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Comparative analysis using a mouse model of the immunogenicity of artificial VLP and attenuated *Salmonella* strain carrying a DNA-vaccine encoding HIV-1 polyepitope CTL-immunogen

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Abstract

Two systems have been examined for delivery of DNA-vaccine encoding a HIV-1 polyepitope CTL-immunogen (TCI). One is intended for i.m. injection and is in the form of an artificial virus like particle containing eukaryotic expression plasmid pcDNA-TCI encapsulated within a spermidine–polyglucin conjugate. The other is intended for mucosal immunization and is based on attenuated *Salmonella typhimurium* strain 7207, which can deliver pcDNA-TCI directly into professional antigen-presenting cells (APC). After immunization, the artificial VLP and recombinant *Salmonella* induced an enhanced HIV specific serum antibody, proliferative and CTL responses compared to those induced by naked pcDNA-TCI. The most significant responses were produced when pcDNA-TCI was delivered by *Salmonella*.

Keywords: Human immunodeficiency virus (HIV); Polyepitope CTL-immunogen; DNA vaccine; Artificial virus like particles; Recombinant attenuated *Salmonella* strain

1. Introduction

Among the new trends in the development of vaccines against HIV, the ones aimed at DNA vaccines inducing a virus-specific cytotoxic response are thought to offer considerable promise [1–4]. Cytotoxic T lymphocytes (CTL) associated with HIV infection are important mediators of antiviral immunity. Evidence supporting this statement has been accumulated; for example, a reliable negative correlation has been found between the amount of HIV-specific CTLs and virus RNA in the blood plasma of HIV-infected individuals [5].

We had previously designed a polyepitope CTL-immunogen (TCI, T cell immunogen) 392 amino acids in length and containing a selection of about 80 optimal CTL epitopes. The TCI included the epitopes that induce both $CD8^+$ CTL and $CD4^+$ Th, are highly conserved among the HIV-1 subtypes and include sequences of the major viral proteins: Env, Gag, Pol, and Nef [6].

Delivery of CTL-immunogens is effective when performed using DNA vaccines, because gene vaccination effectively induces the cellular response [7]. However, the earliest DNA vaccination experiments revealed some problems. One of the major problems is one of delivering the plasmid to the host, because unless plasmid DNA has been injected in large amounts, delivery of DNA vaccine through traditional administration (intramuscularly or subcutaneously) would result in a poor immune response. This is because of a low number of APCs present in muscle. For this reason, much attention is being given to the development of systems for targeted delivery of DNA vaccines specific to APCs [8].

We have developed an original system to deliver DNA vaccines using synthetic virus-like particles (VLPs) consisting of the polymer spermidine–polyglucin and plasmid DNA encapsulated within. This morphology allows non-degraded DNA to persist until VLPs are consumed by APC, and thus the effective dose is reduced.

Another promising way is delivery by using attenuated *Salmonella* strains [9–12]. This option has several advantages: first, non-parenteral routes of administration; secondly, in addition to systemic humoral and cellular re-

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sponses, a specific mucosal immunity is exhibited, which prevents virus from entering the organism. The mucosal immunity is especially important in cases of infection transmitted by direct contact, like HIV-1 [13,14].

Here we report a comparison of the immunogenicity of artificial vaccine constructs developed using VLP and an attenuated *Salmonella* strain carrying a DNA vaccine with a synthetic protein, TCI. While plasmid DNA and VLP were administered by intramuscular injection, immunization with *Salmonella* was performed by the mucosal route (per rectum).

2. Materials and methods

2.1. Plasmids, bacterial strains, and media

Eukaryotic expression vectors pcDNA3.1 were from Invitrogen Inc., USA. Plasmid pcDNA-TCI was described previously [6]. The auxotrophic *Salmonella typhimurium aroA* strain SL7207 (*S. typhimurium* 2337-65 derivative *hisG*46, DEL407 [*aroA*::Tn105Tc-s6]) was kindly provided by B.A.D. Stocker of the Stanford University, USA [15]. Preparation of media and bacterial cell transformation were as described [16]. DNA-vaccine vector strain SL7207/pcDNA-TCI was obtained by introducing plasmid pcDNA-TCI into *S. typhimurium* strain SL7207 by electroporation. Control *Salmonella* vector SL7207/pcDNA3.1 was constructed by introducing pcDNA3.1 into *S. typhimurium* strain SL7207. For immunization experiments, overnight bacterial culture was diluted 1/100 in LB broth (Difco) under aeration at 37 °C until they reached mid-log phase ($\sim 10^9$ colony-forming units [CFU]/ml). After 5 min of centrifugation at 3000 × *g*, the bacterial pellet was resuspended in phosphate-buffered saline (PBS).

The recombinant TCI protein was prepared as described previously [6].

2.2. The preparation of an artificial virus-like vaccine construct (VLP)

To obtain a polysaccharide matrix, 50 mg of polyglucin (MW 60 kDa) was incubated with 0.5 M sodium periodate for 20–30 min and desalted by gel filtration on Sephadex G-50 in 20 mM phosphate buffer (pH 7.6). Activated polyglucin was combined with 2.5 mg of spermidine (MW 145 Da). The mixture was incubated overnight, combined with sodium borohydride, and stirred for 2 h. Free components were removed by gel filtration on Sephadex G-50; the column was equilibrated with 50 mM Tris–HCl (pH 8.3). To assembly the construct, 3 mg of pcDNA-TCI in saline was combined with the conjugate and incubated at 4 °C for 2 h. The resulting complex was purified by gel filtration on Sepharose CL-6B. Spermidine (Sigma, USA) and polyglucin (Krasfarma, Russia) were used.

2.3. Atomic force microscopy

For atomic force microscopy [17], VLP preparations were adsorbed on freshly cleaved mica, a buffer salt was removed by secondary dissolution. Preparations were analyzed under a SolverP47BIO atomic force microscope (NT-MDT, Russia) in intermittent-contact mode in air.

2.4. Immunizations

BALB/c (H-2d) mice, 6 weeks of age, were purchased from Vector's animal breeding facilities. The immunization dose for naked plasmid pcDNA-TCI or pcDNA3.1 was 100 μg per animal and for the VLP construct was 20 μg of plasmid DNA per animal. Immunization was carried out once by intramuscularly injection.

Attenuated *Salmonella* strains was introduced by the mucosal route. Mice were immunized per rectum with a single dose of SL7207/pcDNA-TCI or SL7207/pcDNA3.1 (30 μl bacterial suspension, containing 10^7 CFU per mouse).

Blood and splenocyte samples were collected 7, 10, 14, 21, 30, and 48 days after immunization and assayed by ELISA, blast transformation and ELISPOT. All the work with animals met the protocols on animal use approved by the IACUC of the SRC VB Vector (NIH OLAW registration no. 01A5505-01).

2.5. Proliferative response

The cell response was assessed by blast transformation using a routine technique [18]. Spleens were removed from four immunized mice and pooled in a single-cell suspension prepared by mechanical dissociation. The recombinant TCI protein in a concentration of 2 μg/ml and peptides N15—DRVIEVVQGAYRAIR and N16—KQIINMWQEVGKAMYA (the H-2d restricted epitope from gp41 HIV-1 and gp120) were used as the specific antigens. The EHEC antigen (uninfected *E. coli* strain O:H157) in a concentration of 2 μg/ml was used as the non-specific antigen (negative control). Concanavalin A (Sigma) in a concentration of 5 μg/ml was used as the mitogen. The proliferative activity of the splenocytes was estimated using the stimulation index, which was calculated as the ratio of mean absolute Delta OD of stimulated to non-stimulated splenocytes.

2.6. Measuring antibody titers by ELISA

Three types of antigen (a mixture of the recombinant Gag and Env HIV-1 proteins, inactivated HIV-1 virus (Vector collection) and recombinant TCI protein) were used for adsorption on polystyrene plates. Titration was performed at

serum dilutions from 1:10 to 1:10,000. Anti-mouse IgG antibodies conjugated to horseradish peroxidase (Sigma) were used. The OD was measured using Titertek at 450 nm.

2.7. ELISPOT assay

ELISPOT assays were performed as described previously [19]. Ninety-six-well microtiter plates (Millipore, Bedford, MA) coated overnight with 100 μl/well of 5 μg/ml rat anti-mouse IFN-γ (BD PharMingen, San Diego, CA) in PBS were washed with PBS containing 0.25% Tween 20 and blocked with PBS containing 5% FBS for 2 h at 37 °C. The plates were washed three times with PBS containing 0.25% Tween 20, rinsed with RPMI 1640 containing 10% FBS, and incubated in triplicate with 1×10^6 splenocytes/well in a 100-μl reaction volume containing a specific protein and peptide. The recombinant TCI protein in a concentration of 2 μg/ml and peptides N15—DRVIEVVQGAYRAIR and N16—KQIINMWQEVGKAMYA (the H-2d restricted epitopes from HIV-1 gp41 and gp120) in a concentration of 1 μg/ml were used as the specific antigens. Non-specific products were identified using the EHEC antigen. The plates were incubated overnight at 37 °C in 5% CO_2 and then washed with PBS. Biotinylated anti-mouse IFN-γ MAb (BD PharMingen) was diluted to 1:500 and added to each well and left to incubate for 2 h at room temperature. After washing, avidin-horseradish peroxidase (BD PharMingen) was added at room temperature for 1 h. Individual IFN-γ-producing cells were detected as dark spots after a 10-min reaction with AEC (Sigma), using a peroxidase substrate. The reaction was stopped by washing with tap water and air-drying.

3. Results

3.1. Physical and chemical properties of the VLPs

Research suggests that the "nucleotide material—polysaccharide insert" complex contains 67 nM of the polysaccharide insert per 1.4 nM of plasmid DNA and, therefore, the ratio of spermidine–polyglucin conjugate to pcDNA-TCI molecules is 48:1. The assembled construct is eluted in the gel-free volume during gel filtration on Sepharose CL-6B. Based on the molecular weights of the components, the diameters of the particles were expected to be 30 nm on average. Electron microscopy suggests that these are spherical particles 25–40 nm in size (Fig. 1). Thus, the dimensions of the construct are comparable with those of poliomyelitis virus and polyoma virus, and are close to those of HIV virus.

In order to see how efficiently the nucleotide material was packaged in the assembled construct, treatment with 0.05 mg/ml RNase solution was performed. It has been demonstrated that the original plasmid DNA, pcDNA-TCI, is totally degraded by incubation for 30 min, whereas the VLP keeps the DNA intact for 24 h.

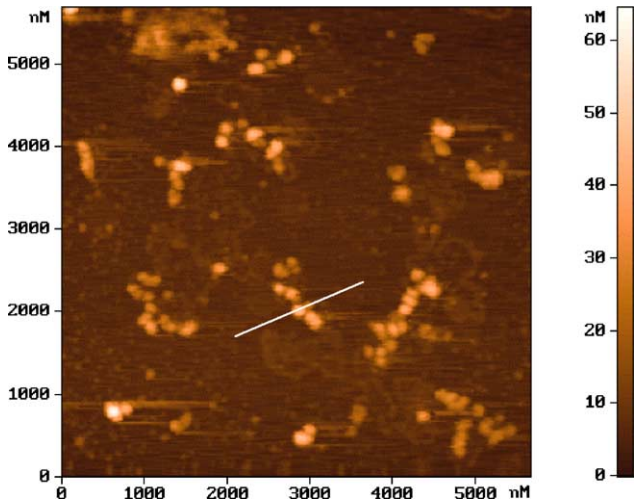

Fig. 1. VLP particles adsorbed on mica and imaged with intermittent contact mode AFM.

3.2. Assessing the stability of plasmid pcDNA-TCI in vitro

Assessment of the stability of plasmid pcDNA-TCI within *S. typhimurium* 7207 cells indicate that the plasmid persists for at least 100 generations following exposure to an agar medium, with and without ampicillin. The authenticity of the plasmid was confirmed by restriction analysis and partial sequencing.

3.3. Comparison of the immunogenicity of vaccine constructs, which contain plasmid pcDNA-TCI encoding the gene of the target immunogen, TCI

In order to compare the immunogenicity of the constructs carrying DNA vaccine, BALB/c mice were immunized using naked plasmid pcDNA-TCI, virus-like particles (VLP) containing plasmid pcDNA-TCI in their nuclei, and an attenuated recombinant strain *S. typhimurium* 7207/pcDNA-TCI. Control animals were immunized with vector plasmid pcDNA3.1 and *S. typhimurium* strain SL7207 transformed by vector plasmid pcDNA3.1.

In order to assess the immunogenicity of the resulting vaccine constructs, the following parameters of the immune response were measured: the CTL response, which was assessed by an ELISPOT assay. Antibody titers were assessed by ELISA using a number of antigens, including the TCI protein, a mixture of recombinant proteins, Gag and Env, and inactivated HIV-1 lysate. The proliferative response of lymphocytes, which was assessed by blast transformation.

3.3.1. Comparison of the CTL responses

Because the TCI protein was designed in the form of a poly-CTL-epitope T cell immunogen, a candidate for use as

DNA-based vaccine against HIV-1 [6], the first-line task was to ensure that the resulting constructs are capable of inducing CTL responses in the immunized animals. These responses were ascertained by the presence of IFN-γ-producing lymphocytes revealed by the ELISPOT assay. The TCI protein and peptides N15 (DRVIEVVQGAYRAIR) and N16 (KQIINMWQEVGKAMYA) were used as the specific antigens. In a murine system, these peptides are multiple class I molecules, which can be presented to CTL. Results are shown in Fig. 2. Based on the comparison of the immunogenicity of the constructs, the following conclusions were made.

First, all the vaccine constructs assayed are capable of inducing a specific CTL response in immunized animals. The use of a full-length TCI protein and separate epitopes (peptides N15 and N16) for re-stimulation of splenocytes in vitro indicate that DNA-immunization incorporates all the stages required for delivery of the target immunogen to the immune system: expression of the TCI gene, processing of the target protein, and presentation of the resulting peptides (determinants) to $CD8^+$ lymphocytes (CTL) together with MHC class I molecules.

Secondly, the highest titer was the same following immunization with the vaccine construct containing 20 μg of plasmid pcDNA-TCI within VLP-(pcDNA-TCI) as with 100 μg of naked pcDNA-TCI. Additionally, a more prolonged response was produced by the plasmid within VLP than naked plasmid DNA. The response reaches its maximum on days 14–21 post-immunization and with comparison with naked pcDNA-TCI immunization is retained until the end of the experiment.

Thirdly, the animals immunized with *S. typhimurium* SL7207/pcDNA-TCI revealed the maximum response and the most delayed CTL responses (48 day), in comparison with groups of animals immunized with naked pcDNA-TCI and VLP. This might be because plasmid pcDNA-TCI effectively replicates in *Salmonella* cells until they are consumed by macrophages.

3.3.2. Antibody titers in the blood sera of immunized animals

The TCI protein was constructed as a poly-CTL-epitope immunogen, which includes several extended fragments of native HIV-1 proteins, Env, Gag, Pol, and Nef. Thus constructed, it consists of overlapping HIV-1 T and B cell epitopes [6] and can induce the synthesis of antibodies, which can bind to both the synthetic TCI protein and the native HIV-1 proteins.

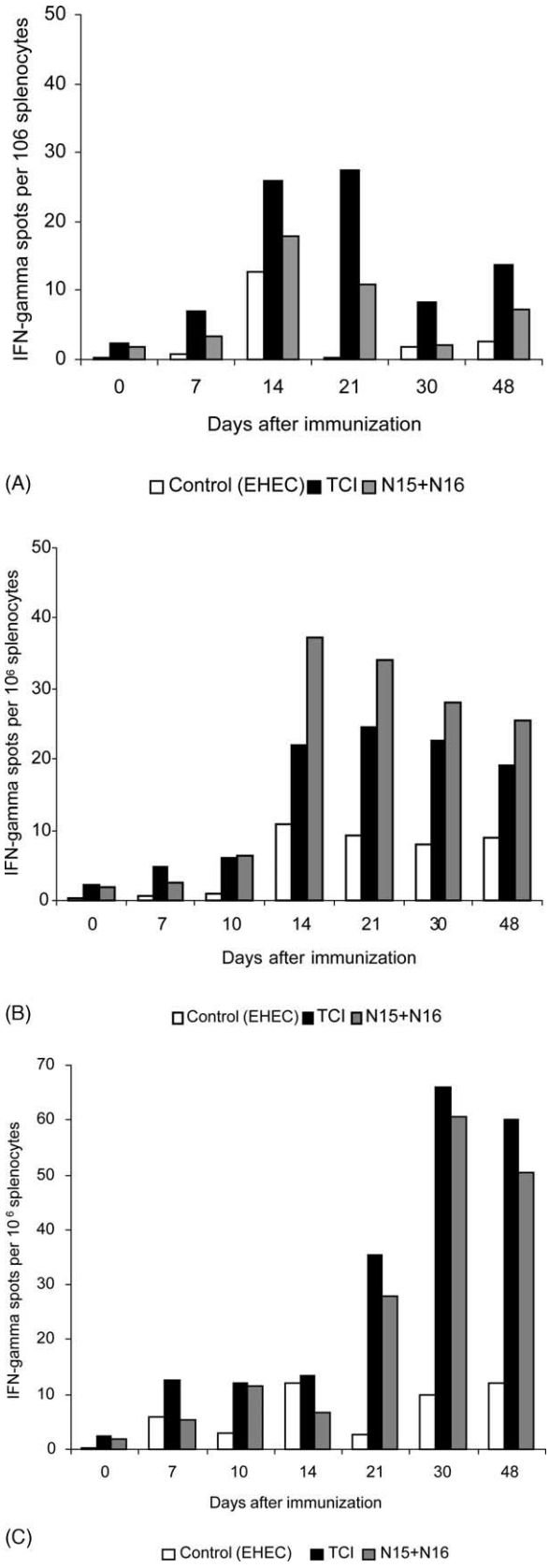

Fig. 2. IFNγ-ELISPOT responses in groups ($n = 4$) of BALB/c mice immunized with naked pcDNA-TCI, dose 100 μg (A); VLP, dose pcDNA-TCI 20 μg (B); and recombinant *S. typhimurium* SL7207/pcDNA-TCI (C). For all animals splenosytes were separately restimulated in vitro with recombinant TCI (black bars), mixture of peptides N15 and N16 (gray bars) and EHEC antigen as a negative control (white bars). The results are expressed as the mean numbers of IFNγ secreting cells (spots) per 10^6 splenocytes.

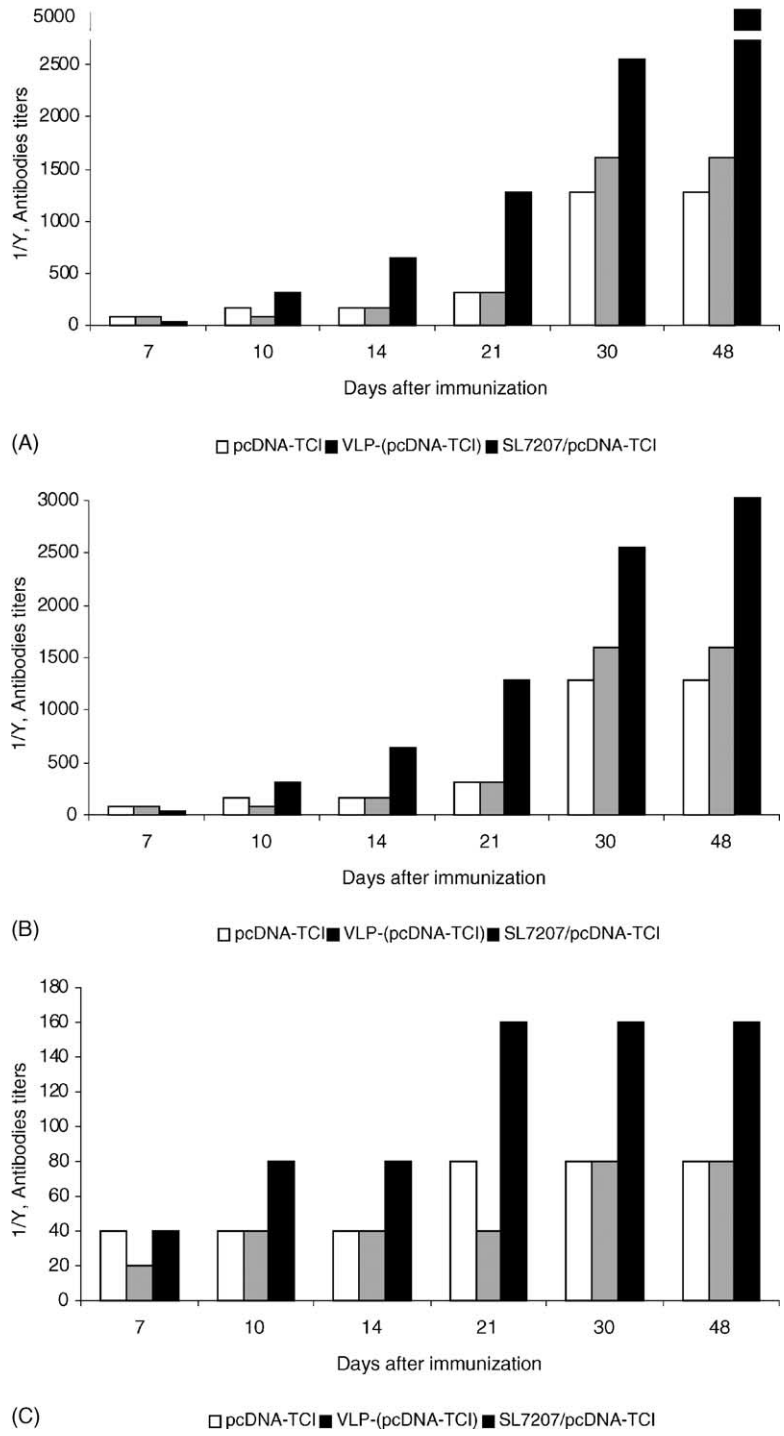

Fig. 3. Serum IgG titer in groups ($n = 4$) of mice immunized with naked pcDNA-TCI, dose 100 μg (white bars); VLP, dose pcDNA-TCI 20 μg (gray bars); and recombinant *S. typhimurium* SL7207/pcDNA-TCI (black bars). Titer of antibodies against recombinant TCI protein (A); mixture of the recombinant Gag and Env proteins (B); and inactivated HIV-1 lysate (C).

Blood samples were collected from four animals for determination of the titers of specific antibodies by ELISA using a number of antigens, including the TCI protein, a mixture of recombinant proteins, Gag and Env, and inactivated HIV-1 lysate. Based on the results obtained (Fig. 3), the following conclusions were made: Immunization with plasmid pcDNA-TCI alone and within VLP and *S. typhimurium* 7207 triggers the production of specific antibodies in mice. These antibodies bind to the TCI protein, recombinant Env and Gag proteins, and HIV-1 lysate. This implies that the artificial TCI protein being synthesized in vivo behaves as a HIV-specific immunogen and

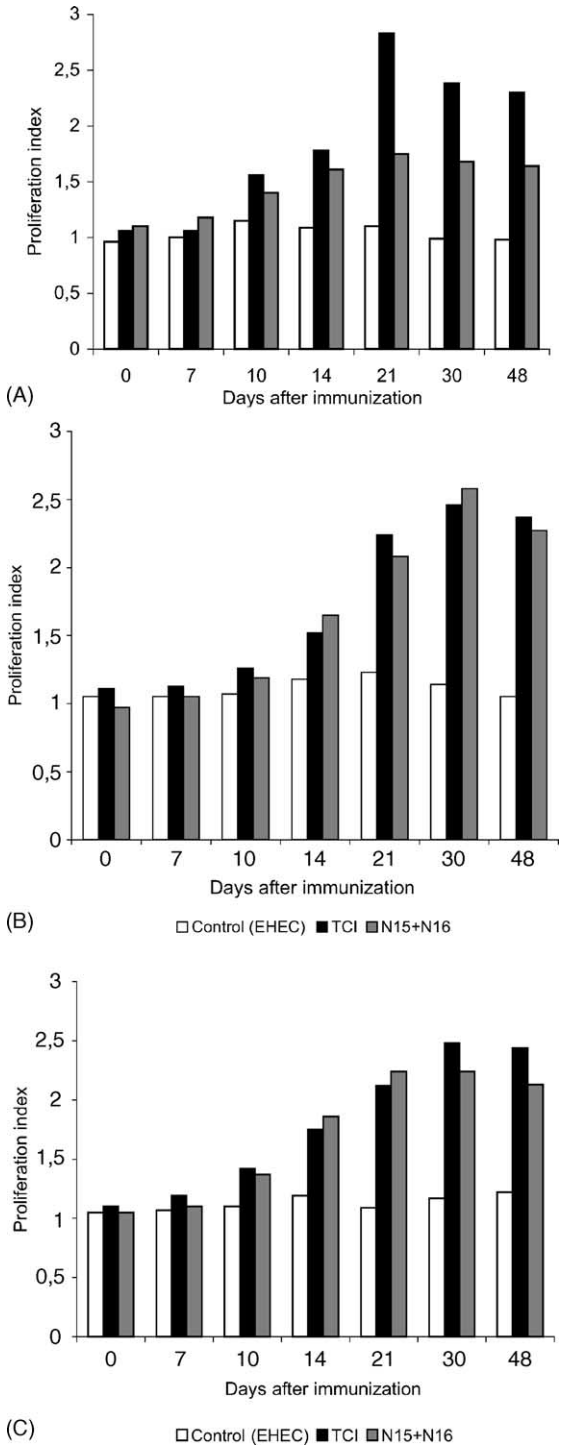

Fig. 4. Proliferative responses in groups ($n = 4$) of BALB/c mice immunized with naked pcDNA-TCI, dose 100 μg (A); VLP, dose pcDNA-TCI 20 μg (B); and recombinant *S. typhimurium* SL7207/pcDNA-TCI (C). Splenosytes from four animals were restimulated in vitro with recombinant TCI (black bars), mixture of peptides N15 and N16 (gray bars) and EHEC antigen as a negative control (white bars). The results are expressed as the index of splenocyte proliferation. All splenosytes demonstrated an identical level of spontaneous proliferation to A-concanavalin mitogen (data not shown).

contains HIV B cell epitopes accessible by the immune system.

It should be noted, however, that the titers of antibodies to the Env and Gag proteins and to the HIV lysate were not too high for a reason—as was mentioned above, the TCI protein was constructed in the form of a poly-CTL-epitope immunogen [6].

Titers of the antibodies to all the antigens began to increase on day 21 post-immunization and reached their maximum by the end of the observation period. The antibody titers in mice immunized with VLP vaccine constructs were comparable with those in mice immunized with naked plasmid pcDNA-TCI. The highest antibody titers were detected in the animals immunized with *S. typhimurium* 7207/pcDNA-TCI (Fig. 3).

3.3.3. Proliferation assay

Analysis of experimental results indicates that there is a proliferative response of lymphocytes in vitro to all variants of the antigen (Fig. 4). Indeed, the splenocytes from all the immunized animals displayed an increased proliferative activity to TCI and peptides N15 and N16 compared to the control animals immunized with vector plasmid pcDNA3.1.

It should be noted that in all groups of immunized animals the proliferative response had stopped increasing by day 48 following stimulation of splenocytes by specific antigens, whereas no significant proliferation whatsoever was observed following in vitro stimulation of lymphocytes by a heterogeneous antigen, EHEC. At the same time, stimulation of lymphocytes from the immunized animals by a mitogen, concanavalin A, caused a strong proliferative response in all the immunized animals at all times of observation (data not shown). This suggests that the constructs did not suppress the immune system.

4. Discussion

We had previously designed and constructed a gene encoding the synthetic TCI immunogen. The TCI protein was designed in the form of a poly-CTL-epitope T cell immunogene (TCI), a candidate for use as a DNA vaccine against HIV-1. The gene encoding TCI was cloned into a series of vector plasmids and expressed in a prokaryotic and a eukaryotic system [6]. It has been demonstrated that pcDNA-TCI can induce both specific T cell responses (CTL and blast transformation) and specific antibodies in immunized animals (Figs. 2–4). This implies that DNA immunization incorporates all the stages required for delivery of the target immunogen to the immune system: expression of the TCI gene, processing of the target protein and presentation of its epitopes (peptides) to T and B lymphocytes.

It is generally recognized that DNA vaccine are often less effective in large animals and nonhuman primates than in mice. For induction antibody and CTL responses in human volunteers high doses of DNA were used [21,22].

Therefore, there is a need to induce effective immunity in humans with lower doses of DNA, as well as to increase the magnitude of the immune responses obtained. There are a number of strategies available that have the potential to improve the potency of DNA vaccines. These strategies include: vector modification to enhance antigen expression; the inclusion of adjuvant, either as a gene or a coadministered agent; or improvements in DNA delivery [1,20,23,24]. Our group has focused predominantly on the use of DNA delivery systems to enhance the response to DNA vaccine.

Here the eukaryotic expression plasmid pcDNA-TCI was used for constructing vaccine constructs based on virus-like particles and an attenuated *Salmonella* strain. A comparative analysis of the immunogenicity of the resulting vaccine constructs has been performed. In particular, the immunogenicity of plasmid pcDNA-TCI was examined using three modes of delivery: immunization with naked plasmid DNA (i.m.), immunization with plasmid DNA within VLP (i.m.), and immunization with attenuated *S. typhimurium* strain 7207 (per rectum).

A VLP is artificial construct in the form of spherical particles with pcDNA-TCI in the center and encapsulated within a spermidine–polyglucin conjugate (Fig. 1).

Positively charged spermidine provides the binding of the conjugate with DNA and tropism to the negatively charged cell membranes. We have demonstrated that immunization with VLP induce the same level of immune response as five such doses of naked DNA vaccine do, and it is more prolonged (Fig. 2). It is possible that the more sizable VLP are more effectively consumed by antigen-presenting cells (APCs). Furthermore, plasmid DNA within VLP is protected from DNases and, therefore, the TCI protein can be expressed, processed, and presented to the immune system for a longer period of time, hence a more prolonged CTL response.

It should be noted that the maximum immune response was detected in the animals immunized with *S. typhimurium* SL7207/pcDNA-TCI (Figs. 2 and 3). This might be because *Salmonella* has the ability to accumulate where the immune response takes action (lymphatic nodes and spleen). Attenuated *Salmonella* cells can be effectively consumed by peripheral phagocyte cells, penetrate through the mucosa M-cells and migrate towards inductive sites of lymphoid organs tuning up the immune response. It has been demonstrated elsewhere that recombinant *Salmonella* strains carrying DNA vaccine can induce both systemic and mucosal immune response [9,13]. This is especially important for development of vaccines against viruses like HIV, which are transmitted parenterally and sexually.

Thus, the weakest immune response was detected in the animals immunized i.m with naked pcDNA-TCI. Thus administered, most of the plasmid DNA end up in the blood or myocytes, which cannot effectively present the antigen. We have shown that delivery using VLP or an attenuated *Salmonella* strain allows the efficiency of presentation of an anti-HIV-1 DNA vaccine to the immune system to be improved.

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