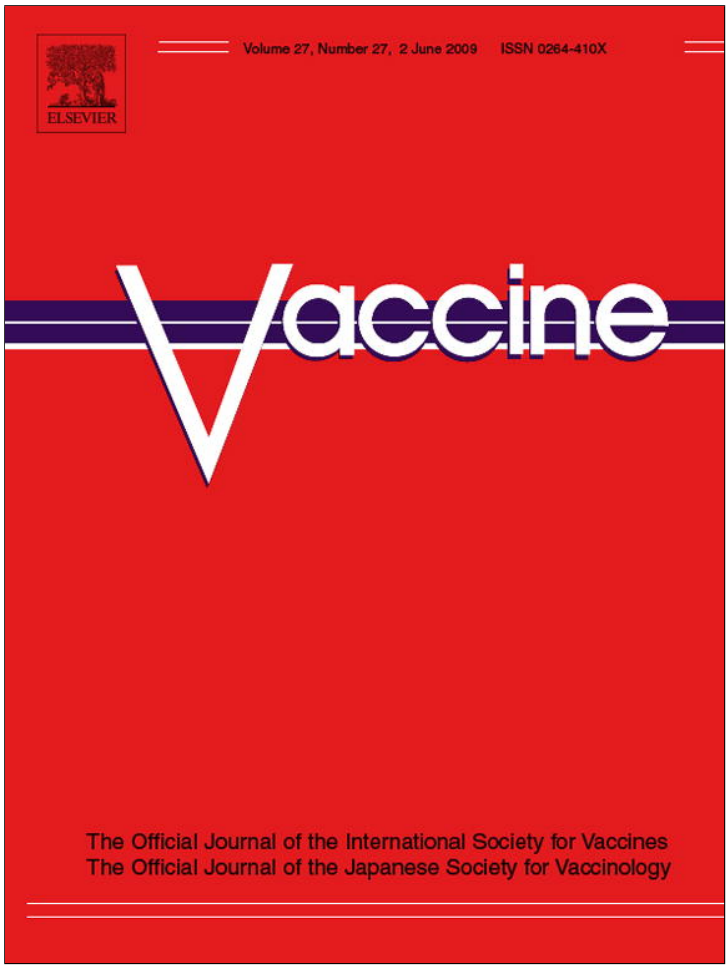

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Induction of humoral and enhanced cellular immune responses by novel core–shell nanosphere- and microsphere-based vaccine formulations following systemic and mucosal administration

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ABSTRACT

Anionic surfactant-free polymeric core–shell nanospheres and microspheres were previously described with an inner core constituted by poly(methylmethacrylate) (PMMA) and a highly hydrophilic outer shell composed of a hydrosoluble co-polymer (Eudragit L100-55). The outer shell is tightly linked to the core and bears carboxylic groups capable of adsorbing high amounts (antigen loading ability of up to 20%, w/w) of native basic proteins, mainly by electrostatic interactions, while preserving their activity. In the present study we have evaluated in mice the safety and immunogenicity of new vaccine formulations composed of these nano- and microspheres and the HIV-1 Tat protein. Vaccines were administered by different routes, including intramuscular, subcutaneous or intranasal and the results were compared to immunization with Tat alone or with Tat delivered with the alum adjuvant. The data demonstrate that the nano- and microspheres/Tat formulations are safe and induce robust and long-lasting cellular and humoral responses in mice after systemic and/or mucosal immunization. These delivery systems may have great potential for novel Tat protein-based vaccines against HIV-1 and hold promise for other protein-based vaccines.

1. Introduction

Several new approaches to vaccine development were proposed in recent years, including protein subunits, peptides and plasmid DNA. These vaccines, although generally safer than the traditional ones (e.g. viral or bacterial vectors), are poorly immunogenic when administered alone. Currently, aluminium salts (generally referred to as alum) and MF59 (a squalene o/w emulsion) are the only vaccine adjuvants approved for human use [1–5]. Consequently, a great need exists for new, safe and potent immunostimulatory adjuvants that may be compatible with the development of new-generation vaccines. Several new adjuvants have been tested in clinical trials. However, most of them were proved to be too toxic for routine clinical application. In addition to a good safety record, other important issues in adjuvant development include biocompatibility, stability, low cost, ease of production and administration (i.e. mucosal vaccines), lack of immunogenicity and applicability to different vaccine antigens. In this scenario, the use of polymeric particulate adjuvants is an expanding research field. It is well-established that antigen encapsulation in biodegradable polymeric matrices protects the antigen from unfavourable conditions encountered after systemic or mucosal administration, and increases its uptake by antigen-presenting cells facilitating the induction of potent immune responses [6–11]. Nevertheless, encapsulation-based approaches may often be accompanied by instability and degradation of the entrapped biomolecules occurring during encapsulation and/or release processes [12–15]. Instead, surface adsorption strategies are claimed to avoid problems of antigen instability and/or incomplete release associated to encapsulation/release in biodegradable microspheres. For instance, very efficient and potent immune responses were induced by antigens adsorbed onto anionic PLG microspheres, polymeric lamellar

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substrate particles or anionic wax nanoparticles [16–25]. However, in most of these systems the presence of adsorbed surfactants or polymeric stabilizers may give rise to irreproducibility of the vaccine formulation, premature release of the charged molecules, and consequently of the antigen, both during preparation of the formulation or after administration, thereby leading to variable efficacy as well as undesirable toxic effects of the free charged molecules [15,26]. Moreover, also the microsphere biocompatibility may be affected by the presence of the surfactant, thus leading to further criticism for future clinical development [27]. To overcome these problems, PLA or PLGA particles with charged groups covalently bound to the particle surface were developed by chemical modification of preformed particles or by using functional polymers or copolymers during particle synthesis [28,29]. In addition, to avoid the still unresolved concerns about the presence of surfactants such as SDS or stabilizers such as PVA in microparticle formulations to be used in humans, new anionic surfactant-free nanoparticles containing only PLA polymers with a carboxylic end group were also developed and shown to be efficient vaccine delivery systems [30].

In this scenario, surfactant-free polymeric core–shell nanoparticles and microparticles were developed by our group with homogeneous size and size distribution and able to bind biologically active macromolecules on their surface without the need for added surfactants and/or detergents during or after the synthetic procedure [31–35]. These novel particles exhibit a core–shell structure, with an inner core constituted by poly(methylmethacrylate) (PMMA) and a highly hydrophilic outer shell composed of a hydrosoluble co-polymer, namely poly(methacrylic acid-*st*-ethyl acrylate) copolymer whose commercial name is Eudragit L100-55, which is tightly linked to the core and bears carboxylic groups. These particles are well-tolerated in mice, even after multiple administrations, and able to accommodate in their shell high amounts (antigen loading ability of up to 20%, w/w) of native proteins, mainly by ionic interactions, while preserving their activity. In particular, we have shown in vitro that anionic nano- and microspheres bind the HIV-1 Tat protein and protect it from oxidation thus increasing the shelf-life of the Tat protein vaccine [34,35]. This is an important feature for a delivery system as it may hold promise for vaccination with Tat, as well as for other subunit vaccines, particularly when a native protein conformation and maintenance of biological activity is required. Tat oxidation leads to protein multimerization, aggregation and loss of the biological and immunological activities [36–38]. In particular, active Tat is required for efficient uptake by and maturation of dendritic cells, for the immunomodulatory effects as well as for vaccine potency [39,40]. The stabilization effect of surface adsorption of Tat on these anionic PMMA nano- and microspheres is likely due to the highly hydrophilic shell which may accommodate native protein molecules in the Eudragit chains, i.e. HIV-1 Tat monomers, preventing protein multimerization and loss of biological activity [33–35].

To gain further insight on the potential use of this technology platform, in the present study we have evaluated in mice the safety and immunogenicity of new vaccine formulations composed of selected samples of these nano- and microspheres and the HIV-1 Tat protein. Vaccines were administered by different routes, including intramuscular, subcutaneous or intranasal and the results were compared to immunization with Tat alone or with Tat delivered with the alum adjuvant.

2. Materials and methods

2.1. Core–shell anionic nano- and microspheres

Core–shell particles with a core constituted by poly(methylmethacrylate) and a highly hydrophilic shell composed of poly(methacrylic acid-*st*-ethyl acrylate) copolymer whose commercial name is Eudragit L100/55, bearing carboxylic groups able to reversibly bind biologically active basic proteins, and characterized by very homogeneous size and size distribution, were generated by dispersion (samples H1D and 2H1B) and emulsion (sample MA7) polymerization procedures, as described in detail previously [33–35]. The physico-chemical properties and surface binding and release kinetics are described in detail elsewhere [33–35] [Sparnacci et al., manuscript in preparation] and briefly summarized in Table 1. The endotoxin content of the particles was tested by the Limulus Amoebocyte Lysate analysis and it was below the detection limit (<0.05 EU/μg).

2.2. Protein and peptides

The monomeric biologically active Tat protein (86 aa) of HIV-1 (HTLVIII-BH10) was produced in *Escherichia coli*, purified as a good laboratory practice (GLP) manufactured product and provided by Diatheva (Fano, Italy). The biological activity of each batch of Tat protein was determined by means of a very sensitive method based on Tat uptake by monocyte-derived dendritic cells, as described previously [36]. Tat is photo-, air- and thermo-sensitive and oxidizes easily (due to the presence of seven cysteines in its sequence) when exposed to air, light and room temperature. Thus, to prevent oxidation, which causes aggregation of the bioactive monomers and loss of biological activity, the Tat protein was stored lyophilized at −80 °C and resuspended (2 mg/ml) in degassed commercial phosphate buffered saline (PBS) in the dark and on ice, immediately before use [37]. Endotoxin concentration of different GLP lots of Tat was below the detection limit (<0.05 EU/μg), as tested by the Limulus Amoebocyte Lysate analysis. The VCF (VCFITKALGISYGRK) Tat peptide containing a K^d-restricted CTL epitope and a CD4+ T cell epitope [41] was synthesized by UFPeptides s.r.l. (Ferrara, Italy), resuspended in H_2O (10^{-2} M) and stored at −80 °C until use.

2.3. Preparation of protein/sphere complexes

Lyophilized nano- or microsphere samples were resuspended in PBS at 2 mg/ml. Complexes between the HIV-1 Tat protein (prepared as described in the previous section) and nano- and microspheres were prepared by mixing appropriate volumes of Tat protein and nano- or microspheres under continuous stirring for 1 h at 4 °C. Tat:particles ratios of 1–10 μg (Tat)/30–60 μg (spheres) which give adsorption efficiency of 100% were used, as previously described [33–35]. Specifically, for intramuscular (i.m.) and intranasal (i.n.) inoculations 1 μg of Tat/30 μg of particles/mouse in 100 μl were used. The immunogen was prepared by mixing 3 μl (6 μg) of Tat and 90 μl (180 μg) of spheres (for groups composed of 6 mice), or 4.5 μl (9 μg) of Tat and 135 μl (270 μg) of spheres (for groups composed of 9 mice). For subcutaneous (s.c.) vaccination, 10 μg of Tat/60 μg of particles/mouse in 100 μl were used, and the immunogen was prepared by mixing 30 μl (60 μg) of Tat and 180 μl (360 μg) of spheres (for groups of 6 mice). After incubation, complexes were collected in a microfuge at 13,500 rpm, resuspended in the appropriate volume of degassed sterile PBS [600 μl for i.m. or s.c. immunization of groups composed of 6 mice (100 μl/mouse); 60 or 90 μl for i.n. vaccination of groups composed of 6 or 9 mice, respectively (10 μl/mouse)] and used immediately.

2.4. Mice studies

Animal use was according to National Guidelines and Institutional Policies. Female BALB/c mice of six to eight weeks of age (Charles River, Italy) were inoculated with formulations composed of Tat protein adsorbed onto the H1D microspheres. Control mice were inoculated with Tat protein alone in aluminium phosphate

Table 1
Summary of physico-chemical characteristics of anionic core–shell nano- and microspheres[a].

Sample	Polymer (stabilizer)	Synthetic procedure	SEM diameter (μm) (±S.D.)	Surface area (m^2/g)	Surface charge (COOH μmol/g)	Antigen loading ability (% wt/wt)	Refs.
MA7	PMMA (Eudragit L100/55)	Emulsion polymerization	0.22 (±0.008)	27.28	64.3	20	[32,35]
2H1B	PMMA (Eudragit L100/55)	Dispersion polymerization	0.63 (±0.06)	9.26	60.2	20	[31,33]
H1D	PMMA (Eudragit L100/55)	Dispersion polymerization	1.99 (±0.17)	2.96	54.7	1–9	[31,33,34]

[a] The synthesis and physico-chemical characterization of polymeric nano- and microspheres composed of an inner core made of poly(methylmethacrylate) (PMMA), and of carboxyl (COOH) functional surface groups derived from Eudragit L100/55 stabilizer, were described in detail elsewhere [31–35].

(alum) adjuvant, and with alum alone. Each experimental group was composed of 6 mice. Immunogens (100 μl) were given at weeks 0 and 4 intramuscularly (i.m.) in the quadriceps muscles (50 μl/leg) or subcutaneously (s.c.) in one site on the dorsal area near the tail. For i.m. vaccination, mice received the H1D/Tat (30 μg/1 μg) vaccine or the same dose of Tat alone in alum. For s.c. immunization mice were injected with H1D/Tat (60 μg/10 μg), or with 10 μg of Tat in alum. The dose of 10 μg was used for s.c. injection based on pilot studies (unpublished results) suggesting that this dose of Tat was optimal for this route of injection. One week later (week 5), 3 mice/group were sacrificed to analyze Tat-specific immune responses. At week 8, all remaining mice (3 animals/experimental group) were boosted i.m. or s.c., respectively, with Tat/alum and sacrificed two (week 10) or four (week 12) weeks later. In other experiments, BALB/c mice ($n = 6$) were inoculated intranasally (i.n.) with Tat (1 μg) protein adsorbed onto the microspheres (H1D) or the nanospheres (MA7, 2H1B) (30 μg), or with Tat alone (1 or 10 μg). Immunogens (10 μl) were administered in the nostrils (5 μl/nostril) 4 times (days 0, 7, 14 and 21). Mice ($n = 3$) were sacrificed 1 week (week 4) and 7 weeks (week 10) after the last immunization. Alternatively, a i.n prime/s.c. boost schedule was used. Mice ($n = 9$) were inoculated i.n. with the complexes (30 μg of spheres/1 μg of Tat), with Tat alone (1 or 10 μg) or with PBS at weeks 0, 3 and 5. Two weeks later (week 7), 3 mice/group were sacrificed to analyze Tat-specific immune responses. The remaining mice were boosted s.c with Tat (1 μg) in alum at week 8 (3 weeks after the last immunization) and sacrificed one (week 9) ($n = 3$) and ten (week 18) ($n = 3$) weeks after the protein boost. During the experiments, animals were controlled twice a week at the site of inoculation and for their general conditions (such as liveliness, food intake, vitality, weight, motility, sheen of hair). At sacrifice mice were anesthetized intraperitoneally with 100 μl of isotonic solution containing 1 mg of Zoletil (Virbac, Milan, Italy) and 200 μg Rompun (Bayer, Milan, Italy). Each immunization experiment was repeated twice. Finally, in some experiments mice were immunized and sacrificed to collect organs for histological and immunohistochemical examinations.

2.5. Serology

The presence of antigen specific antibodies (IgG) in sera and in mucosal (IgA, IgG) vaginal and lung lavages was searched by Enzyme Linked Immunosorbent Assay (ELISA) on mice samples tested individually, as previously described [42–44].

2.6. Cell purification

Splenocytes were purified from spleens squeezed on filters (Cell Strainer, 70 μm, Nylon, Becton Dickinson). Following lysis of red blood cells (RBC) with RBC lysing buffer (Sigma), cells were washed with RPMI 1640 (Cambrex) containing 10% FBS (Hyclone), spun for 10 min at 1200 rpm, resuspended in RPMI 1640 containing 10% FBS, 1% L-glutamine (BioWhittaker, Walkersville, MD), 1% penicillin/streptomycin (BioWhittaker, Walkersville, MD), 1% non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma) and 50 mM β-mercaptoethanol (Gibco, Grand Island, NY). In some experiments, depletion of B lymphocytes and purification of CD8+ T cells were carried out using anti-CD19 and anti-CD8 magnetic beads (BD Pharmingen, San Jose, CA), according to the manufacturer's instructions. Cell cultures were then analyzed by fluorescence-activated cell sorter (FACSCalibur, BD) analysis using rat anti-mouse monoclonal antibodies (α-CD19, α-CD3, α-CD4, α-CD8) and a goat anti-rat FITC-conjugated antibody (all from BD Pharmingen). In some experiments NALT (nasal-associated lymphoid tissue) was also collected (bilaterally on the posterior side of the palate) and processed as the spleens. Cellular responses were analyzed using pools of cells for each experimental group.

2.7. Proliferation assays

Proliferation assays were performed by standard ^{3}H-Thymidine incorporation assays in sestuplicates using splenocytes or NALTs cultures at 2×10^5 cells/200 μl/well (round-bottom plates, NUNC), as previously described [41]. The reported results are expressed as stimulation index (S.I.) determined as the ratio between the mean counts/minute of antigen-stimulated cells and the mean counts/minute of the same unstimulated sample.

2.8. Enzyme-linked immunospot (Elispot) assays

For enzyme-linked immunospot (Elispot) analysis on fresh cells, total splenocytes (5×10^5 cells/well) were added to 96-well Elispot plates pre-coated with the cytokine-specific capture antibody, and incubated at 37 °C for 24 h in the absence (untreated) or presence of the peptide (10^{-6} M). For Elispot analysis after ex vivo restimulation, splenocytes (3×10^6 ml) were cultured with the specific peptide (3 μg/ml) for 5 days, extensively washed with RPMI 1640 containing 10% FBS, placed on ($4–5 \times 10^4$ cells/well) pre-coated Elispot plates and incubated as above. Elispot assays were performed for Th1 (IFN-γ) and Th2 (IL-4) cytokines (duplicate wells), using commercially available murine IFN-γ and IL-4 Elispot kits (BD, Pharmingen), as described [35,41]. Results are expressed as number of spot forming cells (SFC)/10^6 cells. Responses at least 2-fold higher than the mean number of spots in the control wells (untreated cells) and ≥30 (fresh) or ≥100 (after ex vivo stimulation) SFC/10^6 cells were considered positive.

2.9. CTL assays

Cytotoxic (CTL) activity was determined at various effector/target ratios by standard ^{51}Cr release assays, using P815 target cells previously pulsed with the VCF Tat peptide, as described [41]. Results are expressed as percentage (%) of specific lysis that was calculated as 100 × (cpm sample − cpm medium)/(cpm Triton X-100 − cpm medium). Spontaneous release was always below 10%.

2.10. Histological, histochemical and immunohistochemical procedures

At sacrifice mice were subjected to autopsy. Sample of cutis, subcutis and skeletal muscles at the site of injection and other organs

(lungs, heart, lymph nodes, ovaries, intestine, kidneys, brain, spleen and liver) was taken and processed for histologic, histochemical and immunohistochemical examination, as described previously [34,42].

2.11. Statistics

The data related to the antibody responses were analyzed by the unpaired two-tailed *t*-test and the one-way ANOVA test and Dunnett's post-test. Analysis of cellular responses was done by the two-way Anova test and Bonferroni's post-test using the GraphPad software Prism 4 (El Camino Real, San Diego, CA). The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Immunization with H1D/Tat formulations by the intramuscular route

To evaluate the immunogenic potential of the H1D microspheres, groups of mice were immunized intramuscularly (i.m.) with the H1D/Tat (30 μg/1 μg) vaccine or with 1 μg of Tat alone in alum, at weeks 0 and 4. After 1 week (week 5), IFN-γ responses tested on fresh splenocytes were significantly higher in mice vaccinated with the H1D/Tat formulation as compared to immunization with Tat/alum ($p < 0.01$) (Fig. 1A). Also IL-4 responses were higher in the H1D/Tat vaccinated group, although for this cytokine the difference was not statistically significant ($p > 0.05$). Similarly, splenocytes proliferation to increasing doses of Tat protein (0.1, 1 and 5 μg/ml) was significantly higher in mice vaccinated with H1D/Tat as compared to that of the Tat/alum group ($p < 0.01$) (Fig. 1B). With respect to the humoral responses, immunization with H1D/Tat elicited production of antibodies. However, at this time point, IgG titers were lower on the average (562 ± 856) than those detected in the Tat/alum vaccinees ($60{,}932 \pm 60{,}475$) ($p < 0.05$) (Fig. 1C).

To evaluate whether i.m. immunization with the H1D/Tat vaccine generate memory T and B cell immunity, all mice were boosted i.m. with 1 μg of Tat/alum 30 days after the last immunization (week 8), and sacrificed after 4 weeks (week 12). As shown in Fig. 1F, the protein boost had a potent effect on the humoral responses, in particular in the group primed with H1D/Tat, whose IgG titers ($156{,}486 \pm 256{,}003$) increased to levels as high as those in the Tat/alum mice ($443{,}726 \pm 403{,}993$) ($p > 0.05$). Accordingly, in both groups of vaccinees the number of IL-4 secreting cells increased and reached similar levels ($p > 0.05$) (Fig. 1D). After the boost, the number of IFN-γ producing cells (Fig. 1D) and the lymphoproliferative responses to increasing doses of Tat protein (Fig. 1E) were still higher in the H1D/Tat-primed mice.

To assess which T cell subset was the primary source of IFN-γ and IL-4 secretion, cytokine production was tested on CD8+ purified and on CD4+-enriched (CD8-depleted) T cell subpopulations. After ex vivo restimulation with the VCF Tat peptide containing either a K^d-restricted CTL epitope and a CD4+ T cell epitope [41], the frequency of IFN-γ and IL-4-secreting cells was similar in CD4+-enriched cultures of both groups (Fig. 2A). In contrast, in CD8+ purified T cell cultures only IFN-γ producing cells were detected and mainly in mice primed with H1D/Tat ($p < 0.01$) (Fig. 2B).

As a whole the results indicate that the H1D/Tat vaccine increases the antigen-specific cellular responses and CTLs, and primes very efficiently the humoral arm of the immune system as IgG titers were readily detected after 2 i.m. immunizations with the H1D/Tat vaccine and, after one protein boost, they reached high levels in a fashion similar to animals immunized with Tat/alum. These results are in agreement with previous data showing that formulations composed of MA7 nanospheres and Tat, given i.m., increase the antigen-specific cellular responses, with a prevalence of Th1-type responses, and promote an efficient priming of the humoral arm [35]. The results also suggest that a pool of memory antigen-specific B and T lymphocytes were generated after 2 i.m. immunizations with the H1D/Tat vaccine.

3.2. Immunizaton with H1D/Tat formulations by the subcutaneous route

The immunogenic potential of the H1D/Tat vaccine following subcutaneous (s.c.) administration was then compared to vaccination with Tat/alum. Thereby, mice were immunized s.c. with H1D/Tat (60 μg/10 μg), or with Tat/alum (10 μg), at weeks 0 and 4. After 1 week (week 5), IFN-γ and IL-4 responses were significantly higher in mice vaccinated with the H1D/Tat formulation as compared to immunization with Tat/alum ($p < 0.01$) (Fig. 3A). In addition, specific anti-Tat CTLs capable of killing P815 cells pulsed with the K^d-restricted VCF Tat peptide were detected in both groups of vaccinees but, again, immunization with H1D/Tat induced a higher activity (Fig. 3B). The analysis of humoral responses showed that also s.c. immunization with H1D/Tat elicited antibody production. As for the previous i.m. protocol, after 2 s.c. injections of H1D/Tat, IgG titers in the H1D/Tat group (4747 ± 3049) were lower on the average than those detected in the Tat/alum vaccinees ($80{,}993 \pm 37{,}896$) ($p < 0.05$) (Fig. 3C).

To evaluate whether s.c. immunization also induces Tat-specific memory B and T cell responses, all mice were boosted at week 8 with Tat/alum by the s.c. route and sacrificed 2 weeks later (week 10). IFN-γ and IL-4 responses were sustained and still significantly higher in the group primed with H1D/Tat ($p < 0.01$) (Fig. 3D), and these responses again correlated with stronger CTLs activities (Fig. 3E). As for the previous i.m. protocol, the protein boost had a potent effect on IgG responses, in particular in H1D/Tat primed mice whose antibody titers ($83{,}733 \pm 38{,}358$) increased to high levels as in the Tat/alum vaccinees ($628{,}521 \pm 614{,}169$) ($p > 0.05$) (Fig. 3F).

The results suggest that, also after s.c. inoculation, the presence of the microspheres in the vaccine formulation increases both Th1- and Th2-type antigen-specific cellular responses and CTLs, and promotes an efficient priming of the humoral arm of the immune system. In addition, a pool of memory antigen-specific B and T lymphocytes are generated also by this route of immunization.

3.3. Immunizaton with H1D/Tat, 2H1B/Tat and MA7/Tat by the intranasal route

As the mucosal route of immunization is considered a simple, safe, efficacious and less expensive method to deliver antigens, to assess the mucosal immunogenic potential of the technology platform, mice were immunized intranasally (i.n.) 4 times (days 0, 7, 14 and 21) with the H1D/Tat vaccine (30 μg/1 μg). Additionally, mice were immunized also with formulations composed of MA7 or 2H1B nanospheres (30 μg) and Tat (1 μg), as these particles have smaller size (Table 1) which may be more suitable for i.n. inoculation. Control mice were inoculated with 1 or 10 μg of Tat alone. As shown in Fig. 4A, 1 week after the last immunization IFN-γ responses were detected at high levels only in mice immunized with the nano- or the microspheres/Tat formulations, and in mice immunized with 10 μg of Tat, but not in mice immunized with 1 μg of Tat alone ($p < 0.05$). In particular, the nanosphere-based vaccines (MA7/Tat and 2H1B/Tat) induced the most potent IFN-γ responses ($p < 0.001$) which were even slightly higher than those induced by immunization with a 10-fold higher dose of Tat. Overall, IL-4 responses were lower than IFN-γ responses in all groups. However, immunization with MA7/Tat, 2H1B/Tat or H1D/Tat elicited higher IL-4 responses as compared to immunization with 1 μg of Tat. The differences were

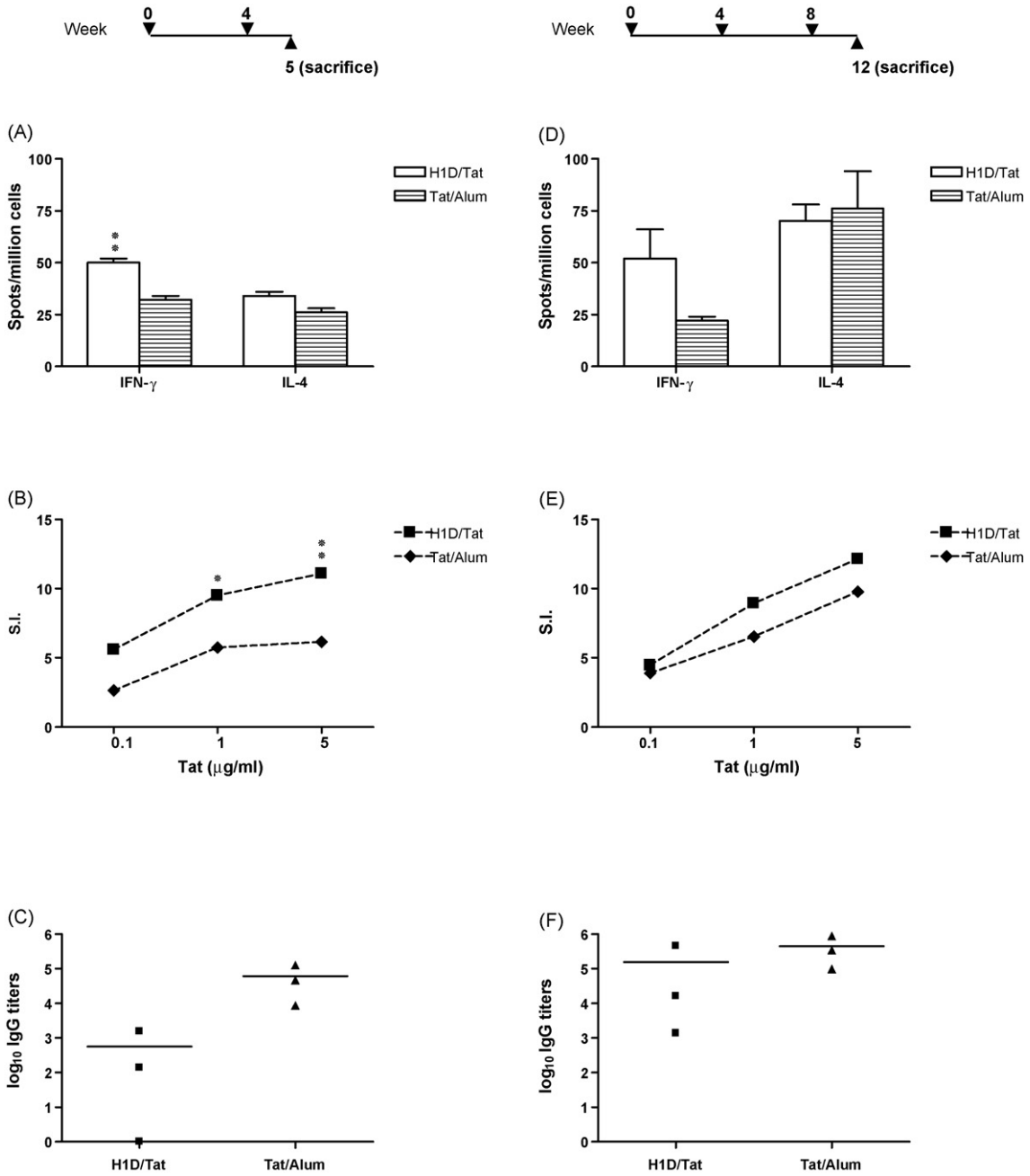

Fig. 1. Analysis of Tat-specific immune responses after H1D/Tat i.m. immunization. Mice ($n = 6$) were immunized with H1D (30 μg)/Tat (1 μg) or with Tat (1 μg)/alum at weeks 0 and 4 and sacrificed ($n = 3$) at week 5 (panels A–C) or boosted ($n = 3$) with Tat (1 μg) and alum at week 8 and sacrificed at week 12 (panels D–F). (A, D) Analysis of Tat-induced IFN-γ and IL-4 secretion by Elispot. Responses were measured on fresh splenocytes. (B, E) Lymphoproliferative responses to Tat. Proliferative responses to increasing doses of Tat protein were measured by ^{3}H-Thymidine incorporation. Results correspond to stimulation indexes. (C, F) Anti-Tat IgG titers. Antibody titers were measured by ELISA. The results are expressed as the $\log_{10}$ of the endpoint titers of mice sera tested individually. The results were reproduced in two independent immunization experiments. The results of one representative experiment are shown. Statistical analysis was carried out in comparison with the results with Tat/alum. $^*p < 0.05$; $^{**}p < 0.01$.

not statistically significant ($p > 0.05$), although IL-4 responses were stronger again after immunization with the nanospheres-based vaccines (MA7/Tat and 2H1B/Tat) (Fig. 4A). In agreement with these results, lymphoproliferation to increasing doses of Tat was higher in mice immunized with MA7/Tat and 2H1B/Tat and comparable to those elicited by vaccination with 10 μg of Tat alone (Fig. 4B). Such an increase was not observed in this assay in the H1D/Tat group.

Cellular immune responses were long-lived, as they were still detected 7 weeks (week 10) after the last immunization in all groups (Fig. 4C and D). In H1D/Tat, MA7/Tat and 2H1B/Tat vaccinees T cell responses were stronger than those of mice immunized with 1 μg of Tat alone, and comparable to those induced by vaccination with 10 μg of Tat. Interestingly, at week 10 also CTL responses were clearly detected, but only in the MA7/Tat and 2H1B/Tat mice and in animals immunized with 10 μg of Tat (Fig. 5), as opposite to the results at week 4 when CTL responses were not detected in any of the vaccinated groups (data not shown). Surprisingly, at any time point after the last immunization, serum IgG and vaginal IgA responses were detected only in few mice after vaccination with 10 μg of Tat alone (IgG mean titers 1983 ± 1535; IgA 1–6%).

As a whole, these results indicate that 4 i.n. vaccinations with microsphere- and, mostly, with nanosphere-based formulations

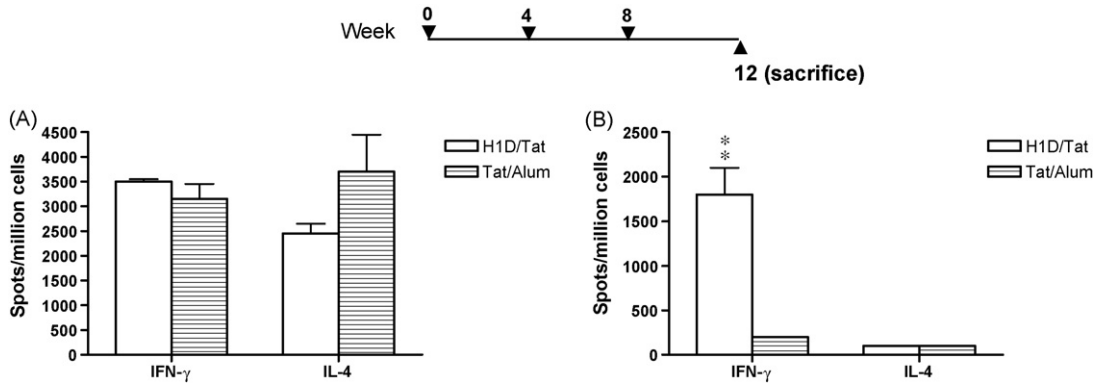

Fig. 2. Analysis of IFN-γ and IL-4 secretion by Elispot on CD4-enriched (CD8-depleted) (panel A) and CD8-purified (panel B) T cell subpopulations. Animals ($n = 3$) were primed i.m. with the H1D (30 μg)/Tat (1 μg) complexes or with Tat (1 μg) and alum at weeks 0 and 4, boosted i.m. with Tat (1 μg) and alum at week 8 and sacrificed at week 12. After in vitro restimulation, cytokine production was determined by Elispot. The results were reproduced in two independent immunization experiments. The results of one representative experiment are shown. Statistical analysis was carried out in comparison with the results with Tat/alum. $^{**}p < 0.01$.

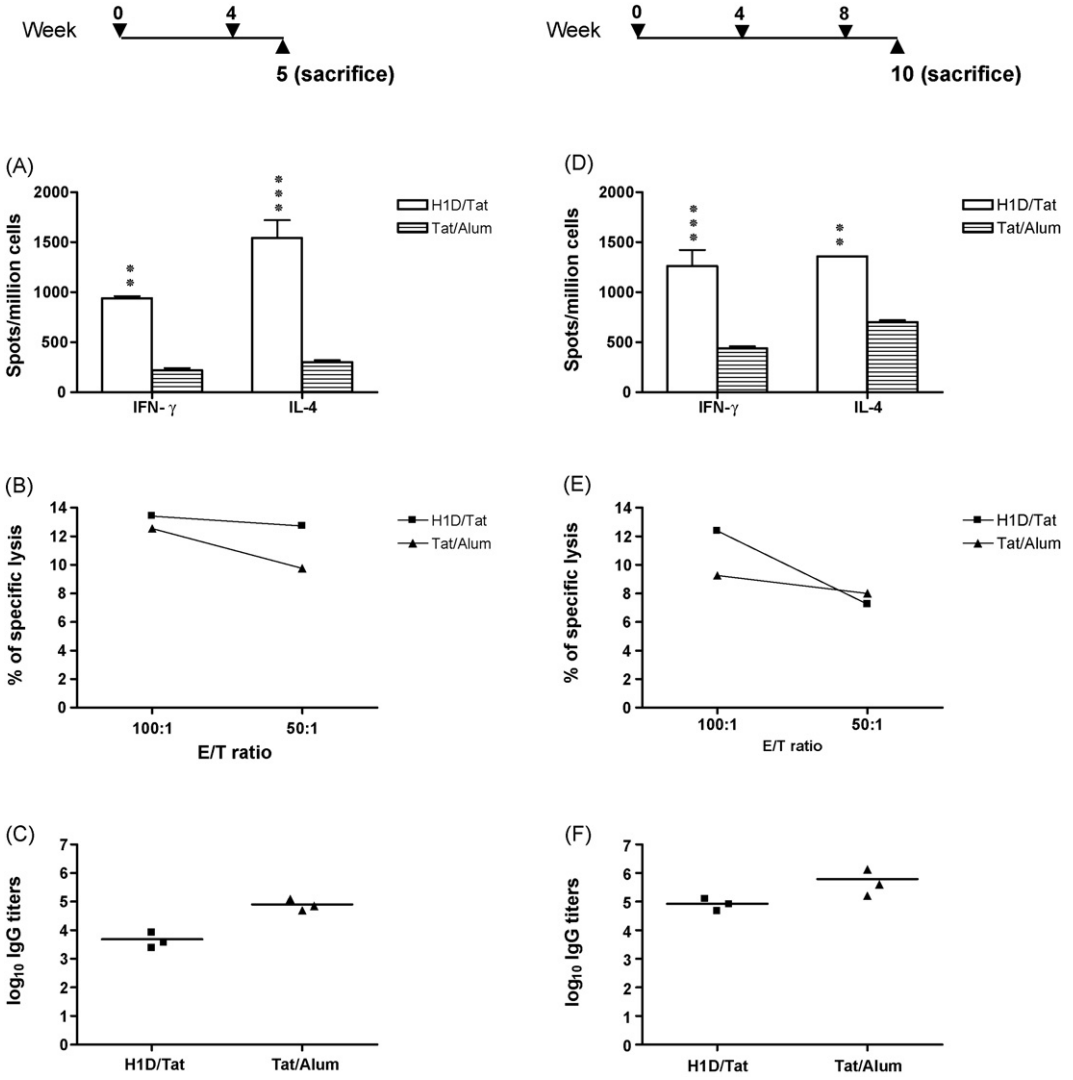

Fig. 3. Analysis of Tat-specific immune responses after s.c. immunization with H1D/Tat. Mice ($n = 6$) were immunized with H1D (60 μg)/Tat (10 μg) or with Tat (10 μg)/alum at weeks 0 and 4 and sacrificed ($n = 3$) at week 5 (panels A–C) or boosted ($n = 3$) with Tat (10 μg)/alum at week 8 and sacrificed at week 10 (panels D–F). (A and D) Analysis of Tat-induced IFN-γ and IL-4 secretion by Elispot. (B and E) CTL responses to Tat. CTL activity was determined, at various effector/target (E/T) ratios, by standard ^{51}Cr release assays using syngenic P815 target cells pulsed with the VCF Tat peptide containing a K^d restricted Tat CTL epitope. The percentage (%) of specific lysis is reported. (C and F) Anti-Tat IgG titers. Antibody titers were measured by ELISA. The results are expressed as the $\log_{10}$ of the endpoint titers of mice sera tested individually. The results were confirmed in two independent immunization experiments. The results of one representative experiment are shown. Statistical analysis was carried out in comparison with the results with Tat/alum. $^{**}p < 0.01$; $^{***}p < 0.001$.

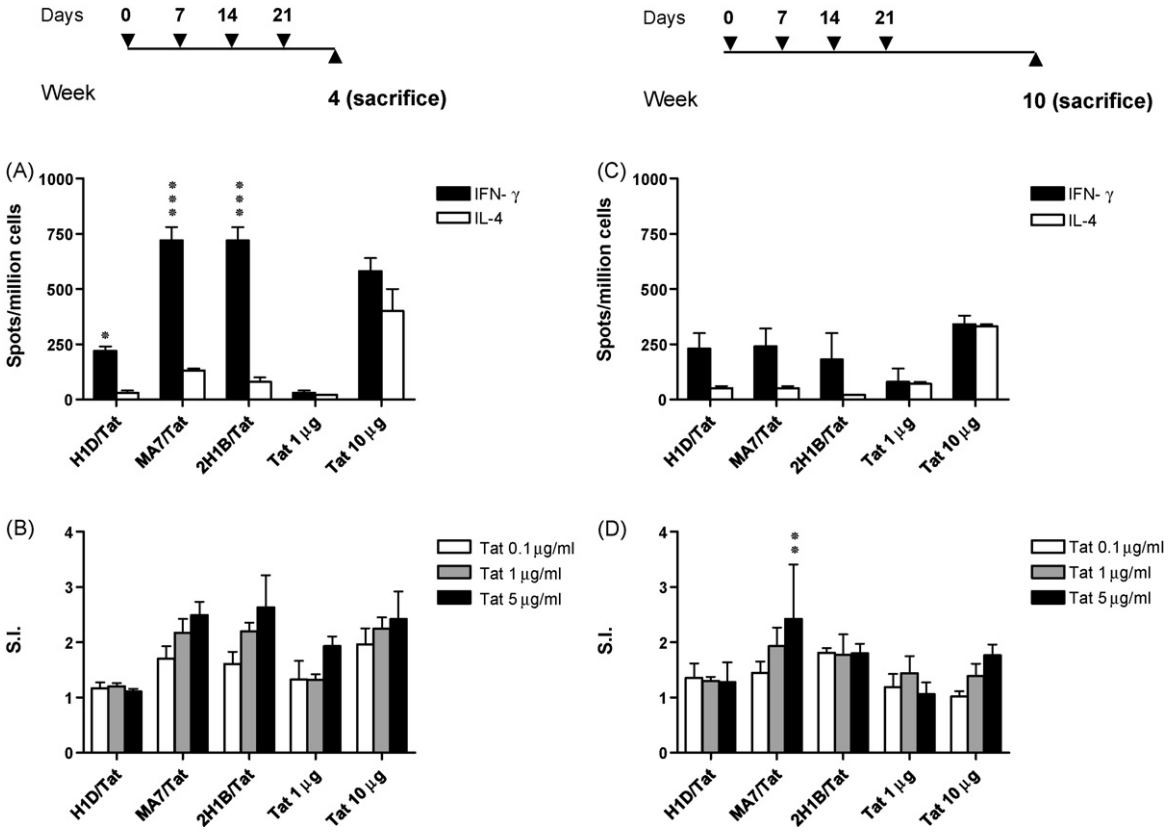

Fig. 4. Analysis of Tat-specific immune responses after i.n. immunization with H1D/Tat, MA7/Tat or 2H1B/Tat. Mice ($n = 6$) were immunized 4 times (1 week interval) with 1 μg of Tat protein formulated with 30 μg of H1D, MA7, or 2H1B particles, or with Tat alone (1 or 10 μg) and sacrificed 1 week ($n = 3$) (panels A and B) and 7 weeks ($n = 3$) (panels C and D) after the last immunization. (A and C) Analysis of Tat-induced IFN-γ and IL-4 secretion by Elispot. (B and D) Lymphoproliferative responses to Tat. Proliferative responses to increasing doses of Tat protein were measured by ^{3}H-Thymidine incorporation. Results correspond to stimulation indexes. Statistical analysis was carried out in comparison with the results with Tat (1 μg). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

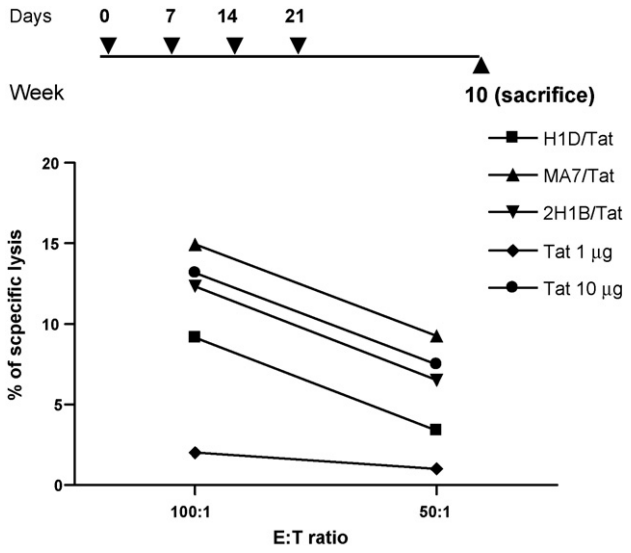

Fig. 5. Analysis of Tat-specific CTL responses after i.n. immunization with H1D/Tat, MA7/Tat or 2H1B/Tat. Mice were immunized as described in legend to Fig. 4 and sacrificed 7 weeks after the last immunization. CTL activity was determined, at various effector/target (E/T) ratios, by standard ^{51}Cr release assays using syngenic P815 target cells pulsed with the VCF Tat peptide containing a K^{d} restricted Tat CTL epitope. The percentage (%) of specific lysis is reported.

increased antigen-specific Th1-and Th2-type cellular responses and CTLs but did not stimulate efficient antibody responses in serum and at mucosal sites.

Thereby, to determine whether i.n. immunization with these Tat/particles formulations primes the humoral arm of the immune system, in a different set of experiments mice were vaccinated i.n. with the various vaccines, at weeks 0, 3 and 5, and boosted s.c. with Tat/alum 3 weeks later (week 8). Control mice were immunized i.n. with Tat alone (1 or 10 μg). Immune responses were evaluated before (week 7) and after (weeks 9 and 18) the protein boost. The protein boost promptly induced antigen-specific IgG responses, which were present in few mice 1 week after the protein boost (week 9) and in all vaccinees 10 weeks after the boost (week 18) at high and comparable titers as in mice receiving Tat alone ($p > 0.05$) (Fig. 6A–C). Accordingly, a pronounced increase of IL-4 responses was observed (Fig. 7A–C). IgA responses in vaginal and lung lavages were instead barely detectable in all groups, including mice immunized with 10 μg of Tat, at any time point (before and after the boost) (data not shown). These results indicate that i.n. immunization with microsphere- and nanosphere-based formulations prime also the humoral arm of the immune system, as all immunized animals developed high serum IgG titers after one protein boost.

After the protein boost, Tat-specific cellular responses generally increased at the systemic level (Fig. 7A–C and D–F). Notably, strong cellular responses were observed mainly at mucosal sites in all immunized animals (Fig. 7G–I). Again priming with the H1D/Tat, MA7/Tat and 2H1B/Tat vaccines induced higher T cell responses, especially in NALT ($p < 0.001$), than vaccination with Tat alone (Fig. 7G–I).

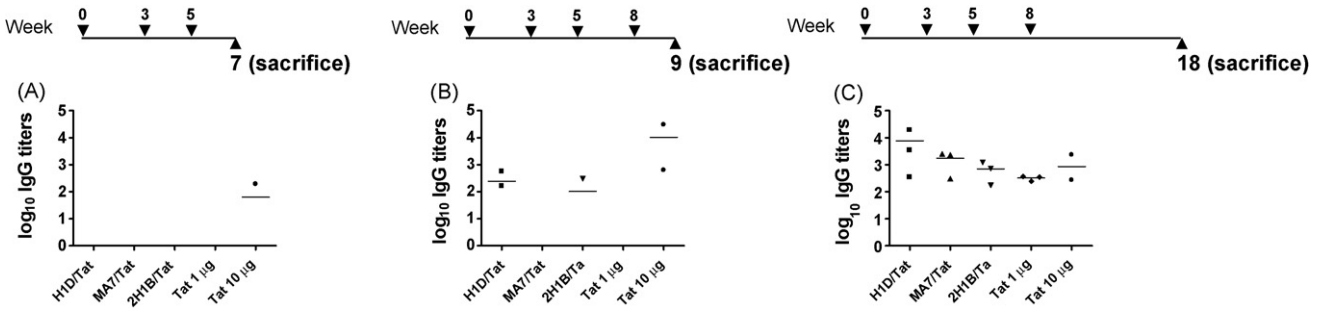

Fig. 6. Analysis of Tat-specific IgG responses after i.n. prime/s.c. boost with H1D/Tat, MA7/Tat or 2H1B/Tat and Tat/alum. Mice ($n = 9$) were immunized (weeks 0, 3 and 5) i.n. with 1 μg of Tat protein formulated with 30 μg of H1D, MA7, or 2H1B particles, or with Tat alone (1 or 10 μg) and sacrificed ($n = 3$) 2 weeks later (A). The remaining mice were boosted s.c. with 1 μg of Tat in alum at week 8 and sacrificed at week 9 ($n = 3$) (B) and at week 18 ($n = 3$) (C). Antibody titers were measured by ELISA. The results are expressed as the $\log_{10}$ of the endpoint titers of mice sera tested individually. The results were confirmed in two independent immunization experiments. The results of one representative experiment are shown.

Altogether the results indicate that the presence of the nano- and microspheres in the vaccine formulations, given with an i.n. prime/s.c. boost regimen, primed efficiently the cellular and humoral arms of the immune system and increased the antigen-specific cellular responses also locally at the mucosal associated lymphoid tissue.

3.4. Histological analysis

No specific signs of visible local or systemic adverse reactions were ever reported during the experiments in mice inoculated i.m., s.c. or i.n. with the various vaccine formulations as compared to mice immunized with Tat alone, with Tat/alum or untreated control mice. At sacrifice samples of cutis at the site of injection and organs were collected for histological and immunohistochemical examination. A total of 52 mice immunized i.m., 42 mice immunized s.c. and 75 mice immunized i.n were analyzed (Table 2). No local reactions were reported after i.n. inoculation. No alterations were detected in all organs examined in all immunization protocols. A visible granuloma at the site of injection was observed few days after injection only in mice injected with Tat/alum or alum alone by the i.m. or s.c., whereas this type of visible inflammatory reaction was never reported after injection of the Tat/particles formulations alone. Histologically, a similar local reaction that could be related

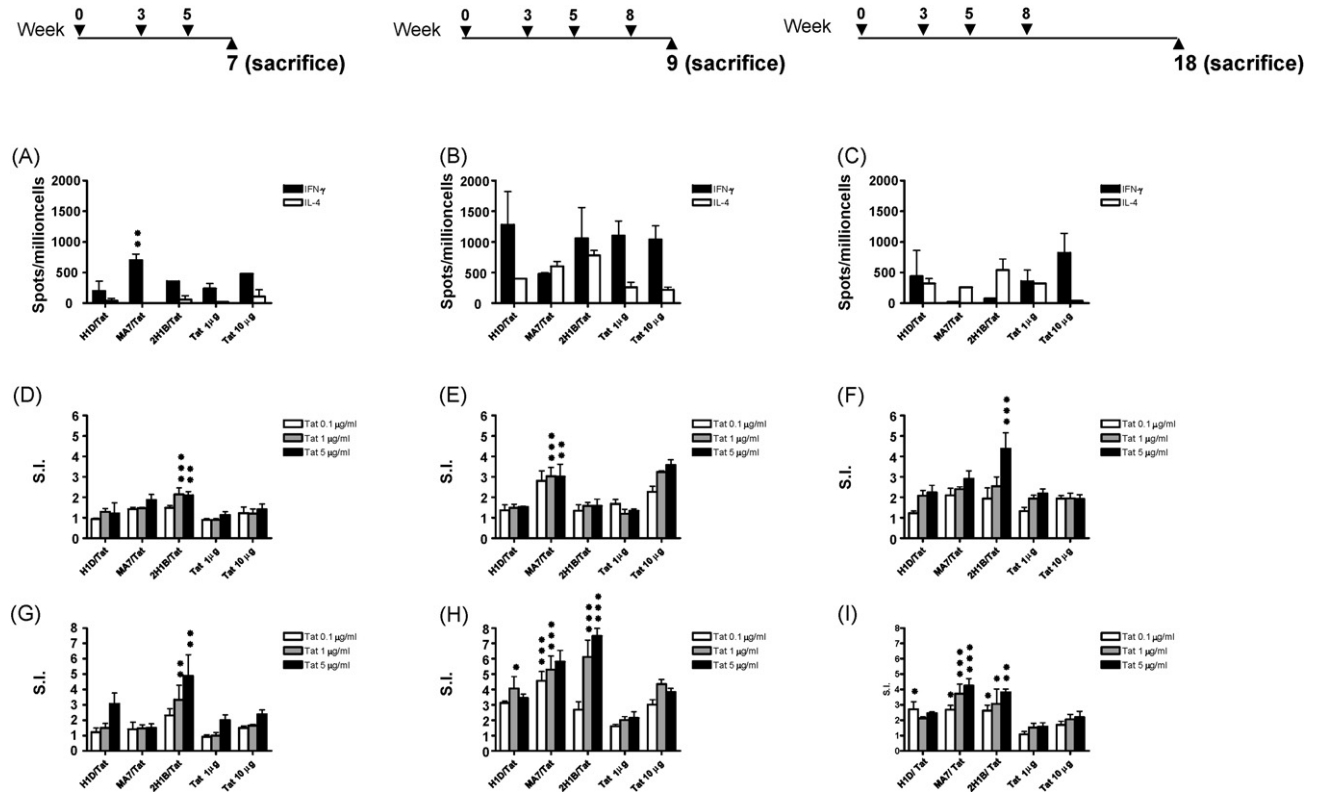

Fig. 7. Analysis of Tat-specific cellular responses after i.n. prime/s.c. boost with H1D/Tat, MA7/Tat or 2H1B/Tat and Tat/alum. Mice ($n = 9$) were immunized (weeks 0, 3 and 5) i.n. with 1 μg of Tat protein formulated with 30 μg of H1D, MA7, or 2H1B particles, or with Tat alone (1 or 10 μg) and sacrificed ($n = 3$) 2 weeks later (panels A, D, G). The remaining mice were boosted s.c. with 1 μg of Tat in alum at week 8 and sacrificed at week 9 ($n = 3$) (panels B, E, H) and at week 18 ($n = 3$) (panels C, F, I). (A–C) Analysis of Tat-induced IFN-γ and IL-4 secretion by Elispot. (D–F) Lymphoproliferative responses to Tat in mice splenocytes. Proliferative responses to increasing doses of Tat protein were measured by ^{3}H-Thymidine incorporation. Results correspond to stimulation indexes. (G–I) Lymphoproliferative responses to Tat in NALT. Proliferative responses to increasing doses of Tat protein were measured by ^{3}H-Thymidine incorporation. Results correspond to stimulation indexes. The results were confirmed in two independent immunization experiments. The results of one representative experiment are shown. Statistical analysis was carried out in comparison with the results of mice immunized with Tat (1 μg). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Table 2
Mice with tissue lesions at the site of inoculation[a].

Immunogen (dose)	Route of inoculation	Mice with histological lesions at the site of inoculation/total mice[b]
H1D/Tat (30 μg/1 μg)	i.m.	7/16 (44%)
H1D/Tat (60 μg/10 μg)	i.m.	3/14 (21%)
Tat/alum (1 μg)	i.m.	8/11 (73%)
Alum	i.m.	6/11 (55%)
H1D/Tat (60 μg/10 μg)	s.c.	9/14 (64%)
Tat/alum (10 μg)	s.c.	14/14 (100%)
Alum	s.c.	11/14 (78%)
H1D/Tat (30 μg/1 μg)	i.n.	0/15 (0%)
2H1B/Tat (30 μg/1 μg)	i.n.	0/15 (0%)
MA7/Tat (30 μg/1 μg)	i.n.	0/15 (0%)
Tat (1 μg)	i.n.	0/15 (0%)
Tat (10 μg)	i.n.	0/15 (0%)

[a] BALB/c mice were inoculated i.m. or s.c. with the H1D/Tat protein formulations, at weeks 0 and 4, and boosted with Tat/alum at week 8. Control mice were inoculated i.m. or s.c. with the Tat protein alone in alum adjuvant, or with alum alone at weeks 0, 4 and 8. Alternatively, mice were immunized i.n. with H1D/Tat, 2H1B/Tat or MA7/Tat at weeks 0, 3 and 5. Control mice were inoculated i.n. with the Tat protein alone. All mice were sacrificed 2 weeks after the last immunization for histological and immunohistochemical examinations of samples of cutis at the site of injection and organs (as described in Section 2).

[b] Histologically, a local reaction that could be related to immunogen injection was reported only at the site of injection, after i.m. or s.c., in mice receiving the Tat/particles formulations and one protein boost, Tat/alum or alum alone. No local reactions were reported after i.n. inoculation. No alterations were detected in all organs examined after i.m., s.c. or i.n. injection.

to immunogen injection was reported only after i.m. or s.c., at the site of injection, either in mice receiving the Tat/particles formulations and one protein boost, Tat/alum or alum alone. In particular, a histological local reaction was described in 10/30 (33%) mice inoculated i.m. with H1D/Tat (two inoculations and one protein boost), in 8/11 (73%) inoculated with Tat/alum (three inoculations) and in 6/11 (55%) inoculated with alum alone (three inoculations). Similarly, after s.c. injection, a histological local reaction was described in 9/14 (64%) mice receiving H1D/Tat (two inoculations and one protein boost), in 14/14 (100%) receiving Tat/alum (three inoculations) and in 11/14 (78%) mice injected with alum alone (three inoculations) (Table 2). Tissue lesions were variable in size and extension; however, they showed the same histologic and immunohistochemical characteristics in i.m or s.c. experimental groups. After i.m. inoculation, lesions showed irregular widening of endomysial connective septa due to a dense and diffuse infiltration of inflammatory cells surrounding the muscular cells. Infiltrative inflammatory cells were predominantly macrophages with large and vacuolated cytoplasm and central nuclei. After s.c. immunization, lesions were localized in the derma and subcutis, and were characterized by a central necrotic core, consisting of amorphous material, nuclear and squamous cell debris, surrounded by a layer of inflammatory cells. The majority of these cells were macrophages with a minority of neutrophil granulocytes. No fibroblast proliferative reaction was observed at the periphery of the lesions (data not shown). These results indicate that these nano- and microspheres-based vaccines are well-tolerated in vivo after systemic and mucosal inoculation, in agreement with previous observations [34,35].

4. Discussion

The HIV-1 Tat protein was used as model antigen to assess the immunogenic potential of this technology platform, as it is a vaccine relevant antigen already proven to be safe, immunogenic and efficacious in preclinical models [39,40,45–47]. In addition, the Tat protein vaccine was recently shown to be safe and immunogenic in phase I clinical trials following intradermal or subcutaneous inoculation, both in healthy seronegative and in seropositive individuals [38,48] and, based on these results, phase II clinical trials have started in July 2008 in Italy [http://www.hiv1tat-vaccines.info/]. Moreover, the HIV-1 Tat protein is considered an optimal co-antigen for anti-HIV/AIDS combined vaccine strategies employing HIV structural genes [36,44,49–51]. Indeed, the Tat protein possesses very peculiar properties rendering it an interesting model antigen to evaluate this technology platform, such as the fact that Tat oxidizes very easily during handling, because of the presence of 7 cysteines in its sequence. Oxidation leads to rapid loss of monomeric conformation and biological activity which are essential for Tat immunogenic [39,40] and immunomodulatory properties [36], and its capability of broadening in vivo the generation of CTL responses to a co-administered heterologous antigens [44,49–51]. Thereby, special procedures are usually followed for purification, handling and storage of Tat to preserve its monomeric form and fully biological activity [36–38].

Based on this knowledge and considering the possibility of simplifying the storage and transport of a Tat protein-based vaccine, we have previously demonstrated in vitro that new surfactant-free anionic PMMA core–shell nano- and microspheres increase the stability of the Tat protein, i.e. they interfer with Tat multimer formation impeding oxidation caused by air, light and high temperature and preserving its biological activity [33–35]. However, the safety and immunogenic potential of new vaccine formulations based on these novel nano- and microspheres and Tat had still to be systematically investigated in preclinical models.

The results presented here show that the described PMMA-based core–shell nano- and microspheres hold great promise as protein vaccine delivery systems and effective adjuvants for inducing antigen-specific humoral and cellular responses. In particular, we have selected for these studies the H1D microspheres ($1.99\,\mu m \pm 0.17$) and two samples of nanospheres, namely MA7 ($0.22\,\mu m \pm 0.008$) and 2H1B ($0.63\,\mu m \pm 0.06$). The selection was based on their previously characterized physico-chemical properties, including small and homogeneous size, high surface loading capability of native proteins and preservation of Tat biological activity, reproducibility, and in vitro and in vivo lack of toxicity ([33–35], and unpublished results).

The results here reported indicate that the H1D/Tat vaccine formulation given i.m. and s.c. is well-tolerated in mice (Table 2), increases the cellular responses, including CTLs, against Tat (Figs. 1–3) and primes very efficiently the humoral arm of the immune system, as IgG titers were readily detected after 2 immunizations with the H1D/Tat formulation and, after one protein boost, they reached high levels comparable to those detected in animals immunized 3 times with Tat and alum, an adjuvant known to induce high antibody titers (Figs. 1 and 3). The results are in agreement with previous data showing that formulations composed of MA7 nanospheres and Tat, given i.m., increase the antigen-specific cellular immunity, with a prevalence of Th1-type responses, and promote an efficient priming of the humoral arm [35]. Additionally, the results are in agreement with the work described by others showing that vaccines based on Tat adsorbed on the surface of anionic wax nanoparticles developed increased Th1-type cellular immune responses as compared to Tat/alum vaccination [17] and levels of anti-Tat IgG titers similar to those elicited by vaccination with Tat/alum [17,52]. Similarly, Guillon and co-workers [53] demonstrated that immunization of rabbits with wild-type Tat adsorbed onto anionic PLA nanoparticles induced high titers of anti-Tat IgG antibodies in a fashion similar to Tat in combination with the MF59 adjuvant, both in serum and faeces. However, the effect of the Tat/PLA vaccine on cellular immunity was not reported in this study. With respect to the H1D/Tat formulation, we wish to highlight that, based on the results here reported, this vaccine has already proceeded to further preclinical testing in monkeys for safety, immunogenicity and efficacy and that the results confirm the promising features of these particles as vaccine delivery system

[Ensoli et al., manuscript in preparation]. In addition, an appropriate selection of experimental parameters allowed the semi-pilot scale preparation of H1D microspheres which was shown to be easy, inexpensive and highly reproducible in terms of microsphere size, surface functionality and biological behaviour [Sparnacci et al., manuscript in preparation]. Moreover, the purification procedure allowed the preparation of microspheres to be used in vivo in agreement with the EMEA guidelines (ICH topic Q3C), which are endotoxin-free, thus achieving important tasks in view of a future clinical development of this technology.

Another interesting results of this study is the observation that the presence of the nano- and microspheres in the vaccine formulations is well-tolerated also by the i.n route of inoculation (Table 2), which indeed primed efficiently the cellular (Figs. 4, 5 and 7) and humoral (Fig. 6) arms of the immune system. A potent effect was observed on antigen-specific cellular responses, in particular with the nanospheres-based formulations, implying that the particle's size has important consequence on induced specific-immunity (Figs. 4, 5 and 7). The factors that regulate the adjuvant effects are under investigation, and at least in part they may be due to a greater capability of the smallest particles to be taken up by the antigen presenting cells [Castaldello et al., manuscript in preparation] and to their higher surface area/gram and loading ability (Table 1) that may allow a more prolonged depot effect. After i.n. immunization, IgG titers were detected only after one protein boost (Fig. 6). Notably, few weeks after the boost, they reached levels similar (2H1B/Tat) or even higher (H1D/Tat, MA7/Tat) than those detected in animals immunized with the same dose of Tat alone (Fig. 6). To our surprise, H1D/Tat, MA7/Tat and 2H1B/Tat vaccination and the schedules of immunizations here described induced barely detectable Tat specific antibodies in lung and vaginal fluids, before and after the protein boost. A possible explanation may be that the dose of 1 μg of Tat used in these experiments is too low to induce mucosal antibody responses at detectable levels, even if Tat is delivered by the particles. This is suggested by the observation that mucosal antibodies were detected at variable degree only in few mice vaccinated with 10 μg of Tat alone (as reported in Section 3), which is in agreement with previously described results [52,54–56]. As an efficacious vaccine against HIV-1 should evoke both humoral and cellular immunity, especially production of IgA and IgG antibodies on the mucous membranes, modification of the nanoparticles and protocol of immunization are under investigation. Nevertheless, locally at mucosal associated lymphoid tissue, strong and long-lasting cellular responses, which represent also an important component of the local immunity, were induced by the nano- and microspheres formulations at levels significantly greater than those elicited by a ten-fold higher dose of Tat alone (Fig. 7), and these responses became even stronger after the protein boost.

In conclusion, the present work demonstrates the ability of Tat-carrying PMMA-based functional nano- and microspheres to be safe and induce robust and long-lasting cellular and humoral responses in mice after systemic and/or mucosal immunization. Several advantages characterize these delivery systems, as earlier underscored [31–35]. They are inexpensive and easy to produce on large-scale under GLP/GMP conditions, can be stored lyophilized or in aqueous solution at room temperature, are easy to transport, safe and not immunogenic. The functional groups are tightly bound to the particle surface, thus limiting the effects of physical desorption of stabilizing and adsorbing agents, as generally reported in the literature and recently reviewed [57]. The synthetic procedure allows the large-scale preparation of reproducible, stable and homogeneous particle batches, and a wide modulation of the outer shell, so that specific and reversible adsorption of antigens with varying hydrophobicity, molecular weight, and isoelectric point can be envisaged. Formation of the complexes is easy and fast, since they spontaneously assemble in aqueous solution after incubation of the two components for 1–2 h, and no purification steps are required. They protect the antigen from enzymatic degradation, and preserve its native conformation and biological activity. Hence, these delivery vectors may have great potential as antigen carrier not only for novel Tat protein-based vaccines against HIV-1 but also for other infectious diseases agents.

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References

[1] Podda A, Del Giudice G. MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile. Exp Rev Vaccines 2003;2:197–203.
[2] Gupta RK. Aluminum compounds as vaccine adjuvants. Adv Drug Deliv Rev 1998;32:155–72.
[3] Singh M, Ugozzoli M, Kazzaz J, Chesko J, Soenawan E, Mannucci D, et al. A preliminary evaluation of alternative adjuvants to alum using a range of established and new generation vaccine antigens. Vaccine 2006;24:1680–6.
[4] Singh M, O'Hagan DT. Advances in vaccine adjuvants. Nat Biotech 1999;17:1075–81.
[5] Ott G, Barchfeld GL, Chernoff D, Radhakrishnan R, van Hoogevest P, Van Nest G. MF59. Design and evaluation of a safe and potent adjuvant for human vaccines. Pharm Biotechnol 1995;6:277–96.
[6] Singh M, Kazzaz J, Ugozzoli M, Malyala P, Chesko J, O'Hagan DT. Polylactide-co-glycolide microparticles with surface adsorbed antigens as vaccine delivery systems. Curr Drug Deliv 2006;3:115–20.
[7] Bramwell VW, Perrie Y. Particulate delivery systems for vaccines: what can we expect? J Pharm Pharmacol 2006;58:717–28.
[8] Langer R, Cleland JL, Hanes J. New advances in microsphere-based single-dose vaccines. Adv Drug Deliv Rev 1997;28:97–119.
[9] Davis SS. The use of soluble polymers and polymer microparticles to provide improved vaccine responses after parenteral and mucosal delivery. Vaccine 2006;24(Suppl. 2). S2-7–S2-10.
[10] Rydell N, Stertman L, Sjoholm I. Starch microparticles as vaccine adjuvant. Exp Opin Drug Deliv 2005;2:807–28.
[11] Espuelas S, Roth A, Thumann C, Frisch B, Schuber F. Effect of synthetic lipopeptides formulated in liposomes on the maturation of human dendritic cells. Mol Immunol 2005;42:721–9.
[12] Sah H. Stabilization of proteins against methylene chloride/water interface-induced denaturation and aggregation. J Control Rel 1999;58:143–51.
[13] Panyam J, Dali MM, Sahoo SK, Ma W, Chakravarthi SS, Amidon GL, et al. Polymer degradation and in vitro release of a model protein from poly(D,L-lactide-co-glycolide) nano- and microparticles. J Control Rel 2003;92:173–87.
[14] Tamber H, Johansen P, Merkle HP, Gander B. Formulation aspects of biodegradable polymeric microspheres for antigen delivery. Adv Drug Deliv Rev 2005;57:357–76.
[15] Singh M, Chesko J, Kazzaz J, Ugozzoli M, Kan E, Srivastava I, et al. Adsorption of a novel recombinant glycoprotein from HIV (Env gp120dV2 SF162) to anionic PLG microparticles retains the structural integrity of the protein, whereas encapsulation in PLG microparticles does not. Pharm Res 2004;21:2148–52.
[16] O'Hagan DT, Singh M. Microparticles as vaccine adjuvants and delivery systems. Exp Rev Vaccines 2003;2:269–83.
[17] Cui Z, Patel J, Tuzova M, Ray P, Phillips R, Woodward JG, et al. Strong T cell type-1 immune responses to HIV-1 Tat (1-72) protein-coated nanoparticles. Vaccine 2004;22:2631–40.
[18] Jabbal-Gill I, Lin W, Jenkins P, Watts P, Jimenez M, Illum L, et al. Potential of polymeric lamellar substrate particles (PLSP) as adjuvants for vaccines. Vaccine 1999;18:238–50.
[19] Singh M, Briones M, Ott G, O'Hagan D. Cationic microparticles: a potent delivery system for DNA vaccines. Proc Nat Acad Sci USA 2000;97:811–6.
[20] O'Hagan D, Singh M, Ugozzoli M, Wild C, Barnett S, Chen MC, et al. Induction of potent immune responses by cationic microparticles with adsorbed human immunodeficiency virus DNA vaccines. J Virol 2001;75:9037–43.
[21] O'Hagan DT, Singh M, Dong C, Ugozzoli M, Berger K, Glazer E, et al. Cationic microparticles are a potent delivery system for a HCV DNA vaccine. Vaccine 2004;23:672–80.
[22] Vajdy M, Selby M, Medina-Selby A, Coit D, Hall J, Tandeske L, et al. Hepatitis C virus polyprotein vaccine formulations capable of inducing broad antibody and cellular immune responses. J Gen Virol 2006;87:2253–62.
[23] Oster CG, Kim N, Grode L, Barbu-Tudoran L, Schaper AK, Kaufmann SHE, et al. Cationic microparticles consisting of poly(lactide-co-glycolide) and polyethylenimine as carriers systems for parental DNA vaccination. J Control Rel 2005;104:359–77.

[24] Kazzaz J, Neidleman J, Singh M, Ott G, O'Hagan DT. Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against recombinant p55 gag from HIV-1. J Control Rel 2000;67:347–56.
[25] Reddy ST, van der Vlies AJ, Simeoni E, Angeli V, Randolph GJ, O'Neil CP, et al. Exploiting lymphatic transport and complement activation in nanoparticle vaccines. Nat Biotech 2007;25:1159–64.
[26] Fischer D, Li YX, Ahlemeyer B, Krieglstein J, Kissel T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. Biomaterials 2003;24:1121–31.
[27] Sesardic D, Dobbelaer R. European union regulatory developments for new vaccine adjuvants and delivery systems. Vaccine 2004;22:2452–6.
[28] Kasturi SP, Sachaphibulkij K, Roy K. Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. Biomaterials 2005;26:6375–85.
[29] Kasturi SP, Qin H, Thomson KS, El-Bereir S, Cha SC, Neelapu S, et al. Prophylactic anti-tumor effects in a B cell lymphoma model with DNA vaccines delivered on polyethylenimine (PEI) functionalized PLGA microparticles. J Control Rel 2006;113:261–70.
[30] Ataman-Onal Y, Munier S, Ganee A, Terrat C, Durand PY, Battail N, et al. Surfactant-free anionic PLA nanoparticles coated with HIV-1 p24 protein induced enhanced cellular and humoral immune responses in various animal models. J Control Rel 2006;112:175–85.
[31] Ensoli B, Caputo A, Gavioli R, Tondelli L, Laus M, Sparnacci K. Patent UK 0325624.5 2003, WO2005/049093.
[32] Ensoli B, Caputo A, Laus M, Tondelli L, Sparnacci K. Patent UK 0325625.2 2003, WO2005/048997.
[33] Sparnacci K, Laus M, Tondelli L, Bernardi C, Magnani L, Corticelli F, et al. Core–shell microspheres by dispersion polymerization as promising delivery systems for proteins. J Biomat Sci Polymer Edn 2005;16:1557–74.
[34] Caputo A, Brocca-Cofano E, Castaldello A, De Michele R, Altavilla G, Marchisio M, et al. Novel biocompatible anionic polymeric microspheres for the delivery of the HIV-1 Tat protein for vaccine application. Vaccine 2004;22:2910–24.
[35] Voltan R, Castaldello A, Brocca-Cofano E, Altavilla G, Caputo A, Laus M, et al. Preparation and characterization of innovative protein-coated poly(methylmethacrylate) core–shell nanoparticles for vaccine purposes. Pharm Res 2007;24:1870–82.
[36] Fanales-Belasio E, Moretti S, Nappi F, Barillari G, Micheletti F, Cafaro A, et al. Native HIV-1 Tat protein targets monocyte-derived dendritic cells and enhances their maturation, function, and antigen-specific T cell responses. J Immunol 2002;168:197–206.
[37] Ensoli B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, et al. Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. J Virol 1993;67:277–87.
[38] Ensoli B, Fiorelli V, Ensoli F, Cafaro A, Titti F, Butto S, et al. Candidate HIV-1 Tat vaccine development: from basic science to clinical trials. AIDS 2006;20:2245–61.
[39] Cafaro A, Caputo A, Fracasso C, Maggiorella MT, Goletti D, Baroncelli S, et al. Control of SHIV-89.6P-infection of cynomolgus monkeys by HIV-1 Tat protein vaccine. Nat Med 1999;5:643–50.
[40] Maggiorella MT, Baroncelli S, Michelini Z, Fanales-Belasio E, Moretti S, Sernicola L, et al. Long-term protection against SHIV89.6P replication in HIV-1 Tat vaccinated cynomolgus monkeys. Vaccine 2004;22:3258–69.
[41] Castaldello A, Brocca-Cofano E, Voltan R, Triulzi C, Altavilla G, Laus M, et al. DNA prime and protein boost immunization with innovative polymeric cationic core–shell nanoparticles elicits broad immune responses and strongly enhance cellular responses of HIV-1 tat DNA vaccination. Vaccine 2006;24:5655–69.
[42] Caputo A, Gavioli R, Altavilla G, Brocca-Cofano E, Boarini C, Betti M, et al. Immunization with low doses of HIV-1 tat DNA delivered by novel cationic block copolymers induces CTL responses against Tat. Vaccine 2003;21:1103–11.
[43] Caselli E, Betti M, Grossi MP, Balboni PG, Rossi C, Boarini C, et al. DNA immunization with HIV-1 tat mutated in the trans activation domain induces humoral and cellular immune responses against wild-type Tat. J Immunol 1999;162:5631–8.
[44] Caputo A, Brocca-Cofano E, Castaldello A, Voltan R, Gavioli R, Srivastava IK, et al. Characterization of immune responses elicited in mice by intranasal co-immunization with HIV-1 Tat, gp140 DeltaV2Env and/or SIV Gag proteins and the nontoxicogenic heat-labile *Escherichia coli* enterotoxin. Vaccine 2008;26:1214–27.
[45] Ferrantelli F, Cafaro A, Ensoli B. Nonstructural HIV proteins as targets for prophylactic or therapeutic vaccines. Curr Opin Biotechnol 2004;15:543–56.
[46] Caputo A, Gavioli R, Ensoli B. Recent advances in the development of HIV-1 Tat-based vaccines. Curr HIV Res 2004;2:357–76.
[47] Titti F, Cafaro A, Ferrantelli F, Tripiciano A, Moretti S, Caputo A, et al. Problems and emerging approaches in HIV/AIDS vaccine development. Exp Opin Emerg Drugs 2007;12:23–48.
[48] Ensoli B, Fiorelli V, Ensoli F, Lazzarin A, Visintini R, Narciso P, et al. The therapeutic phase I trial of the recombinant native HIV-1 Tat protein. AIDS 2008;22:2207–9.
[49] Gavioli R, Gallerani E, Fortini C, Fabris M, Bottoni A, Canella A, et al. HIV-1 tat protein modulates the generation of cytotoxic T cell epitopes by modifying proteasome composition and enzymatic activity. J Immunol 2004;173:3838–43.
[50] Gavioli R, Cellini S, Castaldello A, Voltan R, Gallerani E, Gagliardoni F, et al. The Tat protein broadens T cell responses directed to the HIV-1 antigens Gag and Env: implications for the design of new vaccines against AIDS. Vaccine 2007;26:727–37.
[51] Bråve A, Hinkula J, Cafaro A, Eriksson LE, Srivastava IK, Magnani M, et al. Candidate HIV-1 gp140ΔV2, Gag and Tat vaccines protect against experimental HIV-1/MuLV challenge. Vaccine 2007;25:6882–90.
[52] Patel J, Galey D, Jones J, Ray P, Woodward JG, Nath A, et al. HIV-1 Tat-coated nanoparticles result in enhanced humoral immune responses and neutralizing antibodies compared to alum adjuvant. Vaccine 2006;24:3564–73.
[53] Guillon C, Mayol K, Terrat C, Compagnon C, Primard C, Charles MH, et al. Formulation of HIV-1 Tat and p24 antigens by PLA nanoparticles or MF59 impacts the breadth, but not the magnitude, of serum and faecal antibody responses in rabbits. Vaccine 2007;25:7491–501.
[54] Borsutzky S, Fiorelli V, Ebensen T, Tripiciano A, Rharbaoui F, Scoglio A, et al. Efficient mucosal delivery of the HIV-1 Tat protein using the synthetic lipopeptide MALP-2 as adjuvant. Eur J Immunol 2003;33(6):1548–56.
[55] Borsutzky S, Ebensen T, Link C, Becker PD, Fiorelli V, Cafaro A, et al. Efficient systemic and mucosal responses against the HIV-1 Tat protein by prime/boost vaccination using the lipopeptide MALP-2 as adjuvant. Vaccine 2006;24:2049–56.
[56] Marinaro M, Riccomi A, Rappuoli R, Pizza M, Fiorelli V, Tripiciano A, et al. Mucosal delivery of the human immunodeficiency virus-1 Tat protein in mice elicits systemic neutralizing antibodies, cytotoxic T lymphocytes and mucosal IgA. Vaccine 2003;21:3972–81.
[57] Caputo A, Sparnacci K, Ensoli B, Tondelli L. Functional polymeric nano/microparticles for surface adsorption and delivery of protein and DNA vaccines. Curr Drug Deliv 2008;5:230–42.