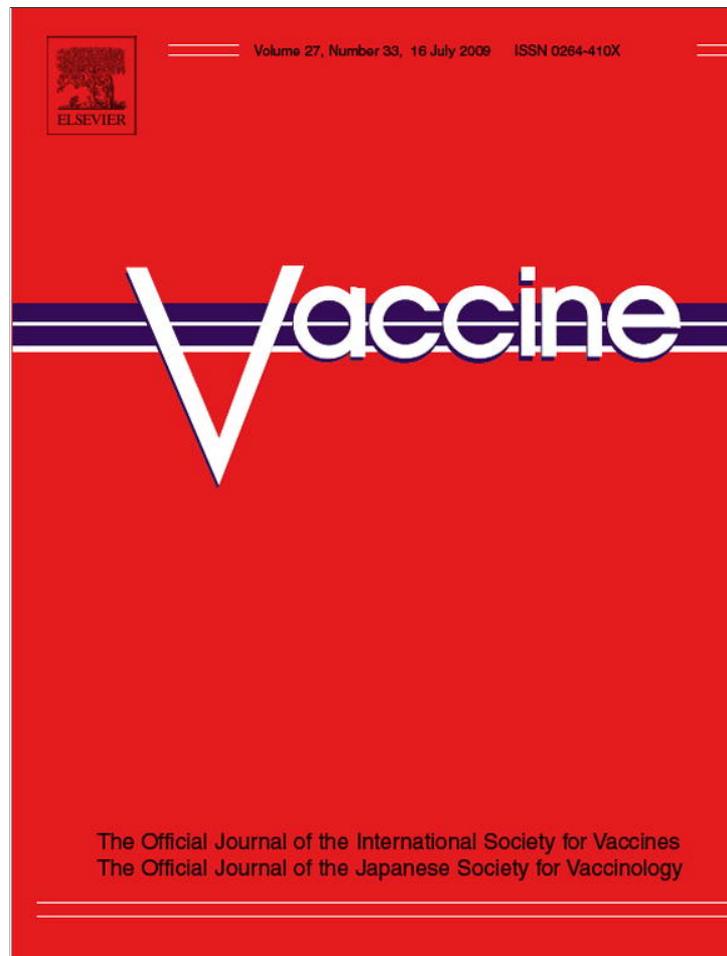


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Priming with a very low dose of DNA complexed with cationic block copolymers followed by protein boost elicits broad and long-lasting antigen-specific humoral and cellular responses in mice

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ABSTRACT

Cationic block copolymers spontaneously assemble via electrostatic interactions with DNA molecules in aqueous solution giving rise to micellar structures that protect the DNA from enzymatic degradation both *in vitro* and *in vivo*. In addition, we have previously shown that they are safe, not immunogenic and greatly increased antigen-specific CTL responses following six intramuscular inoculations of a very low dose (1 μ g) of the vaccine DNA as compared to naked DNA. Nevertheless, they failed to elicit detectable humoral responses against the antigen. To gain further insight in the potential application of this technology, here we show that a shorter immunization protocol based on two DNA intramuscular inoculations of 1 μ g of DNA delivered by these copolymers and a protein boost elicits in mice broad (both humoral and cellular) and long-lasting responses and increases the antigen-specific Th1-type T cell responses and CTLs as compared to priming with naked DNA. These results indicate that cationic block copolymers represent a promising adjuvant and delivery technology for DNA vaccination strategies aimed at combating intracellular pathogens.

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1. Introduction

DNA vaccines represent attractive approaches for the development of novel immunization strategies to combat infectious diseases because they are stable and inexpensive and, therefore, they are also potentially affordable by the developing world [1–4]. However, the high expectations deriving from the results of DNA vaccination in animal models were diminished by several disappointing results when DNA vaccines were tested in humans [1,5,6]. Inefficient delivery, especially to antigen-presenting cells, and high extra- and intra-cellular degradation of naked DNA may be contributing to lack of potency. This may partially explain why

high doses (mg range) and multiple boosts are required to elicit robust immune responses which, in addition to raise the production costs, may impair the success of vaccination particularly in the developing world. Nevertheless, DNA vaccines are easy and fast to prepare on large-scale, and experimental evidence indicate that they can induce broad immune responses against multiple antigens and can be conveniently manipulated for the induction of the desired immunity [1,6–8]. Consequently, the success of DNA vaccines depends on the improvement of DNA delivery and, therefore, requires the development of novel technologies, including the use of polymeric or biological (viral or bacterial) delivery systems.

Several groups have shown that it is possible to increase the transfection efficiency of DNA, to improve the immunogenicity of gene vaccines and to reduce the number of booster shots by using polymeric delivery systems such as cationic and chitosan microparticles, non-ionic block copolymers and cationic liposomes, which also enable the use of lower doses of DNA [4,5,7–10]. These formulations may function as depot adjuvants by protecting the DNA from

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degradation, thereby prolonging antigen presentation and improving immune responses against the antigen [4,5,7–10].

Polycations have been receiving increasing attention since they are not immunogenic, much safer and cheaper than biological vectors, can be produced in large quantities, and have high genetic material carrying capacity. In addition, they commonly carry functional positive charged amine groups that bind negatively charged DNA molecules to form small condensates (100–200 nm), which facilitates DNA transport through the negatively charged cell membrane, usually by endocytosis [7,11].

In this context, we have previously described a novel class of cationic block copolymers, constituted by a neutral hydrophilic poly(ethylene glycol) (PEG) block and a positively charged poly(dimethylamino)ethyl methacrylate (PDMAEMA) block, which are capable of spontaneously assembling with DNA in aqueous solution to give rise to micellar structures of 100–200 nm in diameter. The supramolecular structure of these complexes is a core-shell-type micelle in which the hydrophobic core consists of DNA, linked via electrostatic interaction to the charged PDMAEMA block, and the outer shell is constituted by the neutral hydrophilic PEG block of the copolymer [12]. Subsequently, we have shown in mice that these copolymers protect the DNA from nuclease digestion, are safe and not immunogenic. Moreover, they improve the delivery of DNA to antigen-presenting cells (in particular copolymer K2) and significantly increase antigen-specific CTL responses as compared to vaccination with naked DNA, using a very low dose (1 µg) of DNA and a long-term vaccination protocol (6 intramuscular immunizations) [13,14]. Nevertheless, this dose of DNA and vaccination strategy was not efficient to raise detectable antibody titers against the delivered antigen, in a fashion similar to vaccination with the same dose of naked DNA [14].

Ideally, efficacious prophylactic and therapeutic DNA vaccines should be able to activate both arms of the immune system possibly with a low dose of DNA and one or few immunizations. In this respect, particularly promising approaches are represented by heterologous prime-boost immunization strategies. Such strategies have been shown to enhance humoral and cellular immunity in several different animal and disease models.

Thereby, to gain more insight in the potential use of this technology, in the present study we have further investigated the potency and safety of DNA/K2 vaccines using a shorter immunization protocol based on a DNA prime-protein boost regimen. Consistently with previous studies [15–21], 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 [15–22]. In addition, this allowed direct comparison with the results of our previous studies where the cationic block copolymers were administered with pCV-*tat* DNA (1 µg) 6 times by the i.m. route [14]. Finally, Tat is a vaccine relevant antigen and the Tat-vaccine has recently completed preventive and therapeutic phase I clinical testing [23,24] and, based on the results, a phase II-proof of concept trial as therapeutic vaccine is currently being conducted in Italy [<http://www.hiv1tat-vaccines.info/>]. In addition, due to its immunomodulatory properties [25], Tat (as protein or DNA) will soon be tested in humans in combination with Env and/or other HIV antigens [26–30] within the AIDS Vaccine Integrated Project (AVIP) funded by the European Community [<http://avip-eu.org>].

The results of the present study demonstrate that two inoculations of the DNA/K2 formulation were sufficient to prime efficiently both arms of the immune system since broad and durable humoral responses with high titers were observed after protein boosting. Moreover, the results show that the presence of K2 in the vaccine formulations plays a key difference in inducing stronger Th1-type T

cell responses and CTLs against the delivered antigen as compared to priming with naked DNA.

2. Materials and methods

2.1. Cationic block copolymers

Cationic block copolymer K2 is composed of a positively charged block (approximately 300 positive charged groups per molecule) derived from poly(dimethylamino ethyl methacrylate) (PDMAEMA) fully methylated with methyl iodide, capable of interacting with negatively charged DNA molecules at physiological pH, and of a neutral and highly hydrophilic poly(ethylene glycol) (PEG) block. Cationic block copolymer K2 (Mn 91000) was synthesized and characterized as previously described [12–14], resuspended (10 mg/ml) in sterile phosphate-buffered saline (PBS) without calcium and magnesium (PBS-A), stored at –20 °C until use and diluted at 1 mg/ml in PBS-A immediately before use. The K2 copolymer was selected among other molecules for this study based upon its previously characterized *in vitro* and *in vivo* performance [12–14].

2.2. Preparation of DNA/copolymer complexes

Plasmid pCV-*tat*, expressing the HIV-1 *tat* cDNA (HLTV-III, BH10 clone) under the transcriptional control of the adenovirus major late promoter, and control plasmid pCV-0 (empty vector) were previously described [31–33]. Plasmid DNAs were purified using the plasmid maxi kit provided by Qiagen (Hilden, Germany), according to the manufacturer's instructions, resuspended in sterile PBS-A and analyzed (restriction enzyme digestion, PCR and sequence analysis, protein expression and endotoxin content) as already described [13,15,34]. The DNA/K2 complexes (1 µg DNA/1 µg K2) were prepared immediately before use at defined molar ratio of copolymer quaternary ammonium positive groups to DNA phosphate negative groups (N-to-P ratio) of 1.0 [12–14]. Briefly, the appropriate volume of copolymer K2 (1 mg/ml) and DNA (1 mg/ml) were mixed in PBS-A, incubated 1 h at room temperature to allow complex formation and inoculated (100 µl/mouse) without further processing. Endotoxin concentration of plasmid DNA and K2 copolymer was always below the detection limit (<0.05 EU/µg), as tested by the *Limulus* Amoebocyte Lysate analysis.

2.3. Tat protein and peptides

The 86-aa long Tat protein (HTLVIII, BH-10 clone) was expressed in *Escherichia coli*, as previously described [33,35], and provided by Diatheva (Fano, Italy). 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. To prevent oxidation that occurs easily because Tat contains seven cysteines, the Tat protein was stored lyophilized at –80 °C and resuspended in degassed sterile PBS (2 mg/ml) (for boost immunization) or in PBS containing 0.1% bovine serum albumin (for ELISA and lymphoproliferation testing) immediately before use. In addition, since Tat is photo- and thermo-sensitive, the handling of Tat was always performed in the dark and on ice [33,35]. Peptides were synthesized by UFPeptides s.r.l. (Ferrara, Italy) based on HIV-1 Tat BH10 clone sequence. The VCF (VCFITKALGISYGRK) Tat peptide contains a K^d-restricted CTL epitope and a CD4+ T cell epitope [18,29,30,36], and its derivative peptide CFI (CFITKALGI) contains the minimal K^d-restricted CTL epitope. Peptides (15-mers) were also produced for Tat IgG epitope mapping analysis as previously described [17]. Peptide stocks were prepared in DMSO at 10^{–2} M concentration, stored at –80 °C, and diluted in PBS (for ELISA tests) or RPMI 1640 (for Elispot and CTL assays) before use.

2.4. Mice immunization

Animal use was according to national guidelines and institutional policies. Six to 8-week-old female BALB/c mice (Charles River, Italy) were immunized at weeks 0 and 4 with the pCV-*tat* plasmid (1 µg) complexed with copolymer K2 (1 µg) or with the same dose of pCV-*tat* DNA alone. Both groups of mice were then boosted with the Tat protein (1 µg) in Aluminium phosphate (Alum) adjuvant 3 weeks (week 7) (2 DNA primes-1 protein boost regimen) and 6 weeks (week 10) (2 DNA primes-2 protein boosts regimen) after the last DNA immunization. Control groups included mice injected with (i) pCV-0 (1 µg) associated with K2 or (ii) naked pCV-0 DNA, and boosted with Alum/PBS. Alum adjuvant was prepared from 10% AlCl₃·6H₂O and 16% Na₃PO₄·12H₂O stock solutions. Before immunization the two solutions were diluted in degassed sterile PBS to reach a final concentration of 7 g/l AlCl₃·6H₂O and 11.125 g/l Na₃PO₄·12H₂O, briefly mixed together with an equal volume of the Tat protein and immediately inoculated. Each experimental group was composed of 14 mice. Immunogens (100 µl) were given by intramuscular (i.m.) injections in the quadriceps muscles of the posterior legs (50 µl/leg). 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). During the experiments, animals were controlled twice a week at sites of injection and for their general conditions (such as liveliness, food intake, vitality, weight, motility, sheen of hair). Mice were sacrificed at week 10 ($n=3$) for the two DNA primes-one protein boost regimen, and at weeks 12 ($n=3$) and 28 ($n=8$) for the two DNA primes-two protein boosts regimen, to collect blood and organs for analysis of humoral and cellular responses, and for histological, histochemical and immunohistochemical studies. To confirm the result, the immunization experiment was repeated 3 times independently.

To assess the effect of *tat*/K2 priming, in some experiments mice ($n=4$) were also immunized i.m. with pCV-*tat*/K2 or pCV-0/K2 (same dose as above) at weeks 0 and 4, and control groups with pCV-*tat* or pCV-0. All groups were then boosted with Tat/Alum 4 weeks later (week 8) and sacrificed 2 weeks after the boost (week 10). In addition, to assess the effect of the protein boost, in some experiments mice ($n=4$) were immunized i.m. with pCV-*tat*/K2 or pCV-*tat* at weeks 0 and 4, boosted with Tat/Alum (+Tat boost) or with PBS/Alum (-Tat boost) at week 8 and sacrificed at week 10.

2.5. Serology

Serological responses against Tat (IgG titers, subclasses, and epitope mapping) were measured on individual mice sera (duplicate wells) by an established enzyme-linked immunosorbent assay (ELISA) using 96-well immunoplates (Nunc-immunoplate MaxiSorb) coated with 100 µl/well of Tat protein (1 µg/ml in 0.05 M carbonate buffer pH 9.6–9.8) or with Tat peptides (10 µg/ml in PBS) for 12 h at 4 °C, as previously described [14,17,30,36].

2.6. Splenocytes purification

Spleens were squeezed through a 70-µm nylon cell strainer (BD-Pharmingen, San Jose, CA) using RPMI 1640 supplemented with 10% Hyclone serum (Hyclone, Logan, UT). After red blood cells lysis (RBC lysing buffer, Sigma, St. Louis, MI), splenocytes were washed twice with RPMI medium (BioWhittaker, Walkersville, MD) supplemented with 3% Hyclone serum, and resuspended in RPMI medium supplemented with 10% Hyclone serum, 1% L-glutamine (Sigma), 1% penicillin/streptomycin (BioWhittaker), 0.2% non-essential amino acids (Sigma), 1 mM sodium pyruvate (Sigma), and 50 µM β-mercaptoethanol (Gibco, Grand Island, NY). Splenocytes

were counted and viability was determined by the trypan blue exclusion dye. Pool of spleens per experimental group was used for the analysis of cellular responses, according to well-established protocols, as described below. In some experiments, depletion of B lymphocytes and purification of CD8+ T cells were carried out using anti-CD45R/B220 and anti-CD8 magnetic beads (BD-Pharmingen), according to the manufacturer's instructions. Purified cultures were then analyzed by fluorescence-activated cell sorter analysis (FAC-SCalibur, BD) 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). Purity of CD8+ and CD4+ T cell cultures were 90–95%.

2.7. T cell proliferation to the Tat protein

Splenocytes ($4 \times 10^5/200 \mu\text{l}$) were cultured in 96-well plates (sestuplicate wells) in the presence or absence of affinity-purified and biologically active Tat protein (0.1, 1, or 5 µg/ml) or Concanavalin A (2 µg/ml, Roche, Mannheim, Germany) for 5 days at 37 °C. [methyl-³H]-Thymidine (2.0 Ci/mmol, NEN-DuPont) was added to each well (1 µCi), and cells were incubated for 16 h at 37 °C. [³H]-Thymidine incorporation was measured with a β-counter (Top Count, Packard). The stimulation index (S.I.) was calculated by dividing the mean counts/min of six wells of antigen-stimulated cells by the mean counts/min of the same cells grown in the absence of the antigen. S.I. ≥ 2 were considered positive. S.I. of Concanavalin A control wells were always around 4–5.

2.8. CTL assays

Splenocytes ($3 \times 10^6 \text{ ml}^{-1}$) were cultured (triplicate cultures) with the VCF Tat peptide (3 µg/ml) for 5 days and extensively washed with RPMI 1640 containing 10% Hyclone serum. CTL activity was determined, at various effector/target ratios, by standard ⁵¹Cr release assays using syngeneic P815 target cells, previously labeled with ⁵¹Cr (25 µCi/ 3×10^6 cells; NEN, Du-Pont) for 90 min at 37 °C and pulsed with the CFI Tat peptide (1×10^{-5} M) for 1 h at 37 °C. After 5 h incubation at 37 °C, the percentage of ⁵¹Cr release was determined in the medium. Percent (%) of specific lysis was calculated as $100 \times (\text{cpm sample} - \text{cpm medium}) / (\text{cpm Triton-X100} - \text{cpm medium})$. Spontaneous release was below 10% [25].

2.9. Elispot assays

Enzyme-linked immunospot (Elispot) assays were performed for evaluation of Th1 (IFN-γ, IL-2) and Th2 (IL-4) cytokines, using commercially available murine IFN-γ, IL-2 and IL-4 Elispot kits (BD-Pharmingen), according to the manufacturer's instructions. Briefly, splenocytes ($3 \times 10^6 \text{ ml}^{-1}$) were cultured with the VCF Tat peptide (3 µg/ml) for 5 days and extensively washed with RPMI 1640 containing 10% FBS. Splenocytes ($4-5 \times 10^4$ cells/well) of each treatment group were then added to 96-well Elispot plates (duplicate wells) pre-coated with the cytokine-specific capture antibody, and incubated at 37 °C for 24 h in the absence (untreated) or presence of Tat peptides (10^{-6} M), or Concanavalin A (5 µg/ml) as a positive control for cytokine secretion. Spots were counted using an automated Elispot reader (AELVis 4.0, Hannover, Germany). The numbers of spots counted in the peptide-treated wells minus the number of spots counted in the untreated wells (background level) were considered the specific responses and they were estimated significant when net spots/million cells were >50 and at least 2 folds above the score of the untreated wells. The numbers of spots in the Concanavalin A control wells were always very high and consequently not measurable (out of scale).

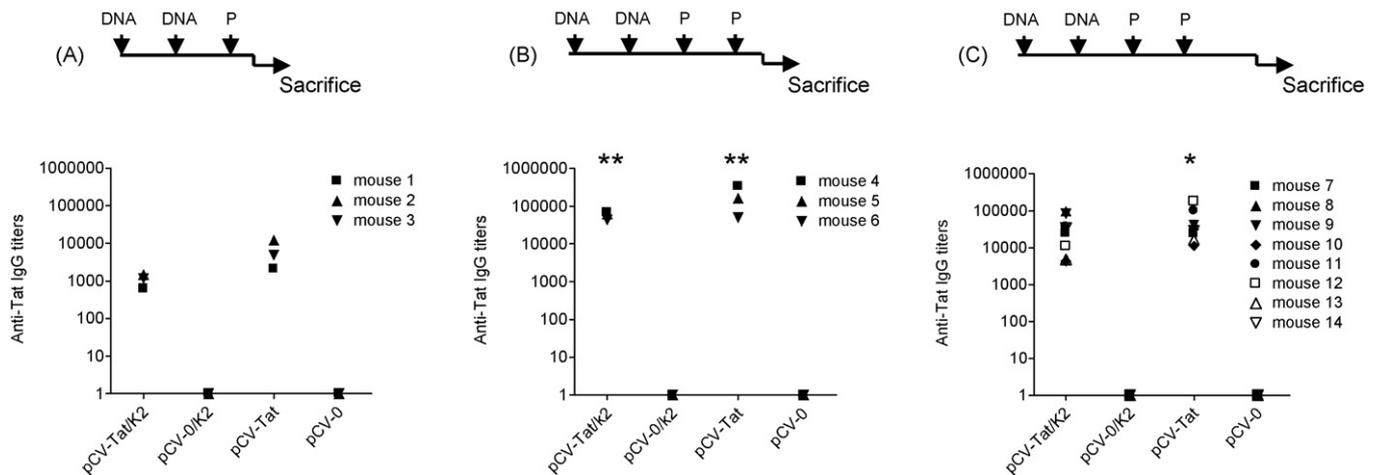


Fig. 1. Anti-Tat IgG responses. Animals were primed i.m. with the DNA/K2 complexes or with naked DNA at weeks 0 and 4, and boosted i.m. with the Tat protein (1 μ g) in Alum, or with PBS/Alum alone (controls), at week 7 (two DNA prime-one protein boost regimen) and at week 10 (two DNA prime-two protein boosts regimen) after the first immunization. Mice were sacrificed at week 10 ($n=3$) for the two DNA primes-one protein boost regimen (A), and at weeks 12 ($n=3$) (B) and 28 ($n=8$) (C) for the two DNA primes-two protein boosts regimen. The results are expressed as the \log_{10} of the endpoint titers of mice sera tested individually by ELISA. The Kruskal–Wallis test was used for statistical analysis. The immunization experiment was repeated 3 times independently. The results of one experiment are shown. Statistical analysis was carried out by comparing *tat*/K2 versus *tat* primed mice (not significant, $p > 0.05$) and by comparing, for each group, the IgG titers at each time point. ** $p < 0.01$, significant increase of IgG titers in *tat*/K2 and *tat* primed mice after 2 protein boosts (week 12) versus IgG titers of the corresponding mice after one protein boost (week 10); * $p < 0.05$ significant decrease in IgG titers in *tat* primed mice as compared to the same group at week 12.

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, intestine, kidneys, brain, liver, spleen, testis, ovaries and draining lymph nodes) were collected and processed for histologic, histochemical and immunohistochemical examination, as described previously [13,14,16,17]. An average of 12 mice per experimental group was analyzed.

2.11. Statistical analysis

The two-way Anova test and Bonferroni post-test, and the Kruskal–Wallis test were performed as described [37]. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Humoral response to Tat after *tat*/K2 priming and protein boost

Previous data of our group showed that the K2 cationic block copolymer associated with a very low dose (1 μ g) of *tat* DNA greatly increased antigen-specific CTL responses after 6 i.m. immunizations, as compared to immunization with naked *tat* DNA. However, 1 μ g of DNA combined to K2 and the 6 i.m. immunizations were not sufficient to elicit detectable antibody responses against Tat, in a fashion similar to immunization with naked DNA [14]. As an extension of these studies and to gain further insight in this technology, here we assessed whether the *tat*/K2 vaccine, in addition to its priming effect on cellular responses, primes also the humoral immunity, using again a low dose of *tat* DNA but this time a shorter immunization protocol based on a DNA prime–protein boost regimen.

Accordingly, animals were immunized twice by the i.m. route with 1 μ g of pCV-*tat* plasmid DNA associated with copolymer K2 (*tat*/K2) at weeks 0 and 4 and then boosted with 1 μ g of native Tat protein in Alum at weeks 7 and 10. Immune responses were analyzed after one (week 10) or two (week 12) protein boosts. Immune

responses were compared to those developed by mice immunized with pCV-*tat* alone (*tat*) and boosted with Tat. Also, control animals included mice injected with the pCV-0/K2 formulation or with naked pCV-0 DNA and boosted with PBS/Alum.

After one protein boost (week 10), anti-Tat IgG titers were detected in all mice primed with *tat*/K2 (mean titers 1088 ± 428) and with naked *tat* DNA (mean titers 6345 ± 5052) (Fig. 1A). After the second protein boost (week 12) (Fig. 1B), anti-Tat IgG titers increased either in mice primed with *tat*/K2 (mean titers 58274 ± 13475) and with naked *tat* DNA (mean titers 184065 ± 145140). The differences between IgG titers of *tat*/K2 and *tat* vaccinees was not statistically significant at any time point ($p > 0.05$), even though titers tended to be slightly higher after *tat* priming. At this time point, for both groups of vaccinees, the increase was statistically significant ($p < 0.01$) as compared to IgG titers observed after one protein boost. The humoral immune responses were long lasting. In fact, 17 weeks after the second protein boost (week 28) high anti-Tat IgG titers were still present in all *tat* immunized mice (*tat*/K2: mean titers 37124 ± 34997 ; *tat*: mean titers 56792 ± 58721) (Fig. 1C). However, at this time point, a significant ($p < 0.05$) decrease in IgG titers was observed in the group primed with naked *tat* DNA as compared to the same group at the earlier time point (week 12), whereas in mice primed with *tat*/K2 IgG levels did not drop when compared to titers in the same group measured at week 12 ($p > 0.05$). These results indicate that the *tat*/K2 vaccine efficiently primes the humoral arm of the immune system, since IgG responses were promptly developed in all mice after one protein boost, in a fashion similar to *tat* priming. However, differently from priming with naked *tat*, *tat*/K2 priming sustains more durable high titers antibody responses.

The epitope reactivity of the antibodies was also determined. It was mainly directed against the immunodominant amino-terminal region of Tat (aa 1–20) (Table 1) in all responding mice, as generally reported for mice, monkeys and humans [38]. However, 17 weeks after the second protein boost (week 28), in the group primed with *tat*/K2 some animals showed also some degree of reactivity against other regions of Tat, including the cysteine-rich region (aa 21–40) (1 out of 8 animal), the glutamine-rich region (aa 57–72 and 65–80) (3 out of 8 mice), and the RGD domain (aa 73–86) (3 out of 8 animals). Conversely, in the group primed with naked *tat* DNA only one

Table 1
Epitope mapping analysis of anti-Tat IgG responses^a.

Peptides (aa)	pCV-tat/K2 (prime)			pCV-tat (prime)		
	I ^h week 10 (2 DNA primes/1 protein boost)	II ^h week 12 (2 DNA primes/2 protein boosts)	III ^h week 28 (2 DNA primes/2 protein boosts)	I ^h week 10 (2 DNA primes/1 protein boost)	II ^h week 12 (2 DNA primes/2 protein boosts)	III ^h week 28 (2 DNA primes/2 protein boosts)
1–20	0.5782 ± 0.4865 (3/3)	2.2978 ± 0.0147 (3/3)	2.1542 ± 0.2834 (8/8)	0.9487 ± 1.0907 (3/3)	2.2192 ± 0.0845 (3/3)	2.1118 ± 0.2306 (8/8)
21–40	0.0 ± 0.0 (0/3)	0.0 ± 0.0 (0/3)	0.0248 ± 0.0344 (1/8) ^b	0.0007 ± 0.0028 (0/3)	0.011 ± 0.0088 (0/3)	0.0279 ± 0.0137 (0/8)
36–50	0.0047 ± 0.0013 (0/3)	0.0088 ± 0.0055 (0/3)	0.0273 ± 0.0143 (0/8)	0.0 ± 0.0 (0/3)	0.0 ± 0.0 (0/3)	0.0097 ± 0.0112 (0/8)
46–60	0.0012 ± 0.001 (0/3)	0.0073 ± 0.002 (0/3)	0.0272 ± 0.0209 (0/8)	0.0 ± 0.0 (0/3)	0.0 ± 0.0 (0/3)	0.0114 ± 0.0111 (0/8)
56–70	0.0013 ± 0.003 (0/3)	0.004 ± 0.0058 (0/3)	0.0236 ± 0.0269 (0/8)	0.0 ± 0.0 (0/3)	0.0 ± 0.0 (0/3)	0.0051 ± 0.0099 (0/8)
57–72	0.0025 ± 0.0015 (0/3)	0.0057 ± 0.0028 (0/3)	0.0411 ± 0.0866 (1/8) ^c	0.0 ± 0.0 (0/3)	0.0 ± 0.0 (0/3)	0.0141 ± 0.0086 (0/8)
65–80	0.0028 ± 0.0033 (0/3)	0.0038 ± 0.0028 (0/3)	0.0556 ± 0.0674 (2/8) ^d	0.0003 ± 0.0039 (0/3)	0.0 ± 0.0 (0/3)	0.0347 ± 0.0534 (1/8) ^f
73–86	0.0048 ± 0.0033 (0/3)	0.001 ± 0.0114 (0/3)	0.1153 ± 0.1391 (3/8) ^e	0.0 ± 0.0 (0/3)	0.0 ± 0.0 (0/3)	0.0487 ± 0.0547 (0/8)

^a Epitope mapping analysis was carried out by ELISA as described in Section 2. Results correspond to the mean OD₄₀₅ nm (±SD) of mice sera tested at each time point at 1:100 dilution. In parenthesis the number of responding animals/total number of mice analyzed at each sacrifice is reported. At each time point, the cutoff values were determined for each peptide and corresponded to the mean OD value of control mice sera (+3SD) immunized with pCV-0/K2 (+PBS/Alum) and pCV-0 (+PBS/Alum) respectively. Cutoff values for each peptide were determined and always resulted <0.05 OD₄₀₅ nm. Responses were considered positive when OD values were at least 2 folds above the cutoff.

^b The OD values of the positive sample and the cutoff are 0.15 and 0.05, respectively.

^c The OD values of the positive sample and the cutoff are 0.26 and 0.04, respectively.

^d The OD values of the 2 positive samples and the cutoff are 0.18 and 0.15, and 0.04, respectively.

^e The OD values of the 3 positive samples and the cutoff are 0.21, 0.22 and 0.38, and 0.06, respectively.

^f The OD value of the positive sample and the cutoff are 0.16 and 0.02, respectively.

animal showed a specific reactivity directed against an additional Tat epitope, namely the RGD domain (aa 65–80) (1 out of 8 mice). Finally, at each time point similar IgG1, IgG2a and IgG3 profiles, with a prevalence of IgG1, were observed in both groups of vaccinees (data not shown). These results suggest that priming with copolymer K2, in addition to induce high titers IgG of both Th1 and Th2-type isotypes and sustain more durable antibody responses, may broaden the anti-Tat IgG epitope specificity.

3.2. Cellular responses to Tat after tat/K2 priming and protein boost

CD4⁺ T cell proliferation in response to the Tat protein was evaluated using the [³H]-Thymidine incorporation test on mice splenocytes cultured for 5 days in the presence of 0.1, 1 or 5 μg/ml of Tat. Antigen-specific and dose-dependent cell proliferation was detected in both groups of mice receiving *tat/K2* or *tat* (Fig. 2), but not in untreated splenocytes nor in lymphocytes of control mice injected with pCV-0/K2 or pCV-0 alone. After one (week 10) (Fig. 2A) and two (week 12) (Fig. 2B) protein boosts, similar stimulation indexes (>2) were detected at the dose of 5 μg/ml of Tat in mice primed with *tat/K2* and with naked *tat* DNA (*p* > 0.05), which were still detected (S.I. = 2) seventeen weeks after the second protein boost (week 28) (*p* > 0.05) (Fig. 2C).

Interestingly, functional anti-Tat CTLs were observed at each time point only after priming with *tat/K2* (Fig. 3), with percentage of specific lysis similar to those reported in few studies [14,15,18,21]. These results indicate an adjuvant effect of K2 priming, in agreement with the previous data showing that the presence of K2 in the vaccine formulation significantly increases CTL responses against the antigen as compared to vaccination with naked DNA [14].

Finally, the generation of Th1-type (IFN-γ and IL-2) and Th2-type (IL-4) producing cells by this vaccination regimen was tested on mice splenocytes using the Elispot technique. IFN-γ, IL-2 and IL-4 secreting cells were always detected at each sacrifice, and in general the numbers of IFN-γ and IL-2 cells were higher than IL-4 responses in both groups (Fig. 4). In addition, after the two DNA primes-one protein boost regimen (week 10) (Fig. 4A) and after the two DNA primes-two protein boosts regimen (week 12) (Fig. 4B), the number of antigen-specific IFN-γ- and IL-2-secreting splenocytes was always higher in mice primed with *tat/K2*, as compared to mice primed with naked DNA, in agreement with the results of a greater cytotoxic activity in the former group (Fig. 3). In particular, statistical significance was observed after one protein boost (*p* < 0.05) for IFN-γ. In contrast, IL-4 responses were similar between the two groups (*p* > 0.05) both at week 10 and 12, in agreement with the results of antibody responses.

Lastly, mice receiving the two DNA primes-two protein boosts immunization were sacrificed 17 weeks after the second protein boost (week 28) (Fig. 4C). At this time point, splenocytes were stimulated *in vitro* with the VCF Tat peptide, depleted of B-cells and Elispot analysis carried out on CD8⁺ (purified) and CD4⁺ (enriched) T lymphocytes subpopulations to assess the nature of cytokine-secreting cells. IFN-γ-, IL-2- and IL-4-producing cells were still present at significant high levels more than 4 months after the last immunization boost. These responses were associated mainly to CD4⁺ memory T cells producing high and similar levels of IFN-γ and IL-2 in both groups of vaccinated mice. Of note, at this time point, IL-4 responses were significantly higher in mice primed with *tat/K2* (*p* < 0.05), in agreement with the results of IgG responses at week 28 showing that antibody titers persisted at high levels after priming with *tat/K2* whereas they significantly dropped in *tat*-primed mice. IFN-γ, IL-2- and IL-4-secreting cells were instead low in the purified CD8⁺ T cells population (data not shown). Since at week 28 specific CTL responses were detected on total splenocytes cultures only in *tat/K2* primed mice (Fig. 3C), the discrepancy in the obtained results

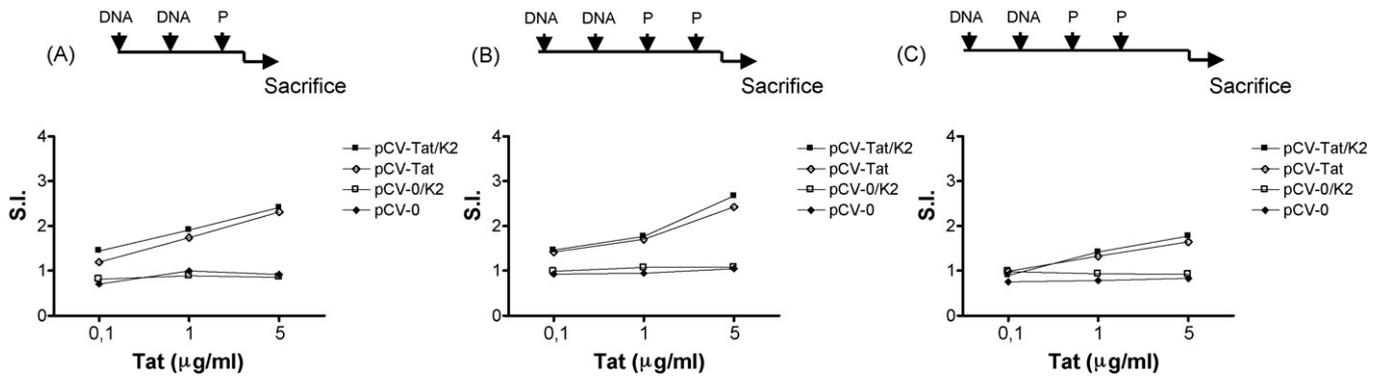


Fig. 2. Analysis of T cell proliferation to Tat protein. Mice were immunized as described in Fig. 1. Immune responses were tested at week 10 (after one protein boost) (A) and at weeks 12 (B) and 28 (C) (after two protein boosts). Values represent the stimulation index (S.I.) of murine splenocytes (pool of spleens) after Tat (0.1, 1 or 5 µg/ml) addition. The immunization experiment was repeated 3 times independently. The results of one experiment are shown. The Anova test and Bonferroni post-test were used for statistical analysis. Statistical analysis was carried out by comparing *tat*/K2 versus *tat* primed mice (not significant, $p > 0.05$).

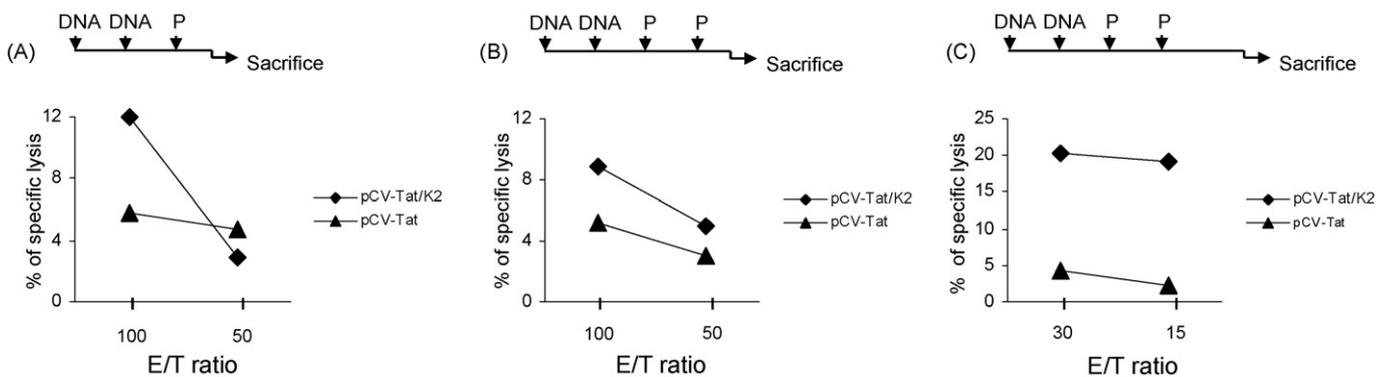


Fig. 3. CTL response to Tat. Mice were immunized as described in Fig. 1. Immune responses were tested at week 10 (after one protein boost) (A) and at weeks 12 (B) and 28 (C) (after two protein boosts). CTL activity was determined (pool of spleens), at various effector/target (E/T) ratios, by standard ^{51}Cr release assays using syngenic P815 target cells pulsed with the CFI Tat 9-mer peptide (CTL epitope). The percentage (%) of specific lysis is reported. The immunization experiment was repeated 3 times independently. The results of one experiment are shown. No cytotoxic responses were observed in cells from control mice (i.e. primed with pCV-0/K2 or pCV-0 and boosted with PBS/Alum) (data not shown).

between ^{51}Cr -release and Elispot assays is likely due to absence of CD4+ T cells in the latter test (performed on purified CD8+ T cells) which are known to be important for CD8+ T cell activation and maintenance of functional memory CTL responses. As a whole these results show that *tat*/K2 primes T helper (both Th1- and Th2-type) and CTL cellular responses, which are long lasting and stronger as compared to priming with *tat* alone.

3.3. Effect of DNA priming

To verify the effect of *tat*/K2 priming on antigen-specific T helper and antibody responses, mice were immunized i.m. with pCV-*tat*/K2 or with pCV-0/K2 at weeks 0 and 4. Both groups were then boosted with Tat/Alum at week 8 and sacrificed at week 10. Additional control groups were mice immunized with pCV-*tat* or

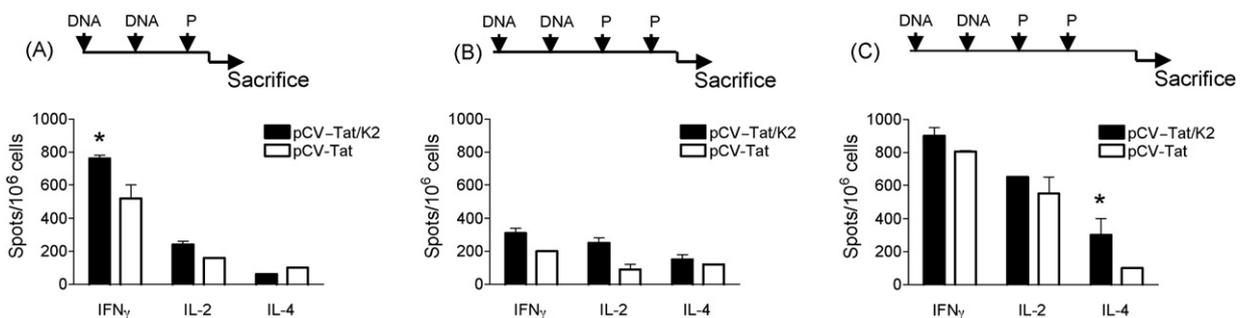


Fig. 4. Analysis of IFN- γ , IL-2 and IL-4 secretion by Elispot. Mice were immunized as described in Fig. 1. Immune responses were tested at week 10 (after one protein boost) (A) and at weeks 12 (B) and 28 (C) (after two protein boosts). Spleens were pooled for each experimental group, stimulated *ex vivo* for 5 days with a Tat 15-mer (VCF) peptide containing a CD4 and CD8 epitope, extensively washed, added to Elispot plates pre-coated with the cytokine-specific capture antibody, and incubated in the absence (untreated) or presence of the VCF peptide (panels A and B). In panel C, *ex vivo* stimulated splenocytes were depleted of B-cells and Elispot assay carried out on CD8+ purified (not shown) and CD4+ enriched T cell subpopulations, and the results correspond to the CD4+ population. Specific responses corresponded to the number of spots counted in the peptide-treated wells minus the number of spots counted in the untreated wells. Responses were considered significant when net spots/million cells were >50 and at least 2 times above the score of the untreated wells. No responses were observed in cells from control mice (i.e. priming with pCV-0/K2 or pCV-0 and boosted with PBS/Alum) (data not shown). The immunization experiment was repeated 3 times and the Anova test and Bonferroni post-test were used for statistical comparison of *tat*/K2 versus *tat* primed mice. * $p < 0.05$.

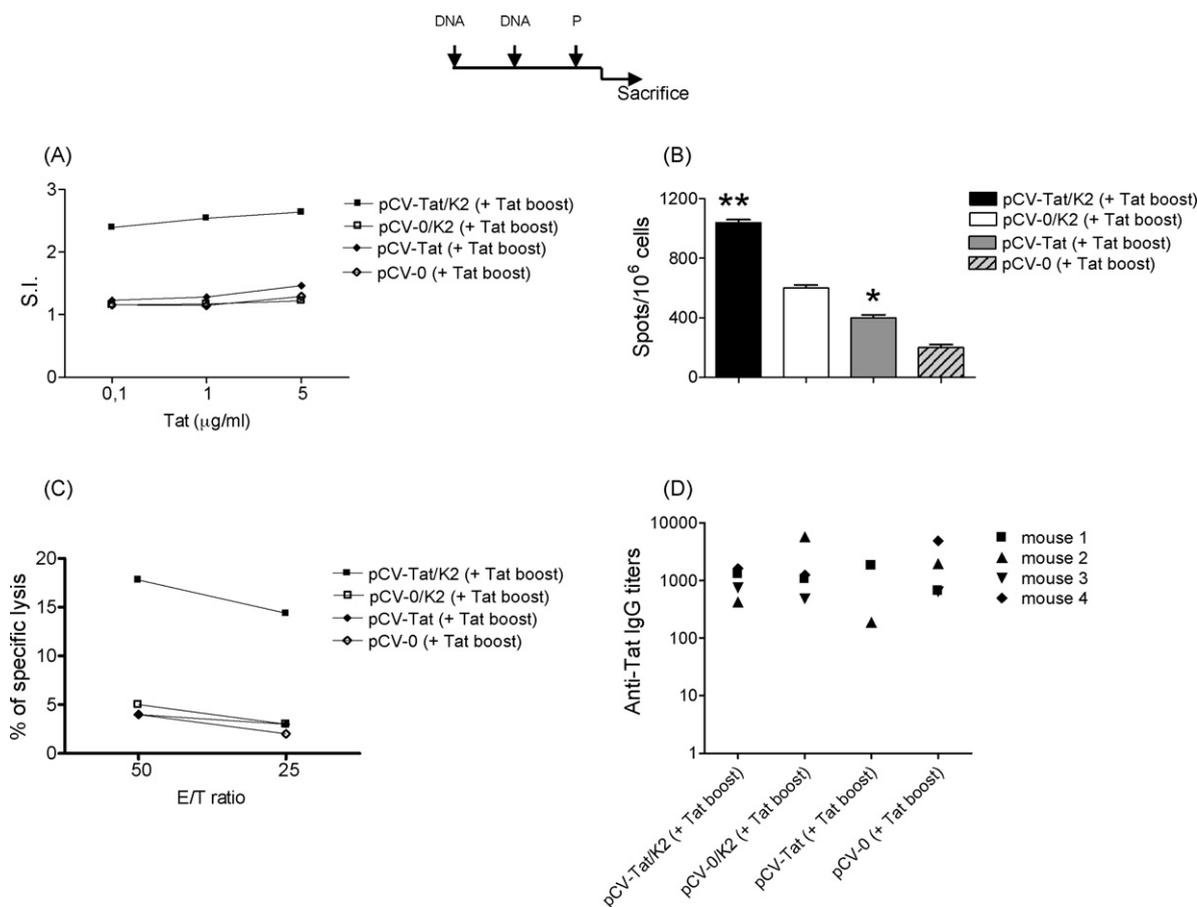


Fig. 5. Effect of *tat*/K2 DNA priming on cellular and antibody responses. At weeks 0 and 4, mice ($n=4$) were immunized i.m. with pCV-*tat*/K2 or pCV-*tat*, and control groups with pCV-0/K2 or pCV-0. All mice were boosted with Tat/Alum (+Tat boost) at week 8 and sacrificed at week 10. The results of (A) lymphoproliferative responses to the Tat protein, (B) IFN- γ and (C) CTL responses to VCF Tat peptide, and (D) Tat-specific IgG titers are shown. The Anova test and Bonferroni post-test were used for statistical comparison of *tat*/K2 (+Tat boost) versus pCV-0/K2 (+Tat boost), and of pCV-*tat* (+Tat boost) versus pCV-0 (+Tat boost). * $p < 0.05$; ** $p < 0.01$.

with pCV-0 alone, both boosted with Tat/Alum. Lymphoproliferative responses to the Tat protein antigen were observed only in *tat*/K2 vaccinees (Fig. 5A), whereas IFN- γ responses were detected in all groups (Fig. 5B). However, in *tat*/K2 primed mice they were significantly higher than those measured in pCV-0/K2 primed animals (receiving only the Tat/Alum boost) ($p < 0.001$), and again stronger than those developed by mice primed with naked *tat* DNA, in agreement with the results shown above. Similarly, IFN- γ responses were higher in *tat* primed mice than those developed by mice primed with pCV-0 (receiving only the Tat/Alum boost) ($p < 0.05$). The results also showed that *tat*/K2 priming elicited high CTL responses, whereas antigen-specific CTLs were undetectable in all other experimental groups (Fig. 5C), in agreement with the previous results. Finally, IgG responses were developed at similar levels in all groups primed with *tat* (\pm K2) or with pCV-0 (\pm K2) (all boosted with Tat/Alum) ($p > 0.05$) (Fig. 5D). The results of these experiments indicate that the presence of K2 in the vaccine formulation plays an important difference in priming cellular responses, independently from the protein boost. Indeed, mice primed with *tat*/K2 and boosted with Tat developed significantly higher CD4 and CD8 T cells responses than mice primed with pCV-0/K2 and boosted with Tat, and as compared to mice primed with naked *tat* or pCV-0 and boosted with Tat. In addition, they confirm the priming effect of *tat*/K2 on humoral responses, which is as efficient as basically one immunization with Tat/Alum (i.e. compared to pCV-0/K2 + Tat/Alum, pCV-0 + Tat/Alum groups), and imply that 2 administrations of DNA/K2 vaccine are sufficient to provide B

cell priming and induction of antigen-specific antibodies after naturally encountering the antigen, in addition to stronger cellular responses.

3.4. Effect of protein boost

To evaluate the effect of the protein boost after *tat*/K2 priming on antigen-specific T helper and antibody responses, immune responses developed by mice primed with *tat*/K2 (weeks 0 and 4) and boosted with Tat/Alum (week 8) (+Tat boost) were compared to those elicited in mice primed with *tat*/K2 but not boosted with Tat/Alum (-Tat boost) (at week 8 they received only PBS/Alum). Other control animals were mice primed with naked *tat* DNA and boosted with Tat/Alum (+Tat boost) or with PBS/Alum (-Tat boost). At week 10, lymphoproliferative responses were detected only in mice primed with *tat*/K2 (+Tat boost) (S.I. > 2) (Fig. 6A). In addition, IFN- γ responses were higher in mice primed with *tat*/K2 (+Tat boost) than those developed in control mice receiving only *tat*/K2 (-Tat boost) ($p < 0.05$) (Fig. 6B). Similarly, *tat* primed mice (+Tat boost) developed higher IFN- γ responses than mice primed with *tat* (-Tat boost) (Fig. 6B). Again the cellular responses (in particular IFN- γ and CTLs) of the *tat*/K2 (\pm boost) groups were stronger than those developed by mice primed with naked *tat* DNA (\pm Tat boost) (Fig. 6). Therefore, the enhancing effect on antigen-specific cellular response of *tat*/K2 versus naked *tat* priming ($p < 0.05$) was not related to the protein boost, indicating that the difference is played by the presence

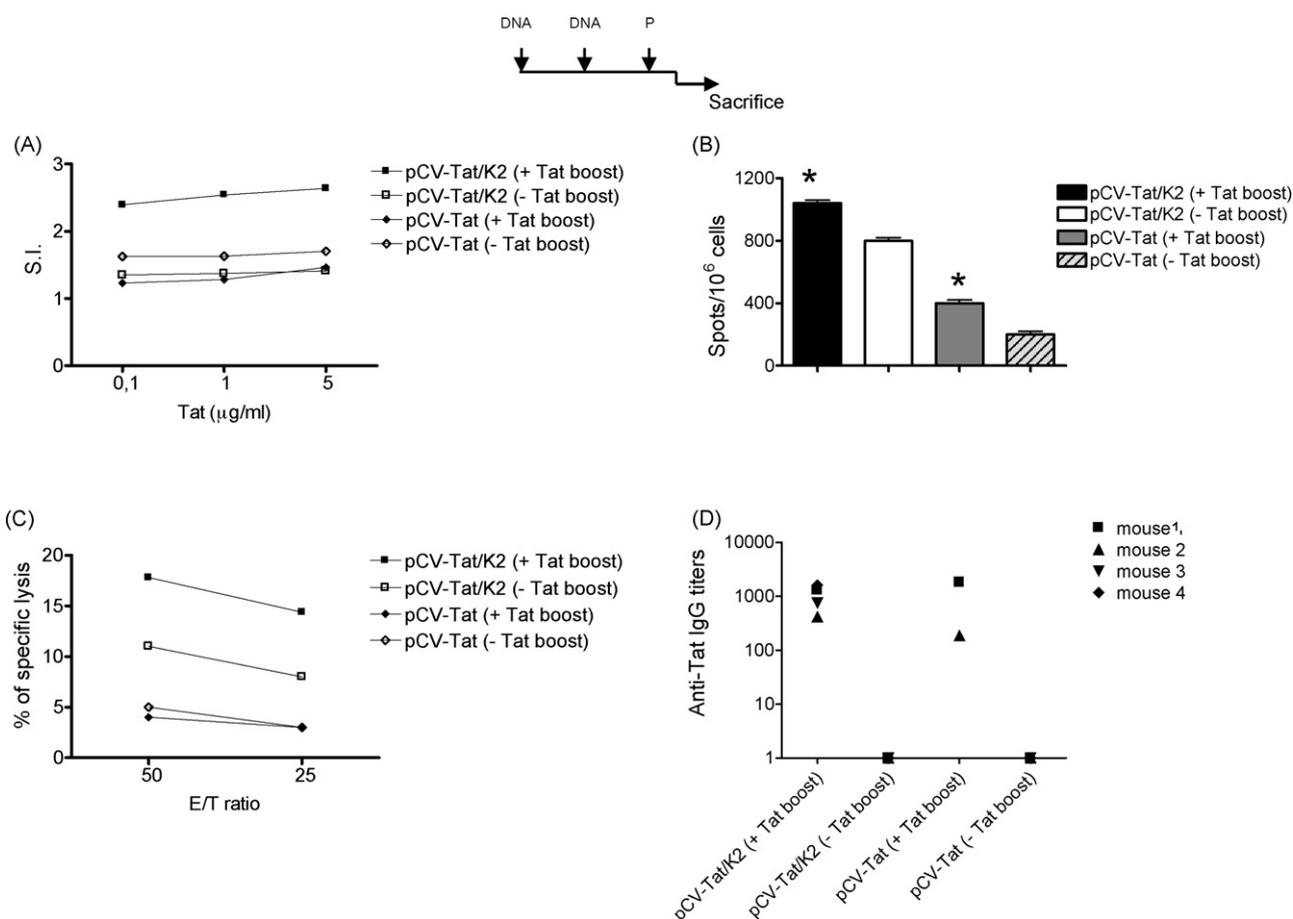


Fig. 6. Effect of Tat protein boost on cellular and antibody responses. Mice ($n=4$) were immunized i.m. with pCV-*tat*/K2 or pCV-*tat* at week 0 and 4 and boosted with Tat/Alum (+Tat boost) at week 8, or not boosted with Tat (-Tat boost), but receiving only PBS/Alum. Immune responses were analyzed at week 10. The results of (A) lymphoproliferative responses to the Tat protein, (B) IFN- γ and (C) CTL responses to VCF Tat peptide, and (D) Tat-specific IgG titers are shown. The Anova test and Bonferroni post-test were used for statistical comparison of *tat*/K2 (+Tat boost) versus *tat*/K2 (-Tat boost) and of *tat* (+Tat boost) versus *tat* (-Tat boost). * $p < 0.05$.

of K2. Nevertheless, the protein boost efficiently increased these responses which were always stronger in *tat*/K2 (+Tat boost) and in *tat* (+Tat boost) mice than in *tat*/K2 (-Tat boost) and *tat* (-Tat boost) mice. Finally, as shown in Fig. 6D, similar levels of Tat-specific antibody responses were detected only in mice receiving the protein boost, independently of whether they were primed with *tat*/K2 or *tat* alone, in agreement with the results shown above and previously [14]. Accordingly, the results show that the protein boost activates memory B cells generated during priming.

3.5. Safety evaluation

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 primed with pCV-*tat*/K2 or pCV-0/K2 complexes (two DNA primes-two protein boosts regimen), as compared to control mice inoculated with naked DNAs. At sacrifice, an inflammatory reaction at the site of injection, mainly characterized by a diffuse macrophage infiltration within and around the muscle fibers (not shown), was observed in 4/12 mice primed with pCV-*tat*/K2 (33%), in 9/12 mice primed with pCV-0/K2 (66%), in 10/12 mice immunized with naked pCV-*tat* (83%) and in 6/12 animals inoculated with pCV-0 DNA (50%), respectively. The central zone of the macrophage infiltrate often showed ischemic necrosis. Importantly, no specific alterations that may be related to injection of DNA/K2 complexes were reported in all other organs

examined. These results indicate that the K2-based vaccine formulations and the immunization protocol are well tolerated *in vivo*.

4. Discussion

This study demonstrates that a vaccine containing a very low amount of HIV-1 *tat* DNA (1 µg) and the cationic block copolymer K2 efficiently primes broad and long-lasting humoral antigen-specific immune responses. In fact, significant IgG titers with a mixed Th1 (IgG2a, IgG3) and Th2 (IgG1) isotype profile were detected after boosting with the Tat protein and Alum, in agreement with previous results showing that *tat* DNA vaccination (higher doses and multiple injections) elicits immune responses with a mixed Th1/Th2 profile [15,17–22,39]. Furthermore, the results indicate that the presence of K2 favors the persistence of high titers antibodies and increased the breadth of the antibody epitope specificity. In fact, a major reactivity directed against the amino-terminal region of Tat (aa 1–20) was detected in all vaccinees, in agreement with previous reports in mice, monkeys and humans [38,40,41]. However, 17 weeks after the second protein boost (week 28), mice sera of the group primed with the *tat*/K2 formulation showed reactivity against other Tat epitopes, including aa 21–40 (cysteine region), aa 65–80 and 57–72 (glutamine-rich region) and aa 73–86 (RGD domain), which were not detected in the group primed with naked *tat* DNA (except for one animal in the latter group which developed an additional epitope specificity against the RGD domain).

tat/K2 priming also induced efficient cellular immune responses with mixed Th1/Th2 profile and, as compared to *tat* priming, significantly stronger Th1-type and CTL responses, which were increased by the protein boost. This is supported by the observation that the number of IFN- γ and IL-2 (Th1-type cytokines) producing cells was generally higher in mice primed with *tat*/K2, and that functional CTL responses were detected only in the group primed with *tat*/K2. Conversely, the number of IL-4 (Th2-type cytokine) producing cells was generally comparable among the two groups of animals. These results and the observation that anti-Tat IgG titers tended to be higher (although without statistical significance) in mice primed with naked *tat* DNA suggest that the presence of copolymer K2 in the vaccine formulation skewed the immune response predominantly toward cellular immunity of Th1-type profile.

This study also demonstrates that *tat*/K2 induced long-lasting antigen-specific immune responses which are mainly sustained by the presence of CD4+ memory T cells producing Th1- and Th2-type cytokines. This is an important feature, since evidence indicates that an inverse correlation between CD4+ T cell responses and viral load exists in HIV chronic infected individuals [42,43], and that strong HIV-specific T helper responses are required for maintaining functional memory CTL responses [44–48]. Finally, the K2 copolymer and immunization protocol were safe, since no toxic effects were ever reported in vaccinees during the experiments, confirming our previous observation [14].

The adjuvant mechanism of copolymer K2 is presently unknown. The enhancement of Th1-type and CTL responses caused by K2 may be a consequence of protection of DNA from enzymatic degradation due to the presence of the PEG block. This, in fact, forms an external shell which reduces the interactions between the DNA and blood or cellular components, as previously shown *in vitro* [13]. It is also possible that, as a consequence of the “proton sponge” effect of the positive charged amine groups, the cationic block copolymer favors lysosome/endosome osmotic swelling and rupture. This could allow release of the polymer/DNA complex in the cytosol and escape from lysosomal degradation, as reported for other systems [7,11], leading to a more efficient and/or prolonged (depot effect) expression/presentation of the DNA. The DNA/K2 complexes and naked DNA may also be internalized by different pathways and/or differently processed by professional antigen-presenting cells (APCs) and presented to different subsets of T cells. This may lead to different polarization of immune response (Th1/Th2 CD4+ helper cell profile). APCs, in particular dendritic cells, can also prime and activate CTLs directly and, additionally, possess an alternative MHC class I pathway that can present peptides derived from extracellular antigens [49,50]. This implies that also extracellular Tat produced and released by transfected muscle cells may be captured and presented with MHC class I molecules by APCs recruited at the site of injection increasing the immune response to the antigen.

Specifically, these results may be relevant for the development of anti-HIV/AIDS DNA vaccine strategies based on Tat. Indeed, Tat possesses very attractive features which make it an interesting antigen for the development of novel anti-HIV/AIDS vaccine strategies [38,40,41,51], including its early expression and key role in the virus life cycle and pathogenesis of AIDS [52–54], conservation among different geographically distinct virus clades [38,55], immunomodulatory properties on heterologous antigens [25,35,56,57], immunogenicity and safety in several animal models and in humans both as protein and DNA [52,58–64] and the evidence that the anti-Tat immune response correlates with non-progression to AIDS [52,58–64]. In addition, although the objective of a preventive immunity still remains a priority, secondary end-points such as block of virus replication and disease onset are being considered at present as more achievable end-points, based on accumulating evidence indicating that a low viral load correlates

with maintenance of immune functions and slow progression to disease, and that cell-mediated immunity plays a major protective role in the absence of sterilizing immunity [41,65–68].

Finally, the adjuvant features of K2, together with its lack of immunogenicity, safety, ease of production on large scale at low-cost and capability to increase the breadth of the immune response, which is considered probably more important than high frequency responses to a limited number of epitopes [51], indicate that this delivery system may represent a new adjuvant and delivery technology for the development of the next generation of DNA vaccines not only against HIV/AIDS but also against other infectious agents or tumors.

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