 weeks [53], a behaviour that differs strikingly from that of non-pathogenic SHIVs or SIVs that resulted in low to undetectable plasma- or cell-associated viremia, and usually do not induce a significant depletion of $CD4^+$ T cells, nor disease onset and death [9, 10, 21, 57].

Taken together, these data prove that the SHIV89.6P generated in cynomolgus monkeys was highly infectious and uniformly pathogenic for all the animals at all the MID_{50} utilized (including 2.8).

Tat vaccine controls infection upon challenge with the pathogenic SHIV89.6P

At week 50, after the initiation of immunization (14–18 weeks after the last boost), all the animals were challenged with 10 MID_{50} of SHIV89.6P. After challenge, all the control monkeys, including the naive animal, and the two monkeys injected with RIBI or alum alone, were infected as indicated by the high levels of p27 antigenemia (Fig. 4) and plasma viremia (Fig. 5). In contrast, only two out of the seven Tat protein-vaccinated monkeys (one given Tat and RIBI and one Tat and alum) were infected as shown by both these assays (Figs. 4 and 5). For all the other five vaccinees, p27 and viral RNA were always undetectable in plasma for up to 46 weeks post-challenge (Figs. 4 and 5). In addition, SIV proviral DNA was undetectable in all the protected monkeys or was only sporadically detected in a few animals and at very low copy

number (Fig. 6). In contrast, PBMCs from the controls and the two vaccinated and infected animals had a very high proviral copy number since week 2 after challenge that remained always detectable. Virus isolation with CD8-depleted PBMCs stimulated with PHA and rhIL-2 or with anti-CD3 antibodies and rhIL-2 was always negative in the five protected macaques vaccinated with Tat (data not shown). In contrast, virus was always isolated and cell-associated viremia (cytoviremia) was high in the three control animals and in the two vaccinated and infected monkeys (Fig. 7).

Anti-SIV antibodies rapidly rose and reached very high titres in all the control monkeys, followed by the two infected and vaccinated animals in which a delayed and lower increase in antibody titres, as compared to the control macaques, was observed (Fig. 8). Low anti-SIV titres (1:2 to 1:100) were detected in the five protected monkeys and they became negative over time (Fig. 8). Anti-HIV-1 Env antibodies were detected only in sera from the infected animals. However, they were detected at week 14 after challenge by IVAP in the supernatants of cultured PBMCs stimulated with pokeweed mitogen from all the monkeys (data not shown).

The number of $CD4^+$ T cells was consistent with the data of the virological assays (Fig. 9). Specifically, in all protected monkeys the CD4 T cells remained in the normal range after the viral challenge and during all the follow-up period, whereas they decreased considerably in all the controls and in the two vaccinated and infected macaques. An increase in $CD8^+$ T cells (Fig. 10) was also observed in all the monkeys after challenge and they remained slightly above or similar to the pre-challenge values [12].

Discussion

Results from immunization of non-human primates with a biologically active Tat protein indicate that this vaccination was safe, induced a broad immunity with antiviral effects *in vitro*, and contained the infection in five out of seven vaccinated cynomolgus monkeys against challenge with the highly pathogenic SHIV89.6P for up to 1 year of follow-up.

The use for vaccination of a biologically active Tat protein is critical since only in this form is Tat taken up very efficiently and at a low concentration by accessory cells, enters the MHC class-I pathway and induces CD8 CTL responses [23, 38] (Barillari et al., in preparation). In fact, cellular

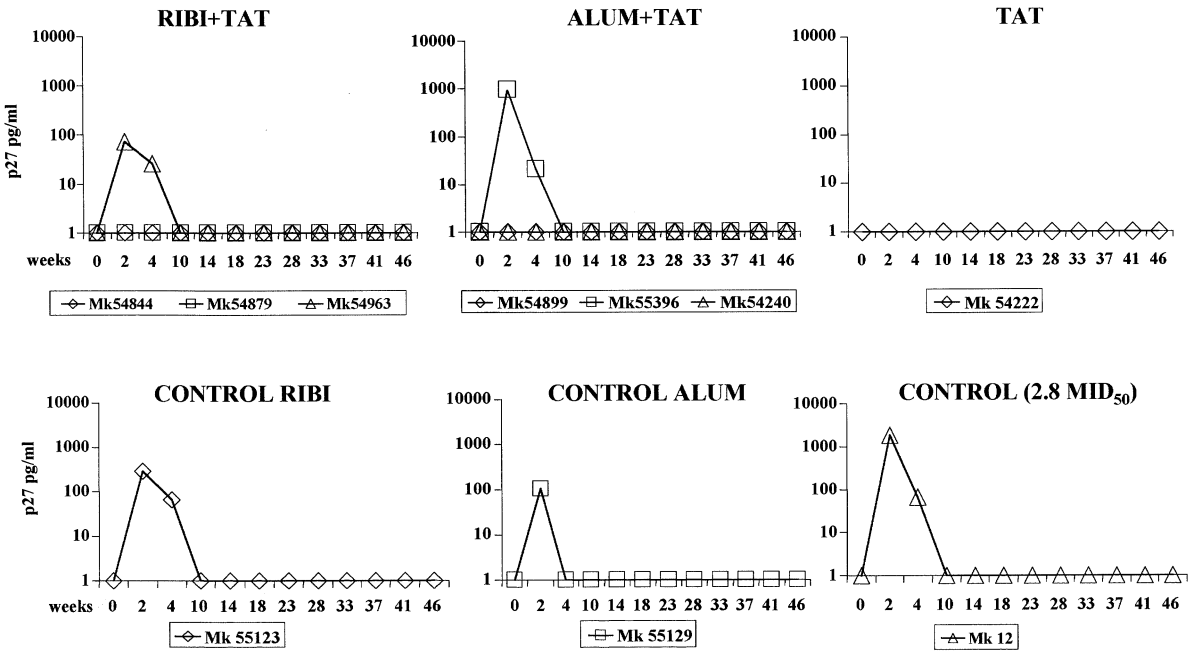

Fig. 4. Measurement of p27-Gag antigenemia (pg/ml, log scale) in cynomolgus monkeys vaccinated with Tat protein and challenged intravenously with 10 MID_{50} of the SHIV89.6P virus stock obtained after passage in a cynomolgus monkey. Shown are the amounts of p27 antigen present in the plasma of macaques vaccinated SC with either RIBI + Tat (monkey 54844, 54879, 54963) or alum + Tat (monkey 54899, 55396, 54240), or vaccinated ID with Tat without adjuvants (monkey 54222). Monkeys 55123 and 55129 are control animals that received only RIBI or alum adjuvant, respectively. Monkey 12 is a naive animal inoculated with a 3-fold lower inoculum (2.8 MID_{50}).

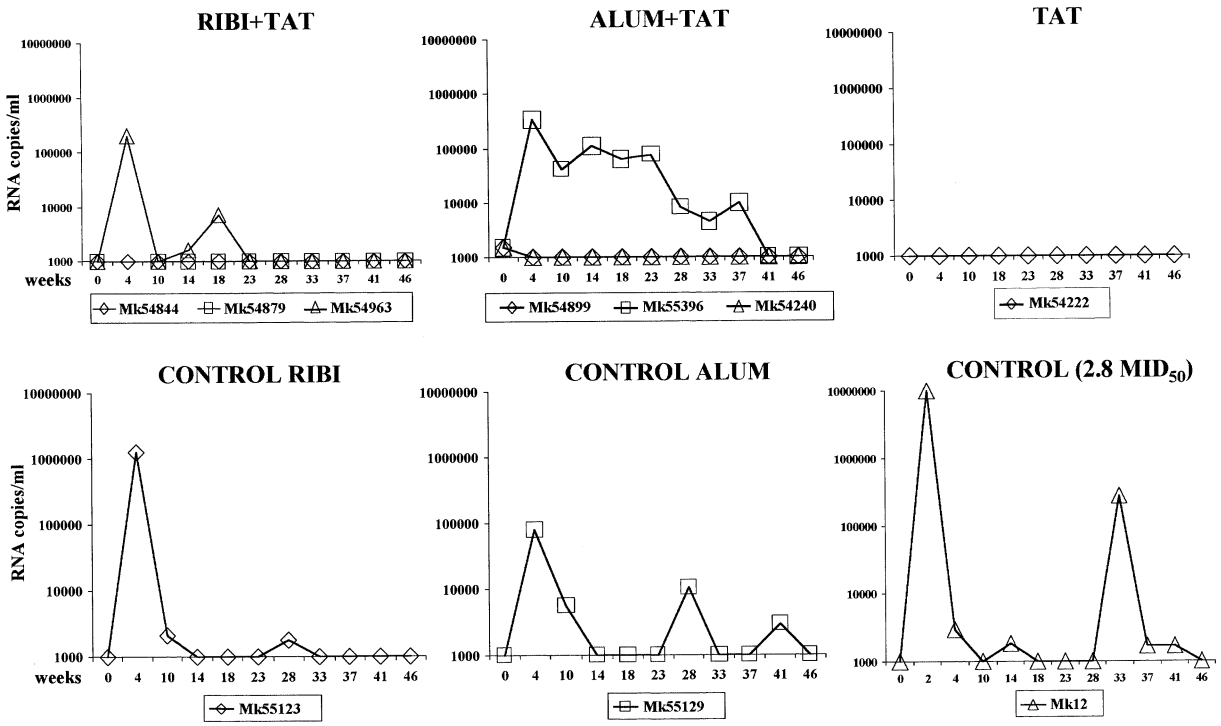

Fig. 5. Plasma viremia (RNA copies/ml) in cynomolgus monkeys vaccinated with Tat protein and challenged intravenously with 10 MID_{50} of the SHIV89.6P virus stock obtained after passage in a cynomolgus monkey. Shown are the levels of viremia present in the plasma of macaques vaccinated SC with either RIBI + Tat (monkey 54844, 54879, 54963) or alum + Tat (monkey 54899, 55396, 54240), or vaccinated ID with Tat without adjuvants (monkey 54222). Monkeys 55123 and 55129 are control animals that received only RIBI or alum adjuvant, respectively. Monkey 12 is a naive animal inoculated with a 3-fold lower inoculum (2.8 MID_{50}).

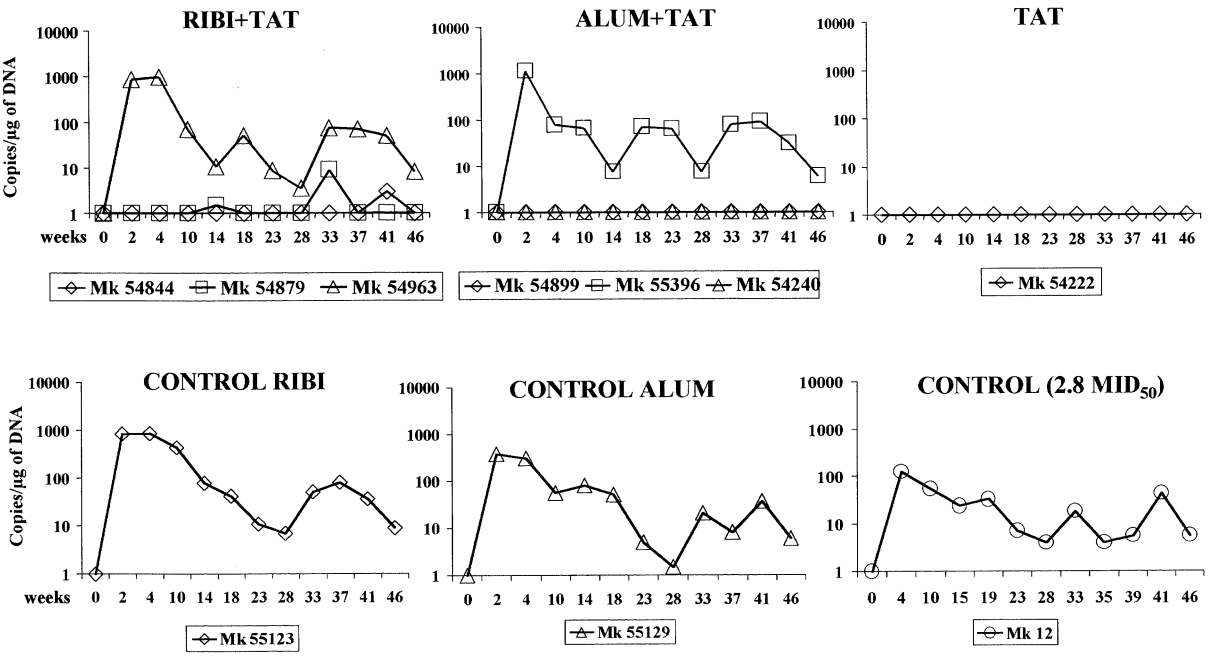

Fig. 6. Measurement of proviral DNA (copies/µg of DNA) in PBMCs from cynomolgus monkeys vaccinated with Tat protein and challenged intravenously with 10 MID_{50} of the SHIV89.6P virus stock obtained after passage in a cynomolgus monkey. Shown are the number of proviral DNA copies present in PBMCs from macaques vaccinated SC with either RIBI + Tat (monkey 54844, 54879, 54963) or alum + Tat (monkey 54899, 55396, 54240), or vaccinated ID with Tat without adjuvants (monkey 54222). Monkeys 55123 and 55129 are control animals that received only RIBI or alum adjuvant, respectively. Monkey 12 is a naive animal inoculated with a 3-fold lower inoculum (2.8 MID_{50}).

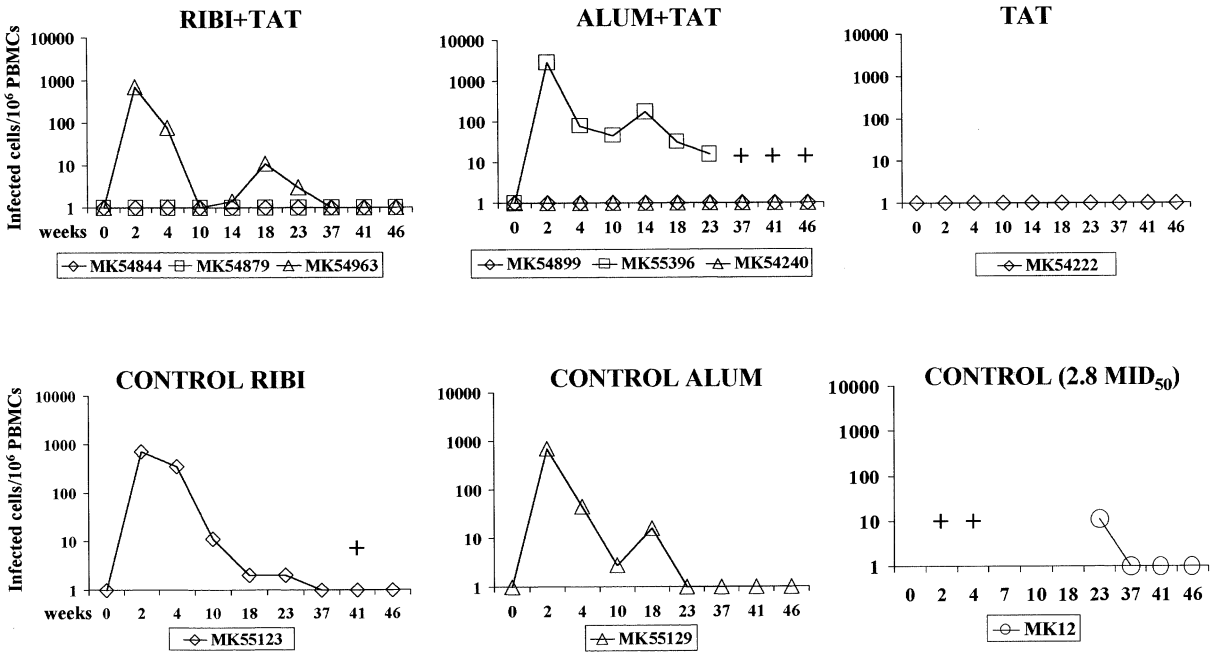

Fig. 7. Measurement of cytoviremia (infected cells per 10^6 PBMCs) and results of virus isolation in cynomolgus monkeys vaccinated with Tat protein and challenged intravenously with 10 MID_{50} of the SHIV89.6P virus stock obtained after passage in a cynomolgus monkey. Shown are the number of infected cells present in PBMCs from macaques vaccinated SC with either RIBI + Tat (monkey 54844, 54879, 54963) or alum + Tat (monkey 54899, 55396, 54240), or vaccinated ID with Tat without adjuvants (monkey 54222). Monkeys 55123 and 55129 are control animals that received only RIBI or alum adjuvant, respectively. Monkey 12 is a naive animal inoculated with a 3-fold lower inoculum (2.8 MID_{50}). + indicates positive virus isolation performed instead of cytoviremia.

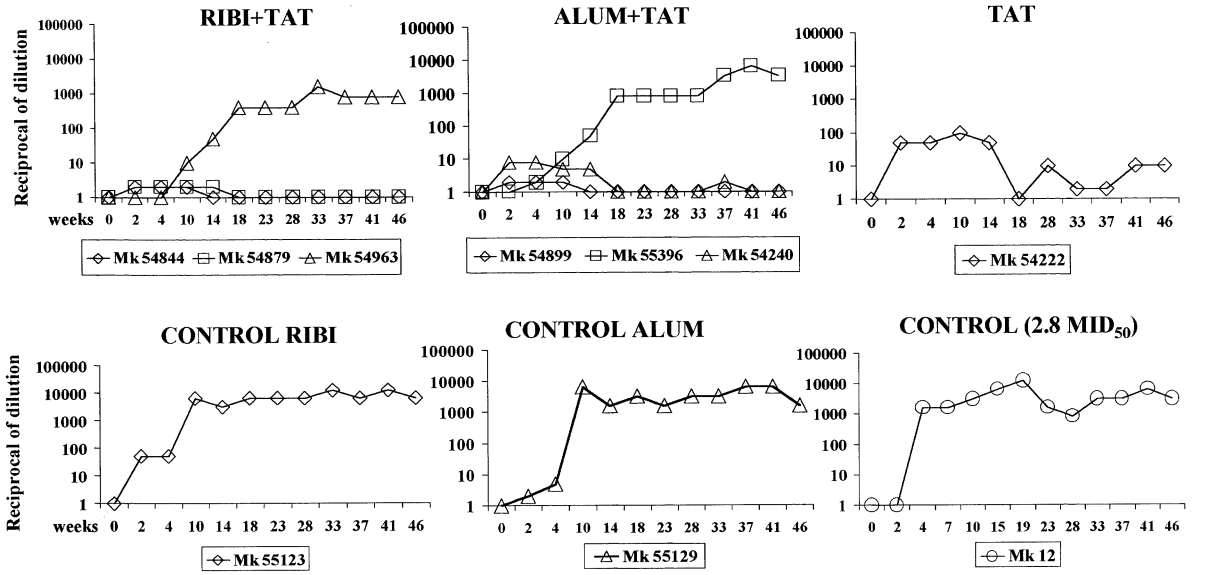

Fig. 8. Measurement of anti-SIV antibody titers in plasma from cynomolgus monkeys vaccinated with Tat protein and challenged intravenously with 10 MID_{50} of the SHIV89.6P virus stock obtained after passage in a cynomolgus monkey. Shown are the anti-SIV antibody titers from macaques vaccinated SC with either RIBI + Tat (monkey 54844, 54879, 54963) or alum + Tat (monkey 54899, 55396, 54240), or vaccinated ID with Tat without adjuvants (monkey 54222). Monkeys 55123 and 55129 are control animals that received only RIBI or alum adjuvant, respectively. Monkey 12 is a naive animal inoculated with a 3-fold lower inoculum (2.8 MID_{50}).

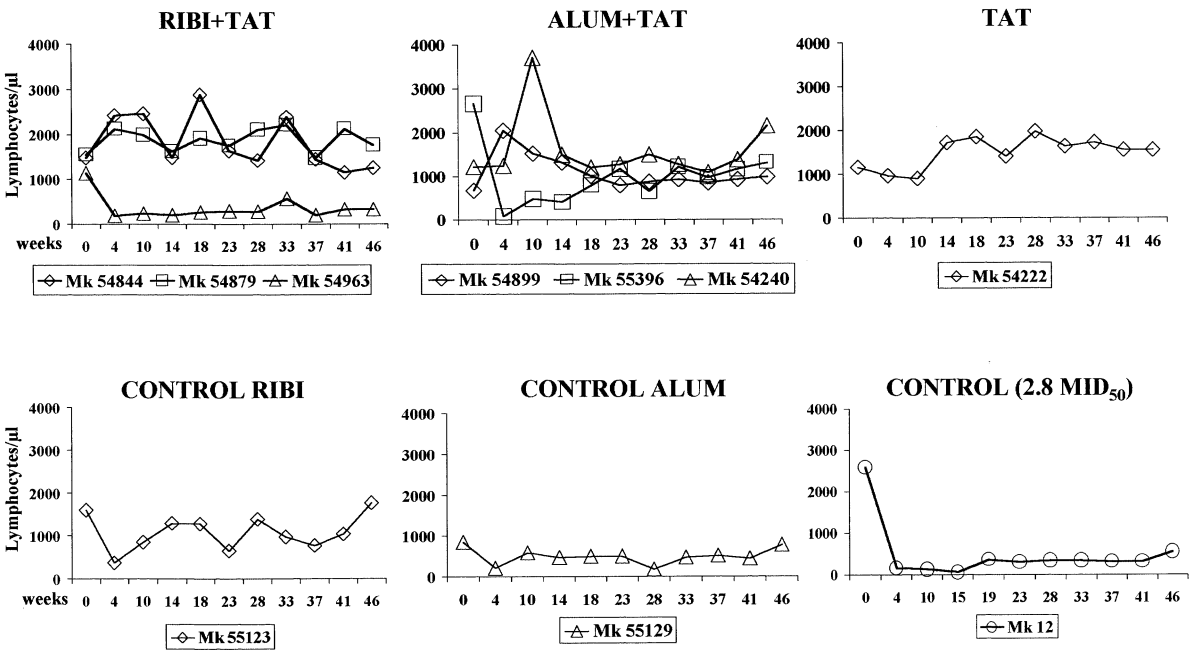

Fig. 9. Absolute CD4 T cell counts per µl in cynomolgus monkeys vaccinated with Tat protein and challenged intravenously with 10 MID_{50} of the SHIV89.6P virus stock obtained after passage in a cynomolgus monkey. Shown are the CD4 T cell counts from macaques vaccinated SC with either RIBI + Tat (monkey 54844, 54879, 54963) or alum + Tat (monkey 54899, 55396, 54240), or vaccinated ID with Tat without adjuvants (monkey 54222). Monkeys 55123 and 55129 are control animals that received only RIBI or alum adjuvant, respectively. Monkey 12 is a naive animal inoculated with a 3-fold lower inoculum (2.8 MID_{50}).

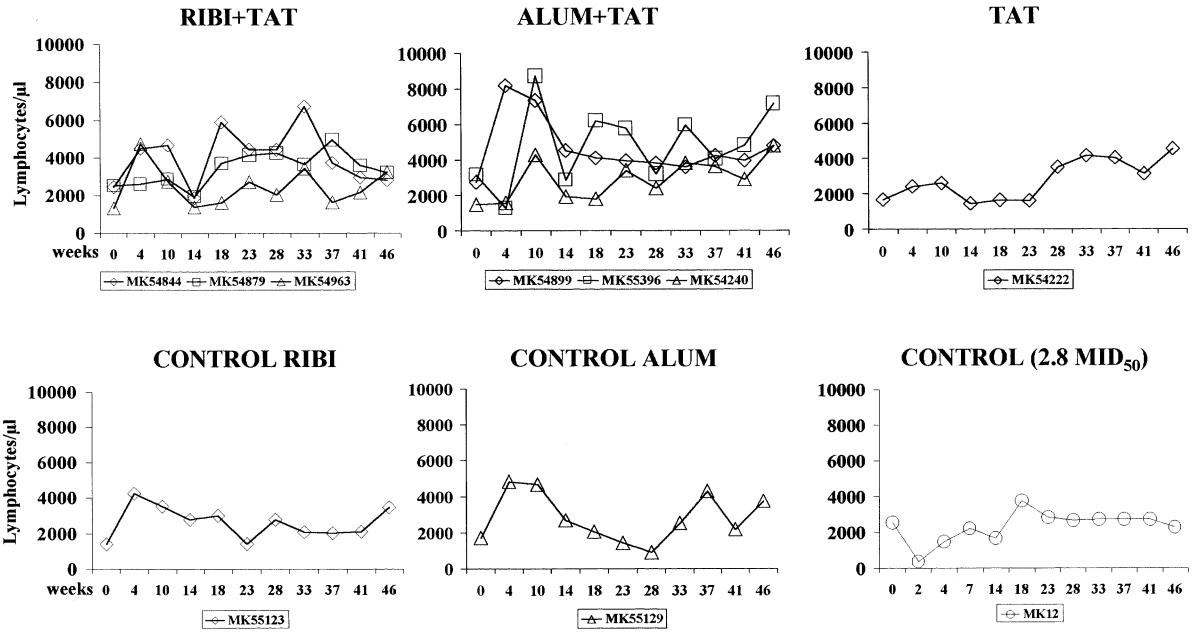

Fig. 10. CD8 T cell counts per µl in cynomolgus monkeys vaccinated with Tat protein and challenged intravenously with 10 MID_{50} of the SHIV89.6P virus stock obtained after passage in a cynomolgus monkey. Shown are the CD8 T cell counts from macaques vaccinated SC with either RIBI + Tat (monkey 54844, 54879, 54963) or alum + Tat (monkey 54899, 55396, 54240), or vaccinated ID with Tat without adjuvants (monkey 54222). Monkeys 55123 and 55129 are control animals that received only RIBI or alum adjuvant, respectively. Monkey 12 is a naive animal inoculated with a 3-fold lower inoculum (2.8 MID_{50}).

uptake of Tat by dendritic cells or cytokine-activated endothelial cells is strongly compromised by inactivation of the protein (Barillari et al., in preparation), that, although it may still induce production of antibodies, it may be unable to induce CTLs. In addition, no toxicity was detected

upon several local injections (10 μg SC or 6 μg ID) of Tat. These data are also in agreement with previous results indicating that Tat alone at these amounts does not induce histological changes upon inoculation in small animals [5, 7, 8, 24, 27, 28]. Similarly, no toxicity was detected in cynomolgus macaques immunized with viral vectors expressing the SIV-Tat and -Rev [44] and the animals were protected upon challenge with a highly pathogenic virus. Finally, results from studies in HIV-infected humans injected with HIV-Tat DNA [13] indicate that Tat was neither toxic nor induced viral replication and was immunogenic in that both humoral and cellular anti-Tat immune responses were induced, as assessed by measuring the antibody titers and proliferative responses to Tat [13, 14].

The immunogenicity of Tat is of relevance, since in the natural course of infection an anti-Tat response can be hardly detected, whereas immunoreactivity to other viral components is readily found. In theory, this may result from poor visibility or presentation of the antigen to the immune system, or from scarce immunodominance. Results from vaccination with Tat, both in naive monkeys [12, 44] and seropositive humans [13, 14], indicate that Tat is fully immunogenic but scarcely immunodominant. However, vaccination is capable of overcoming this problem, as indicated by the induction of anti-Tat responses in HIV-infected patients [13, 14].

While it remains to be elucidated why a minority of individuals upon HIV infection develop a humoral and cellular immune response to Tat (i.e. long-term non-progressors), the present study provides the first *in vivo* evidence of the protective role of an anti-Tat response, explaining the observed correlation of an anti-Tat reactivity with a delayed disease onset [48, 52, 58]. In fact, five out of seven vaccinated animals controlled the infection to undetectable levels and did not experience any CD4 T cell decline or disease development up to 46 weeks post-challenge. In contrast, two out of seven of the vaccinated animals were infected, although the kinetics and the levels of anti-SIV antibody titres were delayed and lower, respectively, as compared to the control animals. Thus, Tat vaccination modified the virus–host interaction that normally leads to immunodeficiency and disease onset.

The search for correlates of protection revealed that the CD8 T cell response is critical for protection. Although Tat vaccine was capable of eliciting in most monkeys high titers of anti-Tat antibodies neutralizing the biological activities of Tat *in vitro*, which were possibly responsible for the delayed and lower anti-SIV or -HIV Env antibody response detected in the two vaccinated and infected animals, no correlation was found with the outcome upon challenge. On the contrary, the CD8 antigen-specific immune response (CTL) strongly correlated with control of SHIV89.6P primary infection. In addition, assessment of the production of TNF-α after stimulation of PBMCs with Tat confirmed that, indeed, CD8 T cells were the main source of TNF-α (about 90%), one of the cytokines released upon stimulation of the T cell receptor of CTLs and a known mediator of CTL activity [2, 35, 45, 47].

A large body of evidence has recently been generated, indicating the presence in the peripheral blood of infected individuals of HIV-specific CTLs that are responsible for the CD8 clonal expansion observed in the chronic phase of HIV infection [46, 63] and the decline along with disease progression [15, 39, 51]. Recent technical advancement, such as the possibility to stain antigen-specific CTLs and to count interferon-γ producing CD8 cells [33] and *in vivo* CD8 depletion experiments in macaques, have provided strong evidence for a protective role played by CTLs. Thus, the detection of CTLs in the protected monkeys confirms that CD8 cellular immunity is critical for controlling the infection.

In summary these data indicate that Tat vaccine is a promising candidate for both preventive and therapeutic vaccine trials in humans. This is also supported by data indicating that sera from Ugandan patients infected with A and D virus subtypes recognize the Tat protein derived from the B clade utilized in the vaccine preparation (Buttò et al., in preparation). This suggests that a vaccine based on Tat can control infection from all or most virus subtypes.

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