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Expression of human immunodeficiency virus type 1 tat from a replication-deficient herpes simplex type 1 vector induces antigen-specific T cell responses

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Abstract

Herpes simplex type-1 virus (HSV-1) based vectors have been widely used in different gene therapy approaches and also as experimental vaccines against HSV-1 infection. Recent advances in the HSV-1 technology do support the use of replication defective HSV-1 as vaccine vectors for delivery of foreign antigens. We have examined the ability of a recombinant replication-defective HSV-1 vector expressing the HIV-1 Tat protein to induce long-term Tat-specific immune responses in the Balb/c murine model. The results showed that vector administration by the subcutaneous route elicits anti-Tat specific T-cell mediated immune responses in mice characterized by the presence of the Tat-specific cytotoxic activity and production of high levels of IFN-γ.

Keywords: HSV-1 replication-defective vector; HIV-tat protein; Vaccine; Immunization

1. Introduction

Despite the strong efforts to halt the HIV pandemic and to reduce the deaths for AIDS, it is clear that it is of extreme importance to develop an HIV vaccine capable of inducing an efficient anti-viral immunity and/or keeping under control the ongoing disease in the infected individuals [1–3]. There is a growing evidence about the importance of both cellular and humoral immune responses against HIV proteins for the control of disease progression during the natural infection [4–6]. It has been previously shown that a broad CTL response against both structural and regulatory viral proteins [7] plays a major role in the clearance of initial viremia [8,9] and in the containment of viral replication during the following stages of infection [10]. The wide genetic variability across the HIV-1 clades and also the high mutation rate of structural genes, resulting in the presence of multiple protein forms even in the same individual [11], has turned the HIV vaccine research direction towards the more conserved regulatory proteins, Tat, Rev and Nef, as possible components of a new combined vaccine [12–16]. In particular, it has been shown that the CTL response against the HIV-1 Tat protein inversely correlates with progression of disease in the infected individuals, i.e. the patients exhibiting stronger cytotoxic activity against Tat are frequently either non-progressors or have a slower disease progression in comparison with the patients showing low or no anti-Tat CTL responses [1,10,17,18]. In addition, recent studies have shown that immunization with HIV-1 Tat (protein or DNA) confers protection against the challenge with the highly pathogenic virus SHIV89.6P in monkeys and that protection correlates with the presence of anti-Tat specific immune responses [12,14,19–22].

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In order to develop new vaccination strategies able to enhance the cellular immunity and elicit specific antibodies towards Tat as well as other HIV-1 proteins [23,24], a large number of viral vectors expressing HIV or SIV antigens are under investigation [25,26], and among them poxvirus-based vectors which have been the most studied as HIV vaccine viral vectors [27], the alphavirus self-replicating vectors [28,29], the adenovirus [30–32], the lentivirus [33–35] or the herpes simplex virus vectors [36–38]. Despite the general concerns regarding safety issues using live viral recombinants, the overall results with these different viral vectors indicate that they might be good candidates for the development of an anti-HIV-1 vaccine. Many of these viral vectors have been reported for their capacity to induce strong *in vivo* Th_1 and CTL responses, as well as high antibody titres, against various HIV-1 gene products [39–41].

In particular, the herpes simplex virus (HSV) vectors show several advantages for prophylaxis against viral infections. They have been shown: (i) to elicit strong and durable immune responses by various routes of inoculation [38,42,43]; (ii) the viral DNA persists inside the host's cell nucleus as an episomal form, thus eliminating the safety concerns deriving from the random integration of the viral genome into the host's DNA; (iii) they carry the *tk* gene, encoding the viral thymidine kinase, that, in case of undesired effects, can be used, in combination with specific antiviral drugs, to kill the virus-harbouring cells.

The use of HSV vectors requires the development of mutated viruses that are genetically stable, incapable of replicating in the CNS and of spreading in immuno-compromised individuals, not transmissible from immunized individual by contacts and, at the same time, capable of inducing protective immunity against disease. Thus, replication-defective herpes simplex viruses characterized by the simultaneous deletion of multiple viral functions, including the immediate-early proteins ICP4, ICP27, ICP22 and the structural protein Vhs (viral host shutoff) have been developed [44,45]. These HSV mutants show a reduced cytotoxicity, due to their inability to replicate and to spread in the host, but maintain the capability to infect a wide range of tissues and host species. In addition, these recombinant replication-defective vectors are able to sustain high expression of the exogenous genes under homologous or heterologous promoters (HSV-1 or HCMV, respectively) [46], and because of their large genome can be engineered to simultaneously express multiple antigens [47]. Moreover, recent studies indicate that the pre-existing immunity against HSV infection does not compromise its efficacy as a vaccine vector [43,48].

In the present study, we investigated the ability of a recombinant replication-defective HSV-1 vector encoding the HIV-1 Tat protein to induce long-term Tat-specific immune responses in a murine model. The results demonstrate that the subcutaneous, but not the intranasal administration, elicited anti-Tat specific T-cell mediated immune responses characterized by Tat-specific cytotoxic activity and high levels of IFN-γ secretion.

2. Materials and methods

2.1. Generation of plasmids and recombinant replication-defective HSV-1 vectors

Plasmid pCV-tat, expressing the HIV-1 *tat* cDNA (HTLV-IIIB isolate, subtype B) has been previously described [49,50]. Plasmid DNA was purified from *Escherichia coli* by using the Qiagen endotoxin-free maxi kit (Qiagen, Hilden, Germany). Plasmid pB410-tat was constructed by introduction of the HIV-1 *tat* cDNA (350 bp) from pCV-tat into the HSV-1 UL41 locus of plasmid pB41 that has been described elsewhere [51]. The *tat* cDNA was inserted into *Eco*RI/*Xba*I sites of pB41 plasmid between the two UL41 HSV fragments (HSV genomic positions 90145–91631 and 92230–93854) [52], under the transcriptional control of the HSV immediate early ICP0 promoter. The T0Z-GFP is a replication-defective HSV-1 viral vector characterized by low cytotoxicity due to the deletion of three immediate early genes (ICP4, ICP27, which are essential for viral replication, and ICP22 which is not) and contains the *gfp* gene in the ICP22 locus and the *lacZ* gene in the UL41 locus as marker genes. Plasmid pB410-tat was constructed to genetically recombine with the genome of the T0Z-GFP viral vector using the previously described Pac-facilitated *lacZ* substitution method [52]. The generation of recombinant viruses was carried out using the standard calcium phosphate transfection procedure with 5 μg of T0Z-GFP viral DNA and 1 μg of linearized plasmid pB410-tat. Transfection and isolation of the recombinant viral progeny was performed in 7b cells, which are Vero cells stably transfected with HSV-1 ICP4 and ICP27 genes, as previously described [44,46].

The recombinant T0-tat virus containing the *tat* cDNA was first identified by isolation of a clear plaque phenotype after X-gal staining. The T0-tat virus was purified by three rounds of limiting dilution technique and the presence of the transgene was confirmed by Southern blot analysis. Viral stocks of the T0-tat and of the control vector T0-GFP (derived from T0Z-GFP without *lacZ* reporter gene in UL41 locus) were prepared and titrated using 7b cells [46].

2.2. Cell lines

The 7b Vero-derived cell line expresses the HSV-1 immediate early genes ICP4 and ICP27 required for virus replication [44,46]. Cells (monkey kidney fibroblast Vero, 7b, P815-1-1 murine ($H\text{-}2^d$) mastocytoma, baby hamster kidney (BHK) and murine fibroblast Balb/c cell lines) were grown in DMEM (Euroclone, Grand Island, NY) supplemented with 10% FBS (Euroclone), 2 mM L-glutamine, 100 μg/ml penicillin and 100 U/ml streptomycin. 7b cells were subjected monthly to 7-days long selection with 1 mg/ml G418 (Sigma). Human HeLa3T1 cells were grown in DMEM and 10% FBS; these cells contain an integrated copy of plasmid HIV-LTR-CAT where expression of the chloramphenicol acetyl transferase (CAT) reporter gene is driven by the HIV-1 LTR promoter

and occurs only in the presence of biologically active Tat protein [53]. Splenocytes from immunized and control mice were cultivated in RPMI 1640 (Euroclone) supplemented with 10% Hyclone (Euroclone), 50 μM β-mercaptoethanol and 10 mM HEPES.

2.3. Western blot analysis

Tat protein expression from the T0-tat vector was analyzed in murine fibroblast Balb/c cell line (1×10^6 cells) infected with T0-tat virus at a multiplicity of infection of 1 (m.o.i. 1). Cell extracts, corresponding to 10 μg of total proteins, were loaded onto 12% SDS-polyacrylamide gel and analyzed by Western blot using a rabbit anti-Tat polyclonal serum (Intracel) at 1:1000 dilution and a mouse anti-rabbit HRP-conjugated secondary antibody (Sigma) at 1:4000 dilution. Immunocomplexes were detected by ECL Western Blot detection kit (Amersham, Biosciences). Controls were represented by uninfected cells and cells infected at m.o.i. 1 with T0-GFP recombinant vector.

2.4. CAT assay

To analyse the expression of functional Tat protein, HeLa3T1 cells (1×10^6) were infected in suspension with T0-tat and control vector T0-GFP, at different m.o.i. (from 0.1 to 1) for 1 h at 37 °C under mild shaking. After infection, cells were washed twice with complete medium to eliminate the virus particles that did not infect the cells, plated onto six-well plates, and cultured at 37 °C, 5% CO_2 for 24 and 48 h. After incubation, cells were disrupted by sonication and cellular debris removed by centrifugation. CAT expression was measured on cleared supernatants after normalization to protein content (100 μg aliquots) as previously described [54].

2.5. HIV-1 Tat protein expression, purification, and manipulation

Recombinant and biologically active HIV-1 Tat protein from the HTLV-IIIB isolate (subtype B) used for *in vitro* experiments was produced and purified as previously described [55]. As Tat is photo- and thermo-sensitive, it was resuspended at 2 μg/ml in degassed phosphate-buffered saline without calcium and magnesium (PBS-A 1X) containing 0.1% BSA immediately before use and handled on ice and in the dark [55,56].

2.6. Virus stock purification

Recombinant replication-defective HSV-1 stocks were prepared by infecting 4×10^8 7b complementing cells with 0.05 m.o.i. of T0-tat and T0-GFP viruses in suspension in 15 ml of medium for 1 h at 37 °C under mild agitation. The infected cells were cultured at 37 °C, 5% CO_2 until a 100% cytopathic effect was evident. The cells were then collected and centrifuged at 2000 rpm for 15 min. The supernatants were spun at 20,000 rpm in JA20 rotor (Beckman) for 30 min to collect the virus. The cellular pellets were resuspended in 2 ml of medium, subjected to three cycles of freeze-thawing (−80 °C/37 °C) and a single burst of sonication, to release the viral particles. The virus was further purified by density gradient centrifugation (Opti Prep, Life Technologies Inc.) and resuspended in PBS-A 1X. Viral stocks were titrated as previously described [46] and stored at −80 °C. Titres averaged between 2×10^8 and 2×10^9 plaque forming units (pfu)/ml.

2.7. Animals and immunization protocols

Animals were handled according to national guidelines and institutional policy. Six-weeks old Balb/c (H-2^d) female mice were purchased from Harlan (Italy) and immunized after 1 week according to the protocols described below. Mice were observed twice a week until sacrifice.

- *First immunization protocol*: to determine the optimal dose of immunogen, mice were immunized with 4×10^4 or 4×10^6 pfu of T0-tat purified virus by subcute (s.c.) injection on the left flank and boosted s.c. with the same dose of virus at weeks 2, 4 and 9 after priming immunization. Control animals were injected with PBS-A 1X.
- *Second immunization protocol*: to compare the immune responses elicited after subcutaneous and intranasal administration, mice were inoculated with 4×10^6 pfu of T0-tat virus or PBS-A 1X, s.c. on the left flank or intranasally (i.n.). The animals were boosted with the same dose of virus s.c. and i.n., respectively, at weeks 2, 4 and 9 after priming.
- *Third immunization protocol*: to determine the effect of different boost-timing on the immune responses, mice were inoculated s.c. with 4×10^6 pfu of T0-tat virus or PBS-A 1X. Animals were boosted with the same dose of virus s.c. at weeks 2, 4 and 9 (time schedule 1), or at weeks 4 and 8 (time schedule 2) after priming.

The above-described doses of recombinant virus were administered in 100 μl for the s.c. route, and in 10 μl (5 μl/nostril) for the i.n. route. On day 14 from the last boost of each immunization protocol, five mice per group were sacrificed to collect their spleens and blood samples for the analysis of individual immune responses.

2.8. Serology

Anti-Tat IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA). The concentration of the recombinant protein used for coating was 1 μg/ml diluted in 0.05 M carbonate buffer (pH 9.6–9.8), and 100 μl/well were added to 96-well immunoplates (Nunc-Immunoplate F96 Polysorp, Nunc, Naperville, IL). The plates were sealed and incubated in the dark for 18–20 h at 4 °C. Prior to use, the plates were extensively washed with 0.05% Tween 20 in PBS-A 1X and blocked for 90 min at 37 °C with 3% bovine serum albumin (BSA) in PBS-A 1X. Sera were two-fold diluted in

PBS-A 1X containing 3% BSA and each sample was run in triplicate wells (100 μl/well). After incubation at 37 °C for 90 min, the plates were washed and immunocomplexes were detected with 100 μl/well of HRP-conjugated anti-mouse IgG (Amersham Biosciences) diluted 1:1000. After incubation at room temperature for 90 min, the wells were washed, and 100 μl/well of ABTS (Sigma) were added as HRP substrate. The reaction was blocked with 100 μl of 0.1 M citric acid per well. The absorbance was measured at 405 nm in an automated plate reader (ELX-800, Bio-Tek Instruments, Winooski, UT).

2.9. Splenocyte purification

Mice spleens were disrupted with 2 ml syringe plungers using 70 μm pores cell strainers (Falcon), resuspended in PBS-A 1X with 2 mM EDTA and, after 15 min centrifugation at 1500 rpm, treated with red blood cell lysis buffer (100 mM NH_4Cl, 10 mM $KHCO_3$, 10 μM EDTA) for 4 min at room temperature and finally washed with RPMI 1640 medium (Euroclone) containing 3% of heat-inactivated FBS. Cells were resuspended in RPMI 1640 complete medium with 10% Hyclone, counted using the trypan blue exclusion method and incubated in a humidified 5% CO_2 atmosphere at 37 °C at the final concentration of 2.5×10^6/ml.

2.10. Lymphoproliferation assay

Splenocytes (2×10^5 cells/well) were cultured in 96-well plates in the presence of 1 and 5 μg/ml of recombinant Tat protein, 10 μg/ml of Concanavalin A (Con A, ICN) or culture medium alone as positive and negative controls, respectively. After 48 h, bromodeoxyuridine (BrdU) was added (10 μM final concentration) to the plates and BrdU incorporation was determined after 16 h using a cell proliferation ELISA system (Amersham Biosciences) according to the manufacturer's instructions. Each sample was run in quadruplicate.

2.11. CTL assay

Mice splenocytes were co-cultivated at 1.5:1 ratio with naive syngeneic stimulator splenocytes, previously irradiated at 30 Gy, in the presence of 1 μg/ml of purified recombinant Tat protein. Recombinant human IL-2 (rhIL-2, 10 U/ml) was added to the cells after 3 days of culture. ^{51}Chromium (^{51}Cr) release assays were performed on day 6 of culture using P815-1-1 target cells, pre-incubated overnight with 2 μg/ml of Tat, as previously described [57], and with additional 10 μg of protein during the ^{51}Cr (100 μCi/target) labelling step. After 4 h incubation of effector and target cells at 37 °C, 5% CO_2, supernatants were harvested and the ^{51}Cr released by the lysed target cells was quantified using a γ-counter. Percent of specific lysis was calculated according to the following formula: % specific lysis = 100 × [cpm (sample release) − cpm (min release)]/[cpm (max release) − cpm (min release)]. The minimum release is represented by the spontaneous release of the ^{51}Cr isotope from the target cells and the maximum release is obtained by addition of 5% solution of Triton X-100 in PBS-A 1X to the target cells. Each sample was tested in triplicate.

2.12. Cytokine ELISA

The cytokine profile was determined in culture supernatants of mice splenocytes (2.5×10^6/ml in 48-well plate) incubated with 1 μg/ml of recombinant Tat protein and 10 U/ml of rhIL-2 (Roche) from day 3. On day 6 of culture standard sandwich ELISA tests were performed, using antibodies and recombinant standard proteins purchased from ENDOGEN. A 100 μl/well of anti-IFN-γ antibody (1 μg/ml diluted in 0.05 M carbonate buffer, pH 9.6–9.8), or of anti-IL-4 antibody (2 μg/ml diluted in PBS-A 1X) were added to 96-well plates (Nunc-Immunoplate). The plates were sealed, incubated 16 h at 4 °C and blocked for 60 min at room temperature with 4% BSA in PBS-A 1X. Cell culture supernatants (50 μl), undiluted or diluted 1:10, were added to the plates in triplicates and incubated for 60 min at room temperature. Without washing the plates, the biotinylated anti-IFN-γ and anti-IL-4 antibodies (0.4 μg/ml diluted in PBS-A 1X + 4% BSA) were added and incubated for 60 min at room temperature. After three washes, HRP-streptavidin was added at 1:6000 (IFN-γ) or 1:20,000 (IL-4) dilutions in PBS-A 1X + 4% BSA and incubated for 30 min. TMB (3,3′,5,5′-tetramethylbenzidine) (100 μl/well) was added as chromogen substrate. Reaction was blocked with 1 N HCl after 5 min. The absorbance was measured at 450 nm in an automated plate reader (ELX-800, Bio-Tek Instruments, Winooski, UT).

2.13. Statistical analysis

The statistical analysis was performed by ANOVA or Student's *t*-test, using the GraphPad Prism 4 Software.

3. Results

3.1. Construction of recombinant T0-tat replication-defective HSV-1 vector and analysis of Tat expression

A replication-defective HSV-1 virus was modified in order to express the HIV-1 Tat protein under the control of the HSV-1 immediate early (ICP0) promoter (T0-tat) (Fig. 1). The Herpesviridae immediate early promoters allow high-level but short-term expression of a transgene [58]. This recombinant virus was generated by homologous recombination between the pB410-tat plasmid and the HSV-1 T0Z-GFP triple mutant virus, and contains the *tat* gene in the UL41 HSV-1 locus. The presence of the *tat* gene in the HSV-1 genome was determined by Southern blot analysis. Expression of the Tat protein was assessed by infecting Balb/c

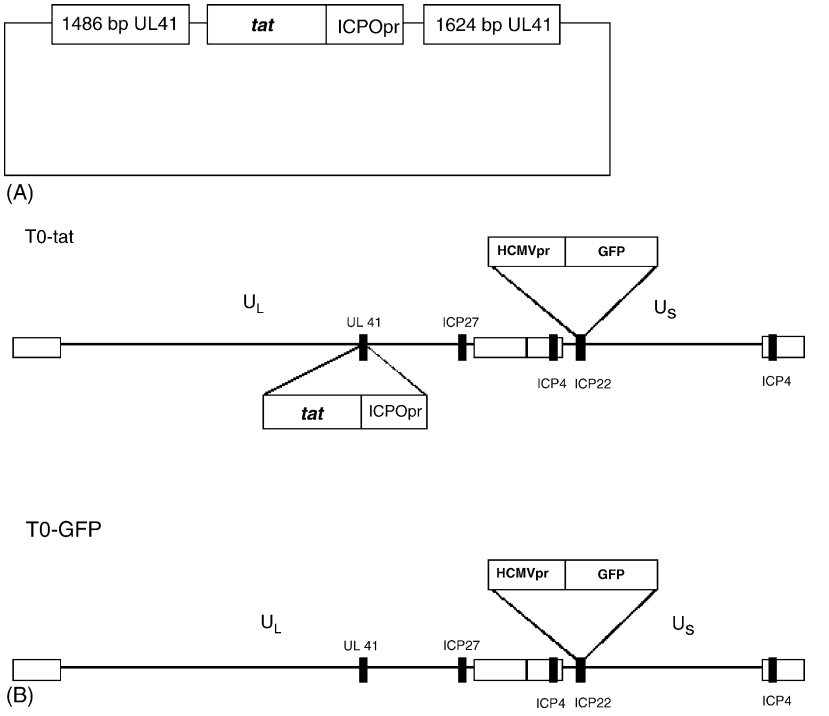

Fig. 1. Schematic representation of pBlueScript plasmid containing UL41-pICP0-tat cassette (A) and of the T0-GFP and T0-tat HSV-1 replication-defective vectors (B). (A) Schematic representation of pBlueScript plasmid, containing *tat* cDNA under the control of HSV-1 ICP0 promoter flanked by HSV UL41 sequences (pB410-tat). The homologous recombination event between viral DNA of T0Z-GFP vector and pB410-tat resulted in the generation of the T0-tat recombinant virus. (B) Expression of GFP gene is driven by HCMV IE promoter. The black squares symbolize the IE genes (ICP4, 27, 22) and other genes that are deleted in the HSV backbone. The white squares symbolize the terminal and internal repeats of the HSV genome delimiting the unique regions (U_L, unique long; U_S, unique short).

fibroblasts with T0-tat replication-defective HSV-1 recombinant vector and analyzing Tat expression by Western blot after 24, 48 and 72 h post-infection. Controls were represented by cells infected with the T0-GFP vector or uninfected cells. As shown in Fig. 2A, the T0-tat vector expressed Tat at high levels. Tat expression was detectable 24 h after infection and increased up to 72 h post-infection. Similar patterns of Tat expression were detected in Vero, BHK, HeLa and P815-1-1 cells (data not shown).

To determine whether T0-tat infected cells release Tat in the extracellular medium and whether extracellular Tat is taken up by uninfected cells, Balb/c fibroblasts were grown for 4, 6, 8, 12 and 18 h with cell-free culture supernatants (collected at 48 h post-infection) of T0-tat infected cells. Internalization of Tat by Balb/c cells was then analyzed at each time-point on cell lysates by Western blot. Balb/c cells cultured for 8 h with supernatant derived from T0-GFP infected cells represented the negative control. As shown in Fig. 2B, T0-tat infected cells released Tat in the medium, and uninfected cells efficiently internalized the extracellular protein, in accordance with the previously published data on Tat uptake [56]. In particular, Western blot analysis showed that Tat protein was promptly detected in cell lysates of uninfected Balb/c cells after 4 h of incubation with Tat-containing supernatants and its uptake increased up to 8 h of incubation. After 12 and 18 h Tat was undetectable (Fig. 2B),

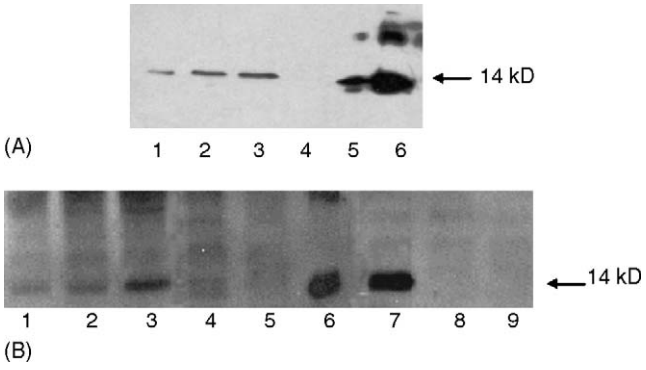

Fig. 2. Western blot analysis of Tat protein expressed by the T0-tat HSV-1 vector. (A) Balb/c cells infected with 1 m.o.i. of T0-tat and analyzed at 24 (lane 1), 48 (lane 2) and 72 (lane 3) h post-infection. Control cells were infected with 1 m.o.i. of the T0-GFP vector (lane 4). Recombinant Tat protein was loaded as the positive control at 20 ng (lane 5) and 50 ng (lane 6). (B) Analysis of Tat protein uptake in Balb/c cells cultured with media obtained from T0-tat infected cells. Cell lysates of Balb/c cells after 4 (lane 1), 6 (lane 2), 8 (lane 3), 12 (lane 4) and 18 (lane 5) h incubation with cell-free medium from T0-tat infected cells are shown. Positive controls were the recombinant Tat protein (50 ng) (lane 6) and Balb/c cell lysates after infection with 1 m.o.i. of T0-tat (lane 7). Balb/c cells cultured with supernatant derived from Balb/c T0-GFP infected cells (lane 8) and lysates of Balb/c cells infected with 1 m.o.i. of T0-GFP (lane 9) were loaded as negative controls. The arrow indicates the 14 kDa Tat protein.

probably because Balb/c cells had already processed it by that time.

Next, to determine whether Tat produced by the T0-tat vector is biologically active, HeLa3T1 cells, containing an integrated copy of the CAT reporter gene under the transcriptional control of the HIV-LTR promoter, and in which CAT expression occurs only in the presence of bioactive Tat, were infected at different m.o.i. (0.01–1) of T0-tat or T0-GFP control vector. CAT expression was measured 24 and 48 h post-infection. As shown in Table 1, high levels of CAT expression were readily detected at 24 h after infection, even at the lowest m.o.i. of 0.01.

In conclusion, these results indicate that the replication-defective HSV-1 T0-tat vector expresses high amount of bioactive HIV-1 Tat upon infection of various cell lines. The Tat protein is released in the extracellular medium and efficiently taken up by neighbouring cells where it is further processed, presumably by entering both MHC class I and class II pathways, suggesting that this vector may represent a useful delivery system for vaccination.

3.2. Analysis of the immune response induced in mice by T0-tat immunization

Different vaccination protocols were tested in order to determine the optimal route as well as the dose of T0-tat recombinant vector required for induction of efficient anti-Tat cell-mediated and humoral immune responses.

To determine the optimal dose of recombinant T0-tat virus, in the first experiment mice were immunized s.c. with 4×10^4 or with 4×10^6 pfu and boosted at weeks 2, 4 and 9 after priming. The analysis of the immune responses demonstrated that only mice immunized with the higher dose of T0-tat recombinant virus mounted a significant T cell-mediated response against Tat (data not shown). From these preliminary studies the dose of 4×10^6 pfu recombinant virus was chosen for the subsequent experiments.

In the second set of experiments we analyzed the immune responses elicited by T0-tat administered parenterally or mucosally. Mice were vaccinated s.c. or i.n. with 4×10^6 pfu of T0-tat and boosted with the same dose of T0-tat s.c. or i.n. at weeks 2, 4 and 9 after priming (Fig. 3A). The results of this experiment, shown in Fig. 3B, indicated that only mice vaccinated with T0-tat by the s.c. route developed specific CTL response against Tat. In addition, high levels of IFN-γ (2150 ± 270 pg/ml) were detected only in splenocytes of mice inoculated s.c. with T0-tat. On the contrary, mice inoculated i.n. with T0-tat did not develop any specific anti-Tat response in a fashion similar to control mice injected with PBS-A 1X. Tat-specific proliferation and IL-4 production, analyzed by BrdU and cytokine ELISA assays, respectively, were not detectable in any of the experimental groups immunized s.c. or i.n. Finally, low but statistically significant ($P < 0.001$) levels of Tat-specific antibodies, with the endpoint titres of 1:100, were detected in the sera of s.c. T0-tat inoculated animals as compared to both i.n. T0-tat and PBS-injected mice (Fig. 4). A possible explanation for the different responses, after s.c. and i.n. immunization, could be that immunization by i.n. route might require a higher dose of recombinant virus in order to induce an antigen specific immune response. Alternatively, the immunization by the i.n. route should be implemented with an adequate mucosal adjuvant to achieve the desired effect.

Finally, in the third set of experiments we tested the immune responses elicited by s.c. vaccination with 4×10^6 pfu of T0-tat using two different time schedules of immunization. Mice were boosted at weeks 2, 4 and 9 after priming (time schedule 1) or at weeks 4 and 8 after the

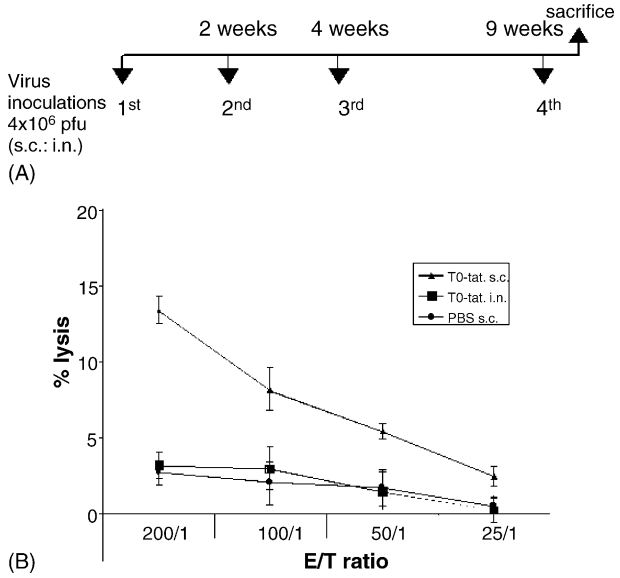

Fig. 3. Analysis of anti-Tat immune responses elicited by vaccination with 4×10^6 pfu of T0-tat HSV-1 vector subcutaneously (s.c.) or intranasally (i.n.). (A) Schematic representation of the immunization regimen. Mice were boosted at weeks 2, 4 and 9 after the first inoculation. (B) Tat-specific CTL response analyzed by ^{51}Chromium release assays on restimulated splenocytes of single mice. The data shown in the chart correspond to the mean % of specific lysis (±S.D.) of five mice per experimental group determined at different effector to target (E/T) ratios.

Table 1
Analysis of the biological activity of Tat protein expressed by T0-tat vector infected cells[a]

m.o.i.	% of acetylation			
	24 h p.i.[b]		48 h p.i.[b]	
	T0-tat	T0-GFP	T0-tat	T0-GFP
1	99.3	0	100	21
0.5	99.3	0	99.5	16
0.1	99.3	0	99.5	2.3
0.05	98	0	99.1	0
0.01	85	0	98	0
Uninfected cells	0	0	0	0

[a] HeLa3T1 cells containing an integrated CAT gene under the control of the Tat-inducible HIV-1 LTR were infected at various multiplicities of infection (m.o.i.).

[b] Results are expressed as percentage (%) of acetylation at 24 and 48 h post-infection (p.i.) with T0-tat or T0-GFP vectors.

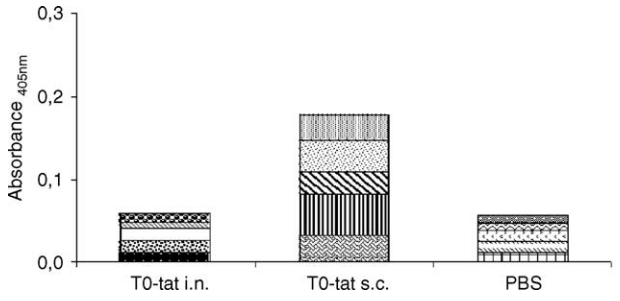

Fig. 4. Tat-specific IgG antibodies in the sera of Balb/c mice immunized with T0-tat replication-defective HSV-1 vector. Mice were injected with 4×10^6 pfu of T0-tat and boosted with the same dose of virus s.c. or i.n. Control mice were inoculated with PBS-A 1X. Sera were collected at sacrifice and tested by ELISA. Results correspond to absorbance values at 405 nm of serum samples (diluted 1:100) of five individual mice per experimental group.

first immunization (time schedule 2) (Fig. 5A). The results, shown in Fig. 5B, indicated that an effective antigen-specific CTL response was developed only in mice receiving the first boost 2 weeks after the priming immunization and not in those receiving the first boost 4 weeks after the priming. Similarly, IFN-γ production was higher, although the difference was not statistically significant ($P > 0.05$), in mice vaccinated according to time schedule 1 (3451 ± 1677 pg/ml) in comparison to mice vaccinated according to the time schedule 2 (1627 ± 540 pg/ml). No IFN-γ was detectable in Tat-restimulated splenocytes of PBS-injected mice. IL-4 production and Tat-specific lymphoproliferation were undetectable in all T0-tat vector or PBS-injected animals. Low levels of Tat-specific IgG were detected only in the sera of mice vaccinated with T0-tat according to time schedule 1 (data not shown).

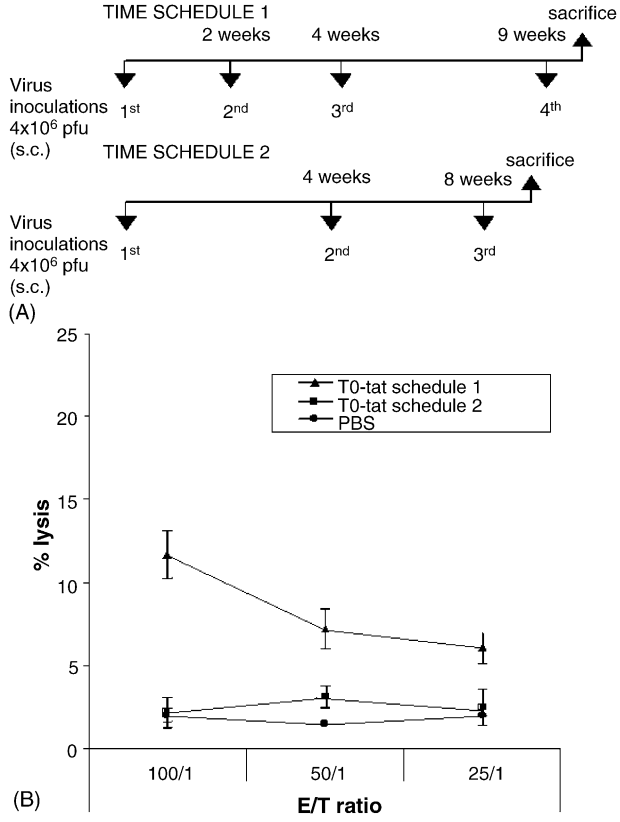

Fig. 5. Analysis of anti-tat immune response elicited by s.c. vaccination with 4×10^6 pfu of T0-Tat virus according to different time schedules of immunizations. (A) Schematic representation of the prime/boost immunization schedule. Mice were immunized with T0-tat and boosted at weeks 2, 4 and 9 (time schedule 1) or at weeks 4 and 8 after the first inoculation (time schedule 2). (B) Tat-specific CTL response measured by ^{51}Chromium release assays. The data shown in the chart correspond to the mean ± S.D. of groups of five mice inoculated with the T0-tat vector according to the time schedules 1 and 2 or with PBS-A 1X.

Since an important issue in vaccine development is the safety of the vaccine formulation, the site of injection and the general health of the mice were monitored twice a week. No signs of local or systemic adverse reactions were ever observed in mice vaccinated s.c. or i.n. with the HSV-1 vectors, as compared to control mice inoculated with PBS-A 1X buffer alone or untreated mice. In addition, no specific alterations that may be related to injection of HSV-1 T0-tat vector were reported in the organs examined at sacrifice (kidney, heart, lungs, intestine, liver, brain and draining lymph nodes). These results indicate that both the replication-deficient HSV-1 vectors and the HIV-1 Tat are safe, in accordance with previously published data [19,59–61] and with the recent results of phase I clinical trials carried out in Italy with a Tat protein-based vaccine [62].

4. Discussion

Currently, many vector systems are being developed for use in vaccine design, and among them those based on herpes simplex virus, adenovirus, poxvirus and alphavirus [27,36,63,64]. The efficacy of all these vectors might potentially be affected by the pre-existing immunity to viral antigens in the host. Nevertheless, herpes simplex viral recombinants have been reported to elicit antigen-specific immune response despite pre-existing immunity against viral antigens in the host [43,48,65]. Moreover, two different recombinant vectors based on herpes simplex virus have been shown to efficiently transduce tumours in mice previously exposed to HSV infection after direct intratumoural inoculation [66,67]. However, intravenous delivery of an HSV-1 recombinant vector has been reported to reduce its capability to transduce a murine tumour [68], which is in accordance with the recent data [69] indicating the C3 fraction of complement as an important factor in serum antiviral activity. These data suggest that the route of administration of the recombinant HSV vector is extremely important in determining the efficacy of transgene delivery to the host cells, whether it is a vaccine antigen, a cytokine or a suicide gene. Another concern regarding the use of HSV recombinants as vaccination vectors is based on the fact that the viral host shutoff (Vhs) protein encoded by the UL41 gene has been shown to block dendritic cell (DC) maturation, and thus to inhibit the immune response

against the vector-delivered transgene [70]. The elimination of the UL41 locus from the viral genome was reported in the same paper to allow DC activation and also to stimulate the antigen specific T-cell response *in vitro*.

In the present study, we describe the construction and the *in vitro* and *in vivo* characterization of a replication-defective HSV-1-based vector deleted of multiple immediate early regulatory genes and also containing a deletion in the non essential UL41 locus, where the transgene was inserted. The replication incompetent HSV-1 vector conserves the capacity to infect both dividing and non dividing cells of different species and origin as efficiently as a wild-type herpes virus, but it is much safer due to the incapacity to replicate and express viral proteins after infection. These characteristics, together with the capability of efficiently infecting and allowing DC maturation, make the multiply-deleted HSV-1 vectors a promising delivery system for vaccine studies in different experimental models. Consistently with previous studies [21,59,71–75], we continued to use as model antigen the HIV-1 *tat* gene which in the form of naked *tat* plasmid DNA induces mainly cellular immune responses, and requires high doses and multiple boosts to elicit an effective immunity in mice, monkeys or humans [61,71–76]. In addition, the Tat-vaccine has recently completed preventive and therapeutic phase I clinical testing in Italy and, based on the results, has been granted for funding for phase II-proof of concept trial as both preventive and therapeutic vaccine.

The results of the present study showed that the T0-tat vector was able to express high amount of HIV-1 Tat protein upon infection of various cell lines and, importantly, the protein maintained its biological activity, as indicated by its ability to trans-activate CAT gene expression. Furthermore, the Tat protein was promptly released in the extracellular medium by T0-tat vector-transduced cells and it was readily taken up by uninfected cells cultivated in the presence of culture medium derived from T0-tat transduced cells. Afterward, the T0-tat vector was injected in Balb/c mice, subcutaneously or intranasally to avoid the antiviral serum activity, to investigate its capacity to induce primary as well as long-term immune response against the HIV-1 Tat protein.

The results showed that the T0-tat vector elicited Tat-specific immune responses, mainly of Th_1-type, although the breadth of the response was different depending on the viral dose, the site of inoculation, and the timing of the boosts. In fact, anti-Tat immune responses were not detected in mice vaccinated with 4×10^4 pfu of T0-tat nor when mice were immunized by the intranasal route. In contrast, the best immune responses were detected in mice vaccinated with 4×10^6 pfu of T0-tat virus administered by the subcutaneous route and boosted at weeks 2, 4 and 9. Indeed, in this experimental group a significant Tat-specific cytolytic activity and higher production of IFN-γ was observed as compared to mice vaccinated s.c. with the same dose of T0-tat and boosted at weeks 4 and 8. In addition, although at low titres, anti-Tat antibody production was observed only in mice immunized s.c. with T0-tat at weeks 0, 2, 4 and 9. Interestingly, no significant Tat-specific T cell proliferation or IL-4 production were observed in T0-tat immunized animals at any viral dose, administration route or vaccination protocol.

Altogether, these results indicate that the T0-tat vector is capable of inducing HIV-1 Tat-specific immune response when administered s.c. in mice. The data we obtained are consistent with previously published results indicating that the same replication-defective HSV-1 delivery system induces a predominant Th_1-type immunity against the delivered antigen [77]. In fact, a single vaccination with the T0-ova vector, carrying the chicken ovalbumin gene, was shown to elicit strong cellular ovalbumin-specific immune responses in mice, characterized by high frequency of primary and memory CTL cells. Although only very low ovalbumin-specific CD4 T cell responses and IgG titres were detected, the single T0-ova i.v. injection was capable of protecting mice from subsequent challenge with lethal doses of ovalbumin-expressing *L. monocytogenes* [77].

Nevertheless, since a growing body of experimental evidence indicates the importance of both cellular and humoral anti-Tat immune response in the control of the HIV-1 replication and in disease progression (reviewed in [78]), the poor anti-Tat antibody induction may hamper the use of the recombinant HSV-1-based T0-tat vector in future vaccination studies aiming at eliciting a broader cellular and humoral Tat-specific immunity. However, a heterologous prime/boost regimen, in which the T0-tat virus would be either preceded or followed by one or multiple inoculations with the recombinant native Tat protein, is likely to improve anti-Tat antibody production, as previously described for other HIV and SIV antigens [79–83].

Of importance, the results of this study indicate that the T0-tat vector is safe since no adverse effects that may be related to its administration either s.c. and i.n. were ever observed during the experiments and at sacrifice, thus confirming the results of previous studies [19,59–62].

The appealing properties of replication incompetent HSV-1-based vectors inducing strong CTL responses against the delivered genes both in murine and in simian models [36,77,84] have made them very promising candidates for potential HIV-1 and also other viral or intracellular bacterial pathogens vaccine development. The capability of these vectors to directly infect the dendritic cells and to allow, in absence of functional UL41 gene product, their maturation and therefore correct antigen presentation necessary for T-cell priming, represent major advantages of HSV-based recombinant live viral particles in designing of future vaccination strategies. Finally, due to the capability of the HSV backbone vector to accommodate up to 50 kbp of exogenous DNA, the possibility to combine multiple vaccine antigens with genes encoding cytokines and/or other immunostimulatory molecules in a unique replication-defective vector represents another important feature in vaccine design since an efficient immune response against a pathogen may often require multi-component vaccine formulations and/or appropriate molecular adjuvants.

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