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Vaccination with DNA containing *tat* coding sequences and unmethylated CpG motifs protects cynomolgus monkeys upon infection with simian/human immunodeficiency virus (SHIV89.6P)

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Abstract

Recent evidence suggests that a CD8-mediated cytotoxic T cell response against the Tat protein of human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) controls primary infection after pathogenic virus challenge, and correlates with the status of long-term nonprogressor in humans. Due to the presence of unmethylated CpG sequences, DNA vaccination can boost the innate immunity driving more potent T cell-mediated immune responses. Therefore, cynomolgus monkeys were vaccinated with a *tat*-expressing vector containing defined unmethylated CpG sequences (pCV-*tat*). Here it is shown that the intramuscular inoculation of the pCV-*tat* contained primary infection with the highly pathogenic SHIV89.6P virus preventing the $CD4^+$ T cell decline in all the vaccinated monkeys. Undetectable virus replication and negative virus isolation correlated in all cases with the presence of anti-Tat CTLs. However, a CD8-mediated non cytolytic antiviral activity was also present in all protected animals. Of note, this activity was absent in the controls but was present in the monkey inoculated with the CpG-rich vector alone that was partially protected against viral challenge (i.e. no virus replication but positive virus isolation). These results suggest that a CTL response against Tat protects against primary infection by blocking virus replication at its early stage, in the absence of sterilizing immunity. Nevertheless, the boost of the innate immunity by CpG sequences can contribute to this protection both by driving more potent CTL responses and by inducing other CD8-mediated antiviral activities. Thus, the CpG-rich *tat* DNA vaccine may represent a promising candidate for preventive and therapeutic vaccination against AIDS.

Keywords: HIV vaccine; Tat; CD8 T cells

1. Introduction

Most AIDS vaccine strategies aimed at blocking virus entry have failed to induce sterilizing immunity and protection against heterologous virus challenge (for review see Refs. [1–3]). Therefore, control of virus infection and block of disease onset are now accepted as more achievable goals of AIDS vaccine development.

The control of infection such as low plasma viremia, limited CD4 T cell depletion and reduced disease progression upon vaccination has been achieved by several strategies [1–3]. In this respect, it has recently been

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shown that a subunit vaccine consisting of a biologically active human immunodeficiency virus type 1 (HIV-1) Tat protein can control replication of the highly pathogenic SHIV89.6P to undetectable levels in most (5 out of 7) of the vaccinated macaques, preventing the $CD4^+$ T cell decline and disease onset for prolonged periods of time [4,5]. Similarly, a *tat-rev* vaccine has been shown to protect rhesus macaques from pathogenic simian immunodeficiency virus (SIV) challenge [6].

The reasons for this protection reside on the features of the HIV-1 Tat protein. In fact, Tat is a regulatory protein expressed very early after infection and required for virus gene expression and replication [7–9]. Of note, early after infection Tat is released extracellularly [10–13] and is taken up by neighbor cells [10,12,14]. By this pathway Tat further activates HIV replication in infected cells, favors transmission of both R5 and X4 HIV strains in uninfected cells, and promotes disease development [8–29]. In addition, extracellular Tat binds specific cell surface receptors [15,17,18,20,21], it is taken up very efficiently by accessory cells, and it is presented with MHC-class I molecules and, therefore, capable of inducing CD8-mediated T cell responses [30].

Of note, CD8-mediated cytotoxic (CTL) immune responses correlated with protection with the Tat protein vaccine. In fact, anti-Tat CTLs and Tat-specific Th-1 cytokine production were present only in the protected animals [4]. These data are consistent with results in humans indicating that an early immune response to Tat is present in long-term nonprogressors and correlates with non-progression to AIDS [[31–36] and our unpublished data], and with results from a very recent study demonstrating that early anti-Tat CTL may be key in controlling early SIV replication in monkeys during primary infection [37].

Due to the presence of unmethylated CpG sequences, DNA immunization is particularly effective in boosting the innate immunity and in enhancing adaptive Th-1-type immune responses by inducing the maturation of dendritic cells and long-lasting cytotoxic T lymphocytes (CTLs) [38]. Of note, the natural immune response induced by the sole CpG sequences can also protect against intracellular bacterial infections in murine systems [38] underlining their importance in protection against pathogens and in vaccine strategies [38,39].

Altogether these results prompted us to verify whether a DNA vaccine utilizing the *tat* gene expressed by a vector containing defined unmethylated CpG sequences would have been capable of enhancing antigen-specific CTL responses against Tat and of inducing an effective protection against AIDS.

2. Materials and methods

2.1. Plasmid DNA preparation

The plasmid pCV-*tat* that contains the cDNA of the HIV-1 *tat* gene (BH-10) under the transcriptional control of the adenovirus major late promoter and the vector pCV-0 have been previously described [7,10–12]. Plasmids were purified on CsCl gradient, according to standard procedures [40] and dialyzed for 48 h against 300 volumes of sterile phosphate buffered saline (PBS) without calcium and magnesium. Before injection in the animals, several plasmid preparations were pooled, analyzed by restriction enzyme digestion and for gene expression in transient transfection assays in HL3T1 cells containing an integrated copy of the reporter plasmid HIV-1-LTR-CAT, as described previously [41].

2.2. Identification of unmethylated CpG sequences in pCV-tat and pCV-0

pCV-0 and pCV-*tat* were sequenced by primer walking strategy [42] with a commercial kit (Dye Terminator Cycle Sequencing, Perkin-Elmer) on an ABI 373 DNA automated sequencer (Applical Biosystem, PE Biosystems, Foster City, CA). Twenty three CpG sequences were found and 12 of them were unmethylated as indicated by restriction endonuclease analysis of the plasmids with the methylation sensitive enzymes Ehe I, Eco 47 III, Hin 1 I, Bsp 143 II, and Psp 1406 I (MBI Fermentas, Vilnius, Lithuania) that recognize the unmethylated forms of GGCpGCC, AGCpGCT, GPuCpGPyC, PuGCpGCPy and AACpGTT, respectively.

2.3. Monkeys and vaccination protocol

Adult cynomolgus monkeys (*Macaca fascicularis*) were housed in single cages within level 3 biosafety facility according to the European guidelines for non-human primate care (ECC, Directive No. 86-609, Nov. 24, 1986). All the inocula and bleedings were performed as previously described [4]. Sites of intramuscular (i.m.) injections were inoculated 24 h prior to immunization with 0.5% Bupivacaine (Sigma, St. Louis, MO) and 0.1% *p*-Hydroxybenzoic acid methyl ester (Methyl paraben; Sigma, St. Louis, MO) in saline buffer. Since week 37 of immunization, this pretreatment was performed 5 days prior to boosting. Monkeys 54920, 55122, 55361 and 54219 received boosts on week 2, 6, 11, 15, 21, 28, 32 and 36 after the first immunization, while monkeys PR2 and 37 were vaccinated with a slightly different schedule (on week 0, 5, 10, 15, 22, 27, 32, 37 and 42). Macaques inoculated with the adjuvant RIBI or Alum were injected with the same schedule of the former group. Sixteen micrograms of Tat protein in

immune-stimulating complexes (ISCOMs [43]) were given i.m. as the last boost to all monkeys, except for the monkey 54219, inoculated with the vector alone, and the monkeys 55123 and 55129, inoculated with RIBI or Alum alone, respectively. At each time-point bleedings were performed for routine hematological, biochemical and immunological determinations. The control macaques injected with the RIBI or Alum adjuvants derive from a simultaneous protocol with the Tat protein and have been included since they were challenged at the same day and with the same virus inoculum of the DNA-injected monkeys. Monkey 12 and 2 were naive animals that received the same virus but 6 weeks earlier. With respect to MK 55123, 55129 and 12, data up to 28 weeks after challenge have already been published [4].

2.4. Anti-Tat antibody detection

Anti-Tat antibodies were detected by ELISA as previously described [4]. The mean of the negative controls plus three standard deviations represented the cut-off value. Plasma from vaccinated monkeys were also tested for neutralization of Tat activity by the rescue assay in which the replication of *tat*-defective HIV-1 proviruses is induced by serial concentrations of Tat added to HLM-1 cells (HeLa $CD4^+$ cells containing a *tat*-defective HIV-1 provirus), as described previously [4].

2.5. Tat-specific proliferative assays

PBMC, purified on a Ficoll-gradient, were cultured in complete medium [RPMI 1640, supplemented of 20 mM Hepes, (Sigma, St. Louis, MO) 100 U/ml Penicillin, 100 μg/ml Streptomycin, 2 mM L-glutamine (Life-Technologies, Paisley, UK) and 15% fetal bovine serum (Hyclone, Logan, UT)] in the presence of Tat (5 μg/ml), PHA (2 μg/ml) or tetanus toxoid (TT) (5 μg/ml) in triplicate wells. After 5 days, 1 μCi of [^{3}H]thymidine was added and the radioactivity measured 16 h later, as previously described [4]. A stimulation index (SI) greater than 3 was considered positive. All monkeys responded to the PHA.

2.6. Anti-Tat CTL assay

CTL assays were performed as described [4]. PBMC 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 PBMC were incubated for 3 h with Tat (1 μg), washed twice and added to the wells containing the PBMC stimulated previously. On day two, 2 IU of recombinant human IL-2 (rhIL-2) were added to the wells. Half of the supernatant was replaced with medium containing rhIL-2 twice a 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 two-fold dilutions (in duplicate) (Effectors). The day before the assay, herpesvirus Papio-transformed autologous B-lymphocytes (BLCL) [44] 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, Turku, Finland) [45], and 5×10^3 cells added to the Effectors. After 2 h, 20 μl of supernatants were mixed with 200 μl of the Europium solution and fluorescence measured after 20 min with a time-resolved fluorescence reader (Victor, Wallac, Turku, Finland). The percent specific lysis was calculated for each E:T ratio as follows: (test release − spontaneous release)/(maximum release − spontaneous release) × 100. The percent specific lysis against unpulsed autologous BLCL was calculated and subtracted from the percent 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.

2.7. Determination of TNF-α production from PBMC

TNF-α production was measured by seeding in duplicate 10^6 PBMC in a 24-well plate in 1 ml of complete medium. Cells were then stimulated with PHA (2 μg/ml) (Purified Phytohaemoagglutinin, Murex Diagnostics, UK) 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 described earlier [4].

2.8. Preparation and titration of the SHIV89.6P

The parental SHIV89.6P [46,47], obtained from Dr N. Letvin (Harvard Medical School, Boston, MA), was expanded in vivo in a *Macaca fascicularis* monkey [4]. Virus pathogenicity in cynomolgus and the 50% monkey infectious dose (MID_{50}) were determined

in a previous study and extensively compared with results from other laboratories [4,5]. 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 seven (dilutions range of 1:50–1:500,000 corresponding, retrospectively, to a range of 28 520–2.85 MID_{50}) monkeys, respectively. High levels of viral replication including p27 antigenemia, plasma viremia, proviral DNA, anti-SIV antibody (Ab) titers, and a profound and persistent decrease in CD4 T cell counts were observed in all monkeys independently from the virus stock utilized, and no differences were found when the data obtained were compared with those of other laboratories including the rate of animal death [5].

2.9. p27 determination in plasma

Levels of p27 SIV-Gag protein were measured in plasma by using an antigen capture ELISA assay (Innotest, Innogenetics, Zwijndrecht, Belgium), as described [4].

2.10. Plasma viremia

Quantitation of SHIV89.6P RNA copies was performed in the Bayer-Chiron Diagnostics reference Testing Laboratory (Amsterdam) by a branched DNA signal amplification assay (bDNA) recognizing the *pol* region of the SIVmac strains, as described [4], with a cut-off of 1500 RNA copies/ml. Results below 10 000 RNA copies/ml were confirmed by a Quantitative Competitive RNA polymerase chain reaction (QT RNA-PCR) as already described [48].

2.11. Proviral DNA detection

SIV proviral copy number was determined in DNA extracted from whole blood (QIAamp Blood Kit, Qiagen GmbH, Hilden, Germany) by a semiquantitative DNA PCR amplifying a 496 bps region of the *gag* gene of SIVmac251, as already described [4].

2.12. Virus isolation and cytoviremia

Virus isolation and cytoviremia were performed by coculturing CD8-depleted PBMC with CEMx174 after PHA and IL-2 stimulation, as already described [4]. Culture supernatants were tested for p27 production twice a week.

Virus isolation was also performed by culturing for three weeks CD8-depleted PBMC in 96-well plates previously coated with an anti-CD3 monoclonal antibody (mAb) (2.5 μg/ml, clone FN-18, Biosource International) in the presence of rhIL-2 (10 IU/ml).

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

Ab titers against the whole SIV or the HIV-1 Env were determined by an HIV-2 ELISA assay (ELAVIA, Ac-Ab-Ak II kit; Diagnostic Pasteur, Paris, France) or by an HIV-1 ELISA assay (HIV-1/HIV-2 Third Generation Plus, Abbott, Chicago, IL), respectively, as described earlier [4]. The IVAP upon Pokeweed Mitogen stimulation was performed as described earlier [4].

2.14. FACS analysis

Phycoerythrin (PE)-conjugated anti-CD4 (Biosource International) was used for staining citrated peripheral blood. Stained cells were then analyzed with a FACScan cytometer and software (Becton-Dickinson) as described earlier [4]. Absolute cell numbers were calculated from the blood cell counts.

3. Results

3.1. The plasmid vector pCV contains unmethylated CpG sequences

The plasmid pCV-0 and pCV-*tat* [7] were chosen since pCV-*tat* is capable of high expression and release of the Tat protein in the absence of cell death [10–12]. The content of unmethylated CpG sequences was determined in both plasmids by sequencing and digestion with methylation-sensitive restriction enzymes. As shown in Table 1, 12 out of 23 different CpG sequences identified were found to be unmethylated. Nine of these sequences are present in the pBR322 sequence, one in the dehydrofolate reductase gene, and two in the Adenovirus genome and consist mainly of GGCpGCC (5 motifs), GGCpGTC (1 motif), AACpGTT (2 motifs) and AGCpGCT (4 motifs) (Table 1).

3.2. Vaccination protocol

As shown in Table 2, four cynomolgus macaques were vaccinated i.m. with 1 mg (monkeys 54920, 55122 and 55361) or 0.5 mg (monkey PR2) of pCV-*tat*; one monkey (37) was immunized with 0.2 mg of the pCV-*tat* intradermally (i.d.); one monkey (54219) was injected i.m. with 1 mg of the CpG-rich empty vector (pCV-0); and two monkeys (55123 and 55129) were inoculated s.c. with the adjuvant RIBI or Alum, respectively. These two macaques were utilized as controls of the immunostimulatory effects of pCV-0. Finally, two naive animals (monkeys 2 and 12) were included in the protocol at the time of the viral challenge and served to demonstrate the infectiveness and pathogenicity of the virus stock utilized for the challenge. The macaques

Table 1
Analysis of the CpG sequences present in the pCV-0 and pCV-*tat* vectors

Position (bp)	Sequence	Region	Vector
232–237	AGCpGCT[a]	pBR322	pCV-0/pCV-*tat*
413–418	GGCpGCC[a]	pBR322	pCV-0/pCV-*tat*
434–439	GGCpGCC[a]	pBR322	pCV-0/pCV-*tat*
494–499	AGCpGCT[a]	pBR322	pCV-0/pCV-*tat*
548–553	GGCpGCC[a]	pBR322	pCV-0/pCV-*tat*
648–653	AGCpGTC	pBR322	pCV-0/pCV-*tat*
775–780	AGCpGCT[a]	pBR322	pCV-0/pCV-*tat*
900–905	AACpGTT[a]	pBR322	pCV-0/pCV-*tat*
967–972	GGCpGTT	pBR322	pCV-0/pCV-*tat*
1205–1210	GGCpGCC[a]	pBR322	pCV-0/pCV-*tat*
1458–1463	GGCpGTT	pBR322	pCV-0/pCV-*tat*
1629–1634	GGCpGTT	pBR322	pCV-0/pCV-*tat*
1676–1681	GACpGCT	pBR322	pCV-0/pCV-*tat*
1725–1730	GGCpGTT	pBR322	pCV-0/pCV-*tat*
1818–1823	GGCpGTT	pBR322	pCV-0/pCV-*tat*
2254–2259	GACpGCT	pBR322	pCV-0/pCV-*tat*
2692–2697	AACpGTT[a]	pBR322	pCV-0/pCV-*tat*
3637–3642	GGCpGTC[a]	Dehydrofolate Reductase Gene	pCV-0/pCV-*tat*
4935–4940	GGCpGTT	SV40	pCV-0/pCV-*tat*
5624–5629	GGCpGTT	SV40	pCV-0/pCV-*tat*
6434–6439	GGCpGCC[a]	Adenovirus	pCV-0/pCV-*tat*
6543–6548	AGCpGCT[a]	Adenovirus	pCV-0/pCV-*tat*
34–39[b]	GGCpGTT	5′ *tat*-gene flanking region	pCV-*tat*

[a] Unmethylated CpG sequences.
[b] This motif is present in the *tat* flanking region of the pCV-*tat* plasmid. Shown are the 5′PuPu-CpG-PyPy3′ motifs contained in the pCV-0 and pCV-*tat* vectors determined as described in Section 2.

were boosted seven times as described in Section 2. The last boost was given intramuscularly with the Tat protein in ISCOMs [43].

At the time of vaccination and during all the follow-up period, neither local nor systemic side effects were observed in all inoculated animals and all the hematological, biochemical and immunological parameters (blood cell counts, blood chemistry and FACS analysis) were always in the normal range (data not shown).

3.3. Humoral and cellular anti-Tat immune responses upon immunization with pCV-tat

All vaccinated and control macaques were monitored for the presence of both humoral and cellular responses to Tat during immunization and prior to challenge.

As shown in Table 3, in all animals vaccinated with pCV-*tat* i.m., anti-Tat antibodies were absent or at low titers and transient since they were not longer detectable prior to challenge. In contrast, in monkey 37, inoculated i.d. with 0.2 mg of *tat* DNA, anti-Tat antibodies appeared later but reached titers up to 1:1600 and remained high throughout the follow-up period. No anti-Tat antibodies were observed at any time in monkey 54219, inoculated with pCV-0, or in the control animals.

Consistent with these data, plasma from the vaccinated macaques were unable to neutralize Tat activity as detected by the rescue assay [4], with the exception of monkey 37 at weeks 48 and 58, for which, however, neutralizing activity was low (30 ng/ml of Tat) (Table 3).

Lymphoproliferative responses to Tat or TT, for which all animals had been vaccinated, were detected in all pCV-*tat*-inoculated monkeys except for monkey PR2 (Table 3). In contrast, skin tests with Tat [4] resulted always negative in all vaccinated macaques,

Table 2
Description of the vaccination protocol[a]

Group	Monkey	Adjuvant	Administration
tat DNA (1 mg)	54920	Pretreatment (24 h, 1 ml) 0.5%	i.m., 500 μl in two sites
	55122	Bupivacaine + 0.1% methyl paraben	(femoral quadriceps)
	55361		
tat DNA (0.5 mg)	PR2	Pretreatment (24 h, 1 ml) 0.5% Bupivacaine + 0.1% methyl paraben	i.m., 400 μl in two sites (femoral quadriceps)
tat DNA (0.2 mg)	37	PBS	i.d., 150 μl in two sites (dorsal area, close to the axillary LN)
Vector (CpG) DNA (1 mg)	54219	Pretreatment (24 h, 1 ml) 0.5% Bupivacaine + 0.1% methyl paraben	i.m., 500 μl in two sites (femoral quadriceps)
RIBI	55123	RIBI (250 μl) + Saline buffer (250 μl)	s.c., 500 μl in one site (dorsal area, near the neck)
ALUM	55129	Alum (250 μl) + Saline buffer (250 μl)	s.c., 500 μl in one site (dorsal area, near the neck)
Naive	2	None	None
Naive	12	None	None

[a] Five macaques were immunized with pCV-*tat* (*tat* DNA), i.m. (four animals) or i.d. (one animal) and one monkey was injected i.m. with pCV-0 (CpG-rich vector DNA). Monkeys 55123 and 55129 were inoculated s.c. with RIBI or Alum adjuvant alone. Monkeys 2 and 12 were naive animals introduced in the protocol at the time of challenge. Boosts were given as described in Section 2. The plasmid vectors were prepared as described in Section 2.

Table 3
Summary of the immunological responses to Tat in vaccinated monkeys

Group	Monkey	Ab titers[a]	Tat Neutr.[b]	Prol. Resp.[c]	DTH[d]	CTL[e]
tat DNA (1 mg)	54920	100	−	+	−	+
	55122	100	−	+	−	+
	55361	0	−	+	−	+
tat DNA (0.5 mg)	PR2	50	−	−	−	+
tat DNA (0.2 mg)	37	1600	+	+	+	−
Vector (CpG) DNA (1 mg)	54219	0	−	−	ND	−
RIBI	55123	0	−	−	ND	−
ALUM	55129	0	−	−	ND	−
Naive	2	ND	ND	ND	ND	ND
Naive	12	ND	ND	ND	ND	ND

[a] Reciprocal of the last positive dilution by ELISA (cut off: mean of preimmune sera + 3 S.D.).
[b] Neutralizing index measured as the capability of sera to block ($\geq$50%) the rescue of a *tat*-defective provirus by the addition of the Tat protein.
[c] Stimulation index (ratio between Tat-specific and the control proliferative response): −, $\leq$3.
[d] Tat-Skin test, ø measured at 48 h: −, <1 mm, +/−, erythema without induration; +, 1–4 mm; ++, $\geq$5 mm.
[e] Specific killing (cut-off: 5%). ND, not done.

except for monkey 37, inoculated with *tat* DNA i.d. (Table 3). The skin tests with TT were positive in all animals.

Anti-Tat CTL activity was determined by labeling the target cells with Europium. CTLs were detected in all the macaques vaccinated i.m. with *tat* DNA (Tables 3 and 4), whereas monkey 37, that was vaccinated i.d., barely reached the cut-off value only at the highest effector: target ratio tested. No CTLs were detected in monkey 54219, injected with pCV-0 and in the control animals (Tables 3 and 4).

The production of TNF-α, a known mediator of CTL activity [49–52], was also evaluated upon Tat or PHA stimulation [4] in those animals for which cells were available. TNF-α was produced upon Tat stimulation in the monkeys 54920 and 55122, vaccinated i.m. with *tat* DNA, whereas no production was observed in monkey 54219, inoculated with pCV-0, and in monkeys 55123 and 55129, inoculated with RIBI or Alum alone, respectively, although they produced TNF-α upon PHA stimulation (data not shown), confirming the CTL data and the presence of an antigen-specific Th-1 response in the *tat*-vaccinated animals.

3.4. Challenge with the SHIV89.6P

All animals were challenged intravenously with the same stock of SHIV89.6P and on the same day. Since the schedules of immunization differed slightly among the arms of the Tat vaccination protocol, the challenge occurred 14–18 weeks after the last boost (at week 50 after immunization for all animals except for monkeys PR2 and 37 that were challenged at week 65 after immunization). The SHIV89.6P is a highly pathogenic virus that contains the *tat* gene of HIV-1 [4,46,47]. Pathogenicity of the virus stocks was previously studied in 13 animals (see Section 2) and the virus was found to be highly pathogenic in cynomolgus monkeys [5] as shown by others in rhesus macaques [46,47]. All animals were, therefore, inoculated with 10 MID_{50} of the virus, except the two naive macaques, 12 and 2, that were inoculated with a 3-fold lower (2.8 MID_{50}) or 3-fold higher (28 MID_{50}) viral dose, respectively, to show the dose of virus inoculum already sufficient to establish a productive infection (2.8 MID_{50}) or capable of inducing a rapid death (28 MID_{50}). In fact, monkey 2 had to be euthanized at week 35 after the viral challenge, due to very low $CD4^+$ T cell counts and severe worsening of the clinical conditions.

Upon virus challenge, all the macaques vaccinated i.m. with pCV-*tat* (monkeys 54920, 55122, 55361 and PR2) were negative for p27 antigenemia up to 40 weeks after challenge (Fig. 1a and b). In contrast, p27 antigen-

Table 4
Tat-specific CTLs in the vaccinated monkeys[a]

Group	Monkey	Specific killing (%) E:T ratios	
		25:1	12.5:1
tat DNA (1 mg, i.m.)	54920	18	5
	55122	11	9
	55361	13	10
tat DNA (0.5 mg, i.m.)	PR2	28	17
tat DNA (0.2 mg, i.d.)	37	6	2
Vector (CpG) DNA (1 mg, i.m.)	54219	0	0
RIBI	55123	0	0
Alum	55129	0	0

[a] CTL assays were performed at week 32 (week 36 for monkeys 55123 and 55129, week 42 for monkeys 37 and PR2) as described in Section 2. The assay was considered positive for values exceeding 5%. This cut-off value was determined by comparative experiments with Europium vs. ^{51}Chromium labeling of the targets as described in Section 2.

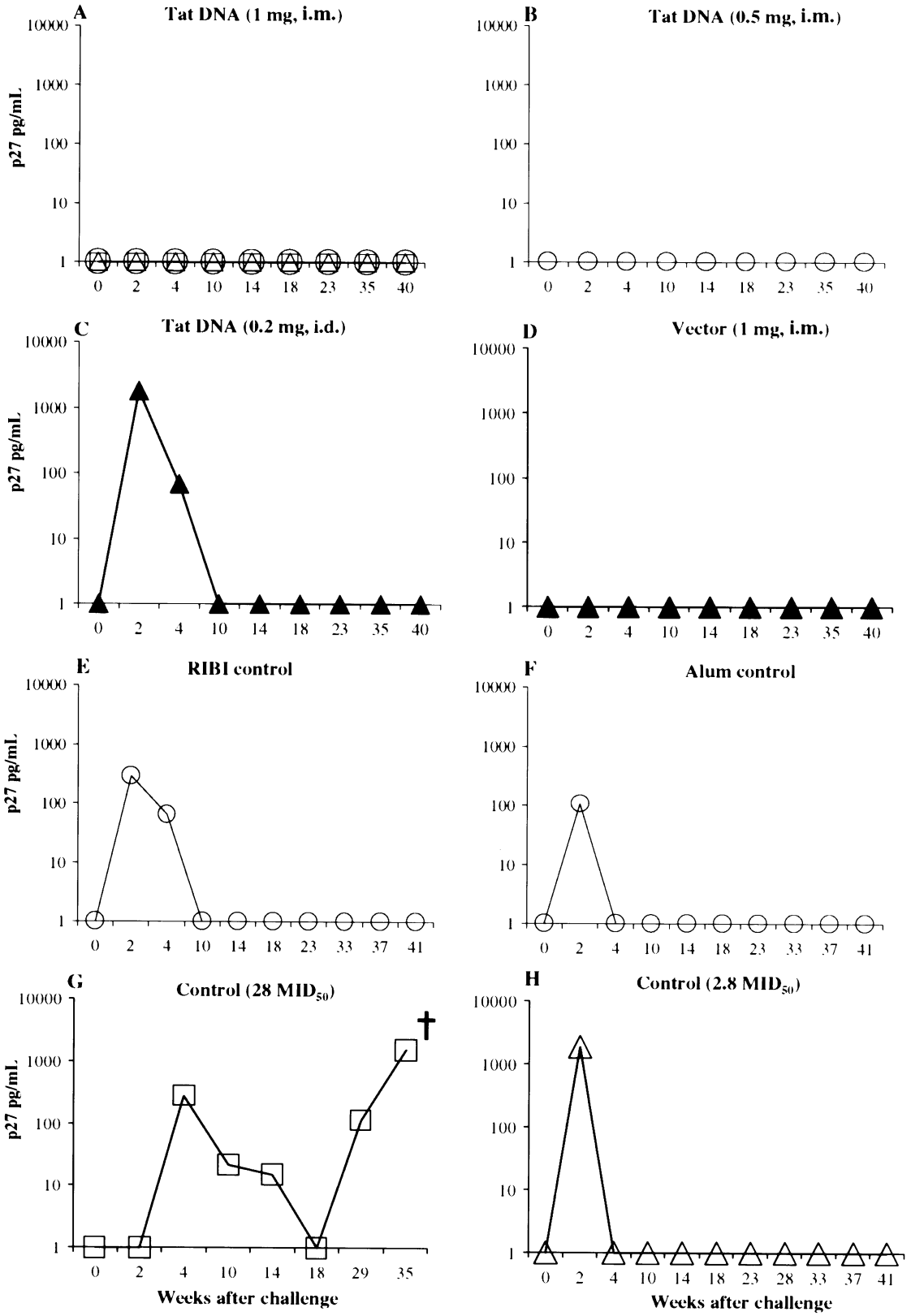

Fig. 1. p27 antigenemia after challenge with the SHIV89.6P. p27 antigenemia levels (pg/ml) up to 40–41 weeks after challenge are shown for the monkeys inoculated with (a) pCV-*tat* 1 mg, i.m.: monkeys 54920 (○); 55122 (□), 55361 (△); (b) pCV-*tat* 0.5 mg, i.m.: monkey PR2 (○); (c) pCV-*tat* 0.2 mg, i.d.: monkey 37 (▲); (d) pCV-0 1 mg, i.m.: monkey 54219 (▲); (e) RIBI alone, s.c.: monkey 55123 (○); (f) Alum alone, s.c.: monkey 55129 (○); (g) nothing: monkey 2 (□) and (h) nothing: monkey 12 (△). Monkey 2 was euthanized at week 35 after challenge.

Table 5A
Virus isolation and cytoviremia after challenge[b]

Group	Monkey	Viral isolation and cytoviremia (infected cells/10^6) Weeks post-challenge							
		2	4	10	14	18	23	29[a]	40
tat DNA (1 mg, i.m.)	54920	−	−	−	−	−	−	−	−
	55122	−	−	−	−	−	−	−	−
	55361	−	−	−	−	−	−	−	−
tat DNA (0.5 mg, i.m.)	PR2	−	−	−	ND	−	−	−	−
tat DNA (0.2 mg, i.d.)	37	+ (2818)	+ (354)	+ (21)	+ (106)	+ (256)	+ (6)	+ (22)	+
Vector (CpG) DNA (1 mg, i.m.)	54219	−	−	−	−	−	−	−	−
RIBI	55123	+ (708)	+ (355)	+ (11)	ND	+ (2)	+ (2)	ND	−
Alum	55129	+ (707)	+ (45)	+ (3)	ND	+ (16)	+ (3)	ND	−
Naive	12	+	+	ND	ND	−	+ (11)	+ (3)	−
Naive	2	+	+	ND	ND	ND	ND	+ (11)	ND[b]

[a] Week 28 for monkeys 55123, 55129 and 12.
[b] Monkey 2 was euthanized at week 35 after the viral challenge. Results of virus isolation (p27 production) above or below the cut−off value were expressed as positive (+) or negative (−), respectively. In parenthesis are shown the values of cytoviremia expressed as the number of infected cells/10^6 CD8-depleted PBMC, as described earlier [4]. ND, not done.

emia was detected both at week 2 and 4 post-challenge in monkey 37 immunized with pCV-*tat* i.d. (Fig. 1c). As for the i.m. vaccinated animals, monkey 54129, injected with pCV-0, was always negative for p27 antigenemia (Fig. 1d). In contrast, the two control macaques injected with RIBI or Alum alone (55123 and 55129), respectively (Fig. 4e and f), and the two naive control monkeys (2 and 12) (Fig. 1g and h) had detectable levels of p27 antigenemia at 2 and/or 4 weeks after challenge. In monkey 2 the p27 antigenemia rose again since week 29 (Fig. 1g).

Similarly, in the macaques inoculated by the i.m. route with pCV-*tat* or pCV-0, SHIV-RNA levels were undetectable throughout the follow-up period (Fig. 2a, b, and d), whereas in monkey 37, inoculated by the i.d. route with pCV-*tat*, a high RNA copy number was evident at week 4 and remained detectable up to week 40 post-challenge (Fig. 2c). Plasma viremia was also detected in all the control animals (Fig. 2e–h). In particular, in the two naive macaques it was very high at week 2, time point that was not available for the other animals. In monkey 2 plasma viremia remained high until the monkey's death (Fig. 2g).

In all the macaques vaccinated i.m. with pCV-*tat*, the SIV proviral DNA was undetectable or sporadically detected at very low levels (range 1–8 copies/μg DNA) (Fig. 3a and b). In contrast, in monkey 37, vaccinated i.d., a high proviral copy number was detected since week 2 post-infection and remained detectable for the entire follow-up period (Fig. 3c). In monkey 54219, injected with pCV-0, proviral DNA was detected at week 14 after challenge (55 proviral copies) (Fig. 3d). All control animals had a high proviral copy number since week 2 that remained always detectable (Fig. 3e–h).

Virus isolation, performed with CD8-depleted PBMC stimulated with PHA and rhIL-2, resulted always negative in all the macaques vaccinated i.m. and in the pCV-0-inoculated monkey (Table 5A). In contrast, virus was repeatedly isolated from monkey 37, injected with *tat* DNA i.d., and from all the control animals. Accordingly, cell-associated viremia (cytoviremia) was high in monkey 37 and in the control macaques that were tested (Table 5A). However, at week 18 post-challenge when virus isolation was also performed with CD8-depleted PBMC stimulated by an anti-monkey-CD3 mAb and rhIL-2, (Table 5B), virus was isolated from the monkey injected with pCV-0 (monkey 54129), but not from those vaccinated i.m. with pCV-*tat*. As expected, virus was also isolated from monkey 37, inoculated i.d. with pCV-*tat*, and from the control macaques that were tested also by this method (Table

Table 5B
Virus isolation from CD8-depleted PBMC after stimulation with anti-CD3 antibodies and rhIL-2[a]

Group	Monkey	p27 (pg/ml)
tat DNA (1 mg, i.m.)	54920	0
	55122	0
	55361	0
tat DNA (0.5 mg, i.m.)	PR2	0
tat DNA (0.2 mg, i.d.)	37	194
Vector (CpG) DNA (1 mg, i.m.)	54219	>305
RIBI	55123	22
Alum	55129	ND
Naive	2	ND
Naive	12	>305

[a] ND, not done. Shown are the results of p27 antigen production at day 11 of culture. Values below the cut-off (20 pg/ml) were given a 0 value. All 4 control animals and monkey 37 were also positive for virus isolation in other assays (Table 5A).

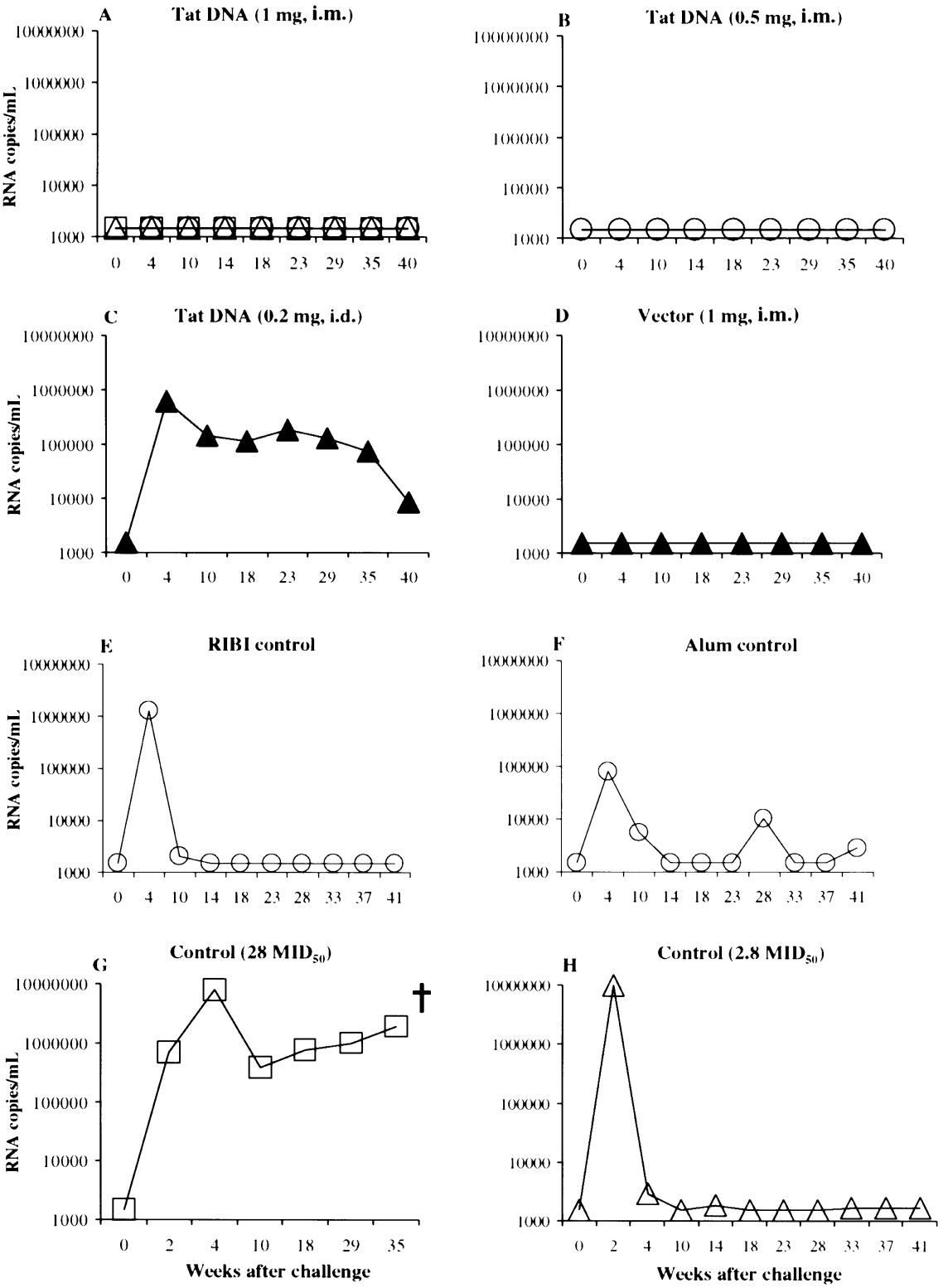

Fig. 2. Plasma viremia after challenge with the SHIV89.6P. Results (SHIV RNA copies/ml plasma) up to 40–41 weeks after challenge are shown for the monkeys inoculated with (a) pCV-*tat* 1 mg, i.m.: monkeys 54920 (○); 55122 (□), 55361 (△); (b) pCV-*tat* 0.5 mg, i.m.: monkey PR2 (○); (c) pCV-*tat* 0.2 mg, i.d.: monkey 37 (▲); (d) pCV-0 1 mg, i.m.: monkey 54219 (▲); (e) RIBI alone, s.c.: monkey 55123 (○); (f) Alum alone, s.c.: monkey 55129 (○); (g) nothing: monkey 2 (□) and (h) nothing: monkey 12 (△). Monkey 2 was euthanized at week 35 after challenge.

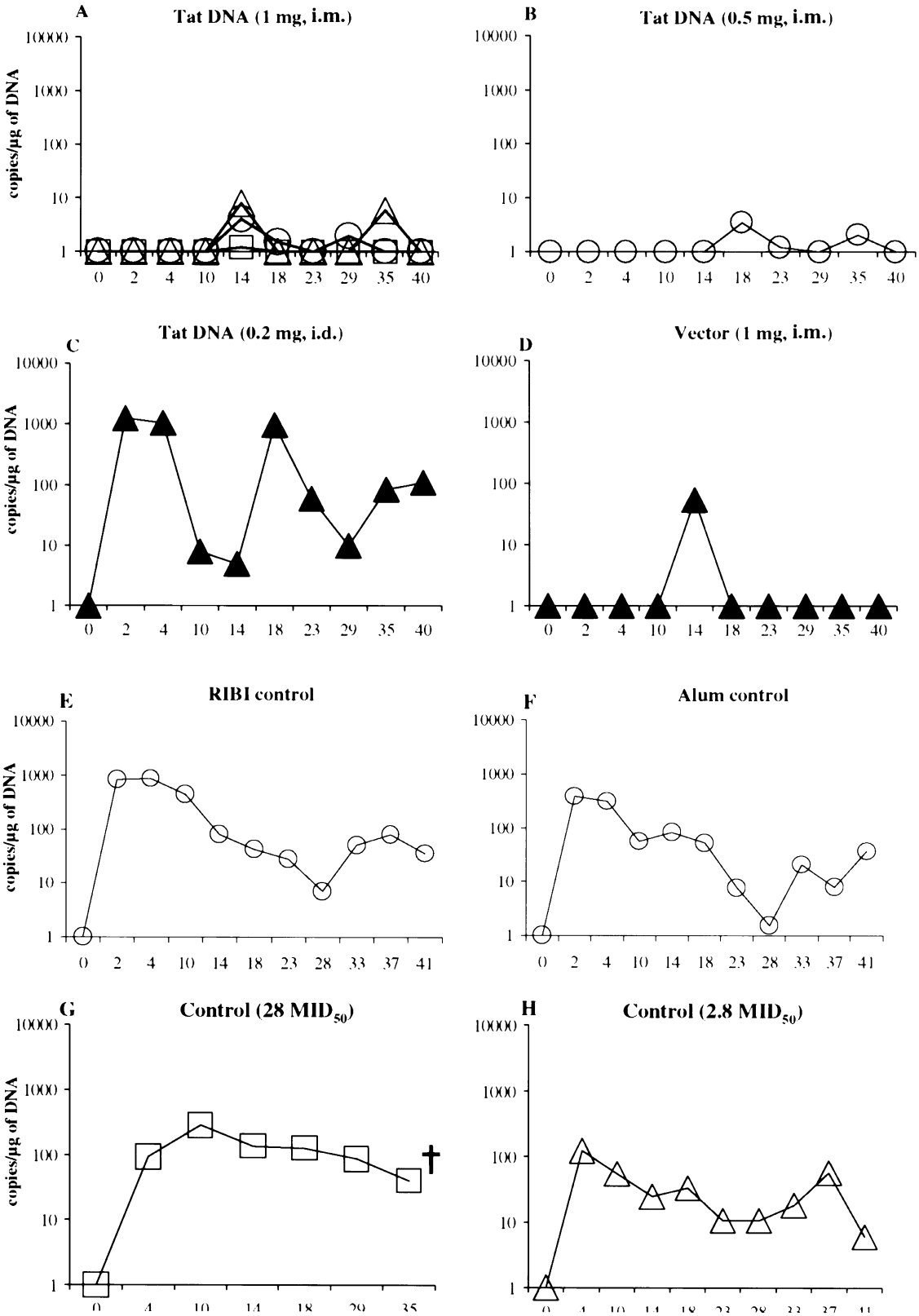

Fig. 3. SIV proviral copy number after challenge with the SHIV89.6P. Results (copies/μg DNA) up to 40–41 weeks after challenge are shown for monkeys injected with (a) pCV-*tat* mg, i.m.: monkeys 54920 (○), 55122 (□), 55361 (△); (b) pCV-*tat* 0.5 mg, i.m.: monkey PR2 (○); (c) pCV-*tat* 0.2 mg, i.d.: monkey 37 (▲); (d) pCV-0 1 mg, i.m.: monkey 54219 (▲); (e) RIBI alone, s.c.: monkey 55123 (○); (f) Alum alone, s.c.: monkey 55129 (○); (g) nothing: monkey 2 (□) and (h) nothing: monkey 12 (△). Monkey 2 was euthanized at week 35 after challenge.

Table 6
Anti-HIV-1 Env Ab titers in plasma and by IVAP after challenge[b]

Group	Monkey	Reciprocal of the last positive dilution Week post-challenge						IVAP
		4	10	23	29[a]	35[b]	40	
tat DNA (1 mg, i.m.)	54920	0	0	0	0	0	0	−
	55122	0	0	0	0	0	0	+
	55361	0	0	0	0	0	0	+
tat DNA (0.5 mg, i.m.)	PR2	0	0	0	0	0	0	−
tat DNA (0.2 mg, i.d.)	37	4	ND	64	512	256	2048	ND
Vector (CpG) DNA (1 mg, i.m.)	54219	0	0	0	0	0	0	−
RIBI	55123	4	64	64	128	128	128	ND
Alum	55129	0	ND	64	256	128	128	ND
Naive	2	0	8	64	512	2056	ND	ND
Naïve	12	0	10	ND	64	256	128	ND

[a] Week 28 for monkeys 55123, 55129 and 12.
[b] Week 33 for monkeys 55123 and 55129. Results are expressed as the reciprocal of the last positive dilution. Values below the cut-off were given a 0 value. IVAP was performed at 14 weeks post-challenge. (−) negative; (+) positive, indicates OD values three times higher than the average of the negative controls. ND, not done.

5B). Further attempts were made by both methods at week 40 after challenge, however, this time point was too late since no virus was isolated from any animal with the exception of monkey 37 that resulted positive in both assays and the monkey inoculated with RIBI alone that resulted positive only in the anti-CD3 stimulation system (data not shown).

All the macaques vaccinated with pCV-*tat* i.m. had undetectable or low (range 1:2–1:100) and transient anti-SIV Ab titers (Fig. 4a and b). In contrast, in monkey 37 (pCV-*tat*, i.d.) anti-SIV titers increased and remained stably high (Fig. 4c). Monkey 54219, injected i.m. with pCV-0, had no anti-SIV antibodies (Fig. 4d). The two macaques inoculated with RIBI or Alum alone (monkeys 55123 and 55129, respectively) and monkey 12 showed a progressive increase of anti-SIV Ab titers (Fig. 4e, f and h), whereas monkey 2 had no detectable anti-SIV antibodies (Fig. 4g), a relatively uncommon feature that correlates with fast progression [53].

No anti-HIV antibodies were detected in plasma from all the animals vaccinated i.m. and from the monkey inoculated with pCV-0, but they were detectable by IVAP in monkeys 55122 and 55361 at week 14 post-challenge (Table 6). High anti-HIV titers were detected in the plasma from monkey 37 and in the control macaques, including monkey 2, that had high and stable anti-HIV titers (Table 6).

Consistent with the data of the virological assays, in all monkeys injected i.m. with pCV-*tat* or pCV-0, the number of $CD4^+$ T cells remained in the normal range after the viral challenge and during all the follow-up period (Fig. 5). In contrast, in the infected animals (monkey 37 and all the control macaques) the $CD4^+$ T cells decreased and remained stably below the baseline values during the following weeks. Monkey 2 had a progressive $CD4^+$ T cell decline reaching a very low value at week 35, when the monkey was euthanized.

4. Discussion

It has been shown that the i.m. immunization with a *tat* DNA expressing vector containing defined unmethylated CpG sequences is safe, induces selectively Th-1 responses and anti-Tat CTL and, most importantly, controls the infection with the highly pathogenic SHIV89.6P to undetectable levels preventing the CD4 T cell decline in all vaccinated monkeys up to 40 weeks after challenge [4]. In contrast, the inoculation of lower *tat* DNA amounts (0.2 mg) by the intradermal route induces Th-2-type responses with antibody production but absence of CTL activity and does not protect from virus replication and disease.

These results are in agreement with our previous vaccination study with the HIV-Tat protein which was carried out in parallel with the present study. In the Tat protein protocol, in fact, anti-Tat CTLs were present prior to challenge only in the protected monkeys (5/7) and correlated with the long-term control of infection against challenge with the same SHIV89.6P virus stock. In contrast, the 2/7 vaccinated animals that had high anti-Tat antibody titers but had no anti-Tat CTL were highly infected [4,5], as shown here for the monkey inoculated i.d. with *tat* DNA. These results support the concept that the presence of anti-Tat CTLs is key to control the early phase of virus replication upon primary infection. In fact, anti-Tat CTLs are directed against early infected cells since Tat is a regulatory protein essential for virus gene expression that is expressed early upon infection and prior to virus produc-

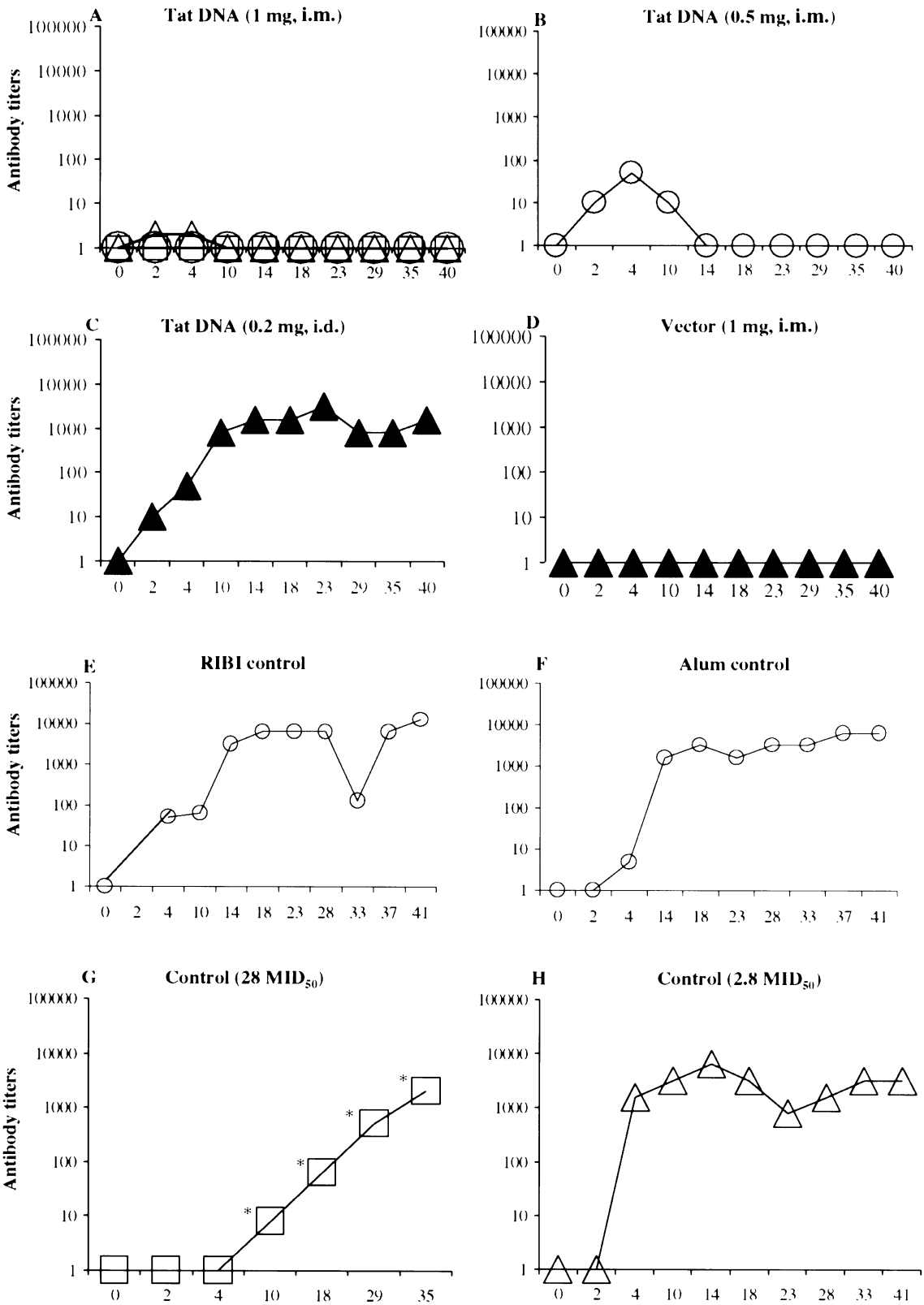

Fig. 4. Titers of antibodies against HIV-2/SIV after challenge with the SHIV89.6P. Anti-SIV Ab titers up to 40–41 weeks after challenge for monkeys inoculated with (a) pCV-*tat* 1 mg, i.m.: monkeys 54920 (○), 55122 (□), 55361 (△); (b) pCV-*tat* 0.5 mg, i.m.: monkey PR2 (○); (c) pCV-*tat* 0.2 mg, i.d.: monkey 37 (▲); (d) pCV-0 1 mg, i.m.: monkey 54219 (▲); (e) RIBI alone, s.c.: monkey 55123 (○); (f) Alum alone, s.c.: monkey 55129 (○); (g and h), nothing: monkey 2 (□), monkey 12 (△). Titers below 1:2 were given a 0 value. Monkey 2 was euthanized at week 35 after challenge. * Values refer to anti-HIV Ab titers (see Table 6).

tion and release. These data are also consistent with results in infected patients showing an inverse correlation between the presence of anti-Tat CTLs and HIV-1 viral load [31,35], and with a very recent study supporting the role of anti-Tat CTL in the early control of primary infection in the SIV model [37].

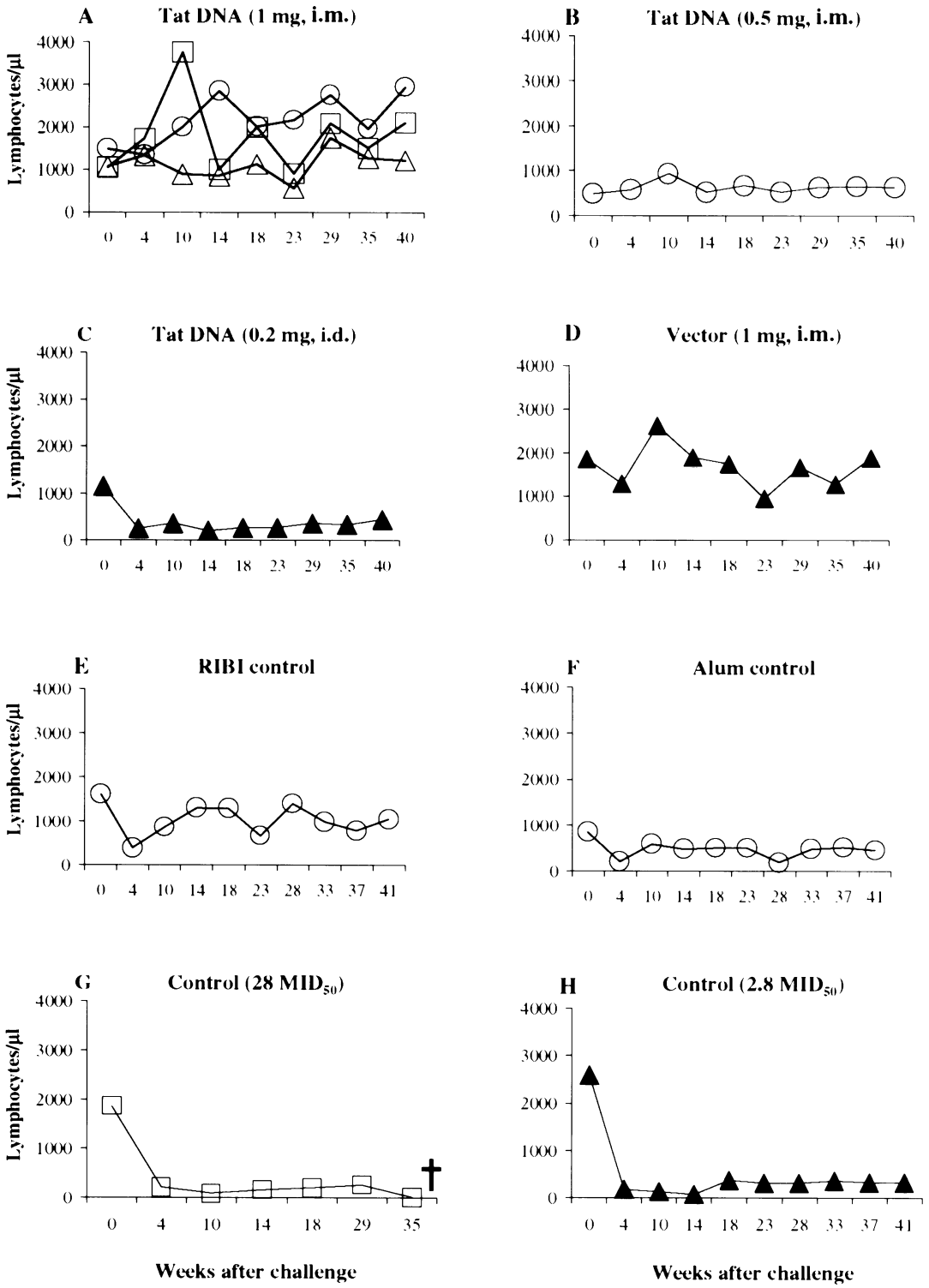

Fig. 5. CD4$^+$ T cell counts after challenge with the SHIV89.6P. CD4$^+$ T cell counts up to 40–41 weeks after challenge are shown for monkeys inoculated with (a) pCV-*tat* 1 mg, i.m.: monkeys 54920 (○), 55122 (□), 55361 (△); (b) pCV-*tat* 0.5 mg, i.m.: PR2 (○); (c) pCV-*tat* 0.2 mg, i.d.: monkey 37 (▲); (d) pCV-0 1 mg, i.m.: monkey 54219 (▲); (e) RIBI alone, s.c.: monkey 55123 (○); (f) Alum alone, s.c.: monkey 55129 (○); (g) nothing: monkey 2 (□) and (g) nothing: monkey 12 (▲). Monkey 2 was euthanized at week 35 after challenge.

In the present study a complete control of infection, i.e. undetectable virus replication and negative virus isolation, correlated with the presence of anti-Tat CTLs and Tat-specific TNF-α production during the immunization and prior to challenge. These responses were higher in the *tat* DNA-vaccinated animals as compared to the Tat protein-vaccinated monkeys [4], likely due to the unmethylated CpG sequences. However, the protective role played by the $CD8^+$ T cells may not be limited to CTLs. In fact, also a potent CD8-mediated non cytolytic antiviral activity, non-MHC-restricted and not mediated by β-chemokines [for review see references [54–57], was detected at week 10 and 18 post-challenge in all the protected and vaccinated monkeys but not in the controls (Goletti et al., manuscript in preparation). Of note, this antiviral activity was also present in the pCV-0-inoculated animal and this correlated with a partial protection from virus replication since virus was isolated from PBMC and proviral copy number was higher in the pCV-0-inoculated monkey as compared to the animals inoculated i.m. with pCV-*tat*. In these latter animals, in fact, virus was never isolated from PBMC and the proviral copy number was only sporadically detected and at very low levels (≤ 8 copies). Such CD8-mediated antiviral activity has already been shown to play a role in conferring protection in vaccination models with attenuated viruses [1–3,58–62] and to be present in long-term nonprogressor individuals [63,64], and appears to belong to the natural or innate immunity [for review see Ref. [37]]. Since the unmethylated CpG motifs present in the vector are known to trigger the innate immune response and to induce a CD8-mediated antimicrobial activity in in vivo model systems, it is tempting to speculate that by boosting the innate immunity, pCV-0 conferred partial protection against primary infection. This is currently being investigated in a new DNA vaccination protocol. Nevertheless, although the boosting of the innate immunity by unmethylated CpG sequences may induce a first block to the primary infection and reduce the levels of virus replication, a full control of the viral infection by vaccination appears to require a Tat-specific CTL response (i.e. pCV-*tat*).

Thus, the CpG-rich *tat* DNA vector has the double advantage of boosting the innate antiviral responses and of driving primarily anti-Tat CTL responses, both to levels capable of fully blocking early virus replication during primary infection.

Tat possesses immunogenic epitopes that are conserved among all the HIV-1 subtypes of group M [65]. In fact, sera from African individuals infected with different HIV strains well recognize the Tat derived from the B strain utilized in the vaccine (Buttò S. et al., submitted). Since *tat* DNA has already been shown to be safe and immunogenic also in seropositive individuals [66], the data shown herein indicate that a CpG-rich *tat* DNA vaccine may represent a promising candidate for preventive and therapeutic vaccinations in humans aimed at controlling virus replication and blocking disease onset.

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