



HIV POLYTOPE CANDIDATE VACCINE FORMULATION WITH N-TRIMETHYL CHITOSAN NANOPARTICLES AS A POTENT DELIVERY SYSTEM

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ABSTRACT

Background: Vaccines have been in use as a prophylactic strategy for more than 200 years and remain the best defense against the infectious diseases. New formulations should be adopted to improve efficacy of HIV polytope vaccine. Lack of efficient delivery system is a major obstacle for up taking and transporting the antigenic molecules to the cytosol of antigen presenting cells present by immune cells. To this end, HIV polytope candidate vaccine was formulated with chitosan nanoparticles as biodegradable delivery system.

Materials and methods: The HIV polytope protein was expressed by prokaryotic expression system. The protein purified by Ni-NTA affinity chromatography and dialyzed against PBS buffer for overnight. Next 6 µg/ml of Trimethyl chitosan and 380 µg/ml of purified protein was mixed and solved by adding tripoly phosphate (TPP) solution, at ambient temperature and pH 7.4 while stirring.

Result: The mean size distribution of nanoparticles were determined by dynamic light scattering using Zetasizer . Formulated nanoparticles showed the potential of 10.3 mV and conductivity of 0.0449 mS/cm. loading efficiency was 70%.

Test Results showed that Immunization with HIV-1 tat/pol/gag/env led to a significant increase in the proliferative responses of lymphocytes, IL-4 and IFN-γ cytokine production and humoral immune response in

Background: Conclusion: These findings demonstrate that N-Trimethyl chitosan nanoparticles may be a potent delivery system for HIV polytope candidate vaccine delivery.

Key Words: AIDS, Vaccine, Chitosan, Formulation, Polytope.

INTRODUCTION

It is more than two centuries that vaccines have been in use as a preventive strategy and remain the best defense against the infectious diseases (1). Most of the principal vaccines that have been developed to until now are based on conventional vaccinology strategies using the Pasteur's principles (2). However they were very prospering in many instances, these approaches were not successful in delivery of vaccines against certain pathogens and on other occasions the vaccines obtained with these classical approaches are no longer adequate due to safety concerns and low efficacy (3, 4). These limitations can be addressed by the development of new technologies to express, deliver and formulate antigens (5, 6). Along this line, the advent of subunit, recombinant and conjugate vaccines has enabled major breakthroughs in expressing antigens (7, 8). In parallel, polysaccharide Nano carriers now offer unique opportunities to exploit non-invasive, mucosal or transcutaneous, routes of administration or to selectively target immuno-competent cells (9-11). We have hypothesized that formulating conjugate vaccines with such Nano carriers will promote their uptakes and potentiate their

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immunogenicity (12, 13). Chitosan which has been shown to have both mucoadhesive and adjuvant properties used as a polymer carrier for our proposed polytope HIV vaccine (14, 18).

Despite extensive efforts of recent researches there is no safe and effective vaccine to prevent HIV-1 infection and the achievement of this goal has been hampered by the fact that the virus is highly variable resulting in its successful evasion of adaptive immune responses (19, 20). To overcome these difficulties various candidate vaccines have been developed including whole inactivated virus, HIV-1 protein subunits and peptides epitopes, DNA vaccines, viral vectors expressing HIV antigens, therapeutic immunization and dendritic-cell-based vaccines (21, 22). While classic vaccines contain whole micro-organisms antigens which induce vast immunologic responses even although only a few make protection, Identification of immunogenic epitopes has led to the production of vaccines incorporating only the critical epitopes in order to elicit the required immunologic response (23, 24). However, due to the mammalian polyclonal immune response system, the rational approach is to include many immunogenic epitopes into a single vaccine (25, 26). These polyepitopic vaccines may induce strong immune responses against immunogenic and protective epitopes and thus may result in reduction of the number of vaccine administrations required (20, 23). Among the HIV-1 antigens *Gag*, *Tat*, *Pol* and *Env* have received considerable attention due to their critical roles in viral life cycle. The *Gag* protein is essential for HIV-1 virus particle assembly (27). Top 4 peptides are the most important antigens for the induction of virus-neutralizing antibodies and subunit vaccines are as potent and efficacious as whole virus and split virus but elicit fewer side effects (27, 28). However most vaccines are not well absorbed when administered as simple solutions. Today carrier systems have been designed to prolong the residence time of the macromolecules in the body (29), to protect entrapped antigens against degradation, to enhance uptake by M-cells, and to target the antigens more specifically to antigen presenting cells (APC) (30, 31). Particulate carriers based on chitosan, a copolymer of glucosamine and N-acetylglucosamine, have received particular interest (32). Besides its favorable biological properties, chitosan is biodegradable and has a very low toxicity (32).

A major drawback of chitosan is, however, its poor solubility at physiological pH, whereas it is

soluble and active as an absorption enhancer only in its protonated form in acidic environments (33). In contrast, N-trimethyl chitosan chloride (TMC), a partially quaternized chitosan derivative, shows good water solubility over a wide pH range. Hence, soluble TMC has mucoadhesive properties and excellent absorption enhancing effects even at neutral pH (34, 35). Because of these properties, TMC is an attractive alternative to chitosan for the design of protein-loaded particles by ionic crosslinking. At present only a few studies have reported the use of TMC solutions and TMC microparticles in vaccine delivery (35). Several studies have shown nanoparticles to offer many advantages over microparticles or other dosage forms (36). TMC nanoparticles have interesting features suitable for antigen delivery, such as high protein loading capacity, low cytotoxicity, and in vivo uptake (35-37).

The most important role of this protein is in the late stages of HIV replication, assembly, maturation and release of a mature viral particle (38, 39). *Tat* in the HIV-1 virus triggers apoptosis, hence, has a significant role in the pathogenesis of this virus and is the first known inhibitor of cytochrome C oxidase (COX) activity (40). The *Pol* gene codes for three enzymes; reverse transcriptase (RT), integrase and protease that are respectively responsible for amplification of viral genome, its integration into the host's genome and hydrolyzing the precursor polypeptides into functional proteins. HIV-1 has been able to escape the effects of traditional vaccine preparations due to its antigenic variations. Thus, new vaccine development strategies must employ multiple epitopes from the HIV conserved immunogens (41).

In this study, we have used *tat*, *env*, *pol* and *gag* sequences based on their importance in the life cycle and pathogenesis of the virus as the selected targets. The recombinant protein corresponding to this polyepitopic sequence was expressed in *E. coli* BL-21 (DE3) to assess its immunogenicity in BALB/c mouse model.

MATERIALS AND METHODS

Ethics Statement:

All the procedures and methods of this study were based on the Institutional Ethical Committee and Research Advisory Committee of Pasteur Institute of Iran According to particular national ethical guidelines for biomedical research by the

Ministry of Health and Medical Education (MOHME) issued in 2005.

min at 9000 RPM. Resin was added to the obtained supernatant and shaken on the rocker in cold room for an hour. The solution was then inserted in the

Table 1. Immunization study design

Groups	Formulation	Ag dose (µg)	Volume (µl)	Immunization schedule (day)	Serum Sampling	Scarifying by cervical dislocation (day)	Route of administration
G 0	control	0	100	1, 14, 28	21, 37	45	subcutaneous
G 1	Polytope loaded TMC	10	100	1, 14, 28	21, 37	45	subcutaneous
G 2	Poly tope loaded TMC + Alum	10	100	1, 14, 28	21, 37	45	subcutaneous
G 3	Poly rope loaded TMC+CPG	10	100	1, 14, 28	21, 37	45	subcutaneous
G 4	TMC + Poly tope	10	100	1, 14, 28	21, 37	45	subcutaneous
G 5	Poly tope + Alum	10	100	1, 14, 28	21, 37	45	subcutaneous
G 6	Poly tope	10	100	1, 14, 28	21, 37	45	subcutaneous
G 7	Poly tope + CPG	10	100	1, 14, 28	21, 37	45	subcutaneous

Groups of 8 mice were immunized with the formulations indicated in the table. All formulations were prepared and/or re-suspended in PBS.

Materials

TMC was a generous gift from Pasteur Institute of Iran. Subunit vaccine against HIV was prepared in Laboratory (Institute Pasteur of Iran, Department of Hepatitis and AIDS). Sodium triphosphate (TPP), Tween 80, and mouse monoclonal anti-HIV envelope IgG were purchased from sigma (sigma, Bornem, Belgium). Ni-NTA was obtained from zist Baran Company (Tehran, Iran). All other material used were of analytical or pharmaceutical grade.

Expression, Purification and Preparation of HIV-1 *tat/pol/gag/env* (Top4) recombinant Antigens

Recombinant expression vector was transformed into *E. coli* BL21 (DE3) cells. A single transformant was cultured and induced for protein expression at mid-log phase (OD₆₀₀=0.6) for 4 hours by addition of 1 mM IPTG at 37 ° C. expressing cells were resuspended in lysis buffer (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH = 8) by gentle vortexing and sonicated for 15 min and then centrifuged for 45

column and was washed with washing buffer at 20, 70 and 90 mM concentrations of imidazole. His-tagged protein was purified in denatured condition using nickel (Ni-NTA) affinity chromatography column (Qiagen, USA), according to the manufacturer's instructions. Protein was collected with 500 mM washing buffer and a linear polytope protein (*tat, env, pol, gag*) of HIV-1 virus was purified. Identity and purity of the eluted protein was evaluated using SDS-PAGE and western blot [11] with anti His-tag monoclonal antibody (Invitrogen, USA). The purified protein was quantified with Nanodrop (Thermo 1000) and stored at -70°C until use.

Preparation and characterization of HIV Antigen-loaded TMC nanoparticles

6 mg of Trimethyl chitosan was dissolved in 3 ml of phosphate buffer (2.5 mM Na₂HPO₄, 4.5 mM KH₂PO₄, 0.8 mM KCl, 34 mM NaCl; pH 7.4.) containing 380 µg Top 4 antigens and 0.5% (w/w) Tween 80. Thereafter, 1.5 ml of an aqueous TPP

Figure1: SDS-PAGE Results of Top4 purification.

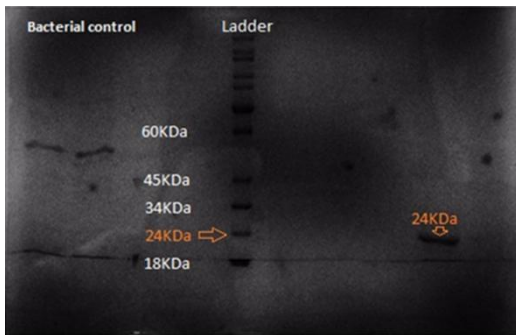


Figure1. Coomassie blue-stained SDS-PAGE electrophoresis (C). First and second lanes from left indicate Top 4 bacterial control; third lane indicates ladder and the last lane shows top 4 protein.

solution (1 mg/ml) was added dropwise to the TMC-antigen solution while stirring. Aliquots of 1 ml of the resulting antigen-loaded TMC nanoparticle suspensions were centrifuged for 15 minutes at 10000 g and 4 °C on a 10-μl glycerol bed. The supernatants were then discarded and the pellets were resuspended in 100 μl of phosphate buffered saline (PBS: 10 mMNa₂HPO₄, 18 mMKH₂PO₄, 3 mMKCl, 138mM NaCl; pH 7.4). For immunizations, 10 μl of suspension containing one dose of HIV-1 antigen-loaded TMC nanoparticles in PBS (pH 7.4) was administered in mice. The size and zeta-potential of the nanoparticles were measured with a Zeta-sizer 3000 (Malvern Instruments Ltd., Malvern, UK) in 5 mM HEPES (pH 7.4). The particle size distribution of the nanoparticles is reported as a polydispersity index, ranging from 0 for an entirely monodisperse suspension to 1 for a completely heterodisperse system. The amount of protein entrapped in the nanoparticles was determined as Loading efficiency (LE) and loading capacity (LC). LE and LC of Top4 were calculated as follows:

$$LE = \frac{\text{Total amount of Top4} - \text{free Top4}}{\text{Total amount of Top4}} \times 100\%$$

$$LC = \frac{\text{Total amount of Top4} - \text{free Top4}}{\text{nanoparticles dry weight}} \times 100\%$$

To eliminate background interference, a blank sample consisting of only nanoparticles resuspended in PBS was used.

Figure 2: IgG1 results of immunized groups.

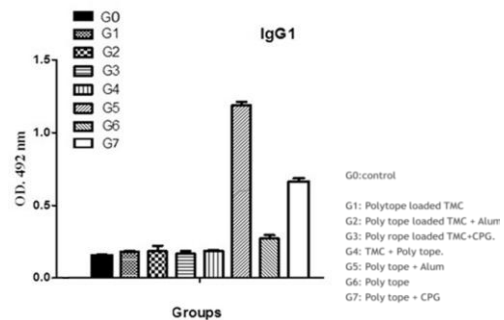


Figure 3: IgG2a results of immunized groups.

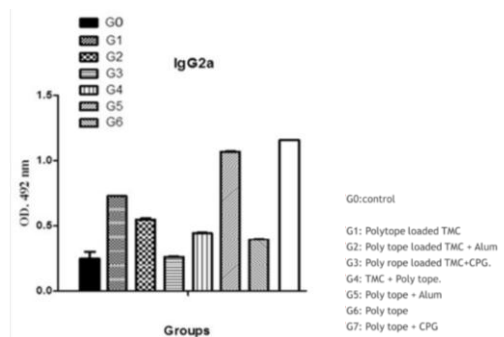


Figure 2 and3. Robust IgG antibody responses are induced against Top4. Blood samples were collected through the retroorbital plexus on day 7 after the last booster injection and total IgG1 and IgG2a subclass antibody titers were determined using an ELISA. The data represent the mean IgG titers in mice. PBS-inoculated animals had a background IgG titers. As shown, most mice vaccinated with various formulations of Top-4 .produced a high titer of IgG subclasses. More importantly, IgG2a, a prototypical Th1-induced isotype, had a higher titer compared to IgG1, a Th2-induced isotype.

Immunization studies

Animals

6-8 weeks old female BALB/c mice (inbred strains) were purchased from the Pasteur Institute of Iran (Karaj,Iran) and were allowed to acclimate to our animal facility for one week before starting the experiments given *ad libitum* access to food and water. The mice were maintained on a 12h light/12h dark cycle. All experiments were according to the Animal Care and Use Protocol of Pasteur Institute of Iran.

Experimental groups and immunization

BALB/c mice were divided into eight groups (n = 8) and were immunized subcutaneously with 100 μ l of each formulation containing 10 μ g of candidate vaccine. Some groups adjuvanted in CPG and Alum adjuvants (Sigma, USA). Experimental groups were immunized three times with 2 weeks intervals. Control group (PBS) were immunized under the same conditions. Two weeks after the last immunization, immunologic responses were assessed.

Figure 5: IL-4 results in immunized mice.

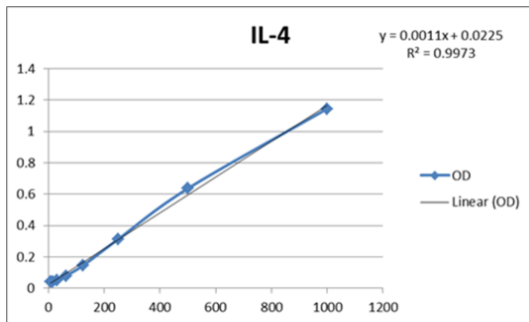


Figure 5: Analysis of Top4 peptide responses. (A) Linear graph of Top4 T cell responses evaluated by IL4- ELISA. Two weeks after the last boost, mice were sacrificed and their spleens harvested. Concanavalin A was used as high control.

ELISA Measurement of IgG Isotypes

One week following the final immunization, mice sera were collected by bleeding through the retro-orbital plexus. The complement was heat-inactivated for 30 min at 56°C and the sera were stored at -20°C until analysis.

Specific antibodies were determined by an optimized indirect ELISA method. 100 μ l of 10 μ g/ml of recombinant HIV-1 *tat/pol/gag/env* protein in 50 mM carbonate-bicarbonate buffer (pH 9.6) was added to 96-well ELISA Maxisorp plates (Nunc, Naperville, IL) and incubated for 24 h at 37°C. Plates were washed by PBS containing 0.05% Tween 20 (TPBS) and blocked at 37°C for 1 h with 5% skimmed milk in PBS. After washing the wells, 100 μ l of diluted sera (1/50 to 1/100) were added to each well and the plate was incubated for 2 h at 37°C. Wells were washed five times with wash buffer and incubated for 2 h with 100 μ l of 1/8000 dilution of goat anti-mouse isotypes of IgG1 and IgG2a (Sigma, USA).

Plates were washed five times and incubated for 30 min with 100 μ l of TMB substrate in the dark and the reaction was stopped with 2N H₂SO₄ and color intensity was measured at A450 nm with an ELISA plate reader.

Splenocyte (Lymphocyte) Culture

Two weeks after the third and last immunization, mice were sacrificed through cervical dislocation and spleens were aseptically dissected and homogenized in complete RPMI-1640 medium (10% FBS, 100U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate and HEPES buffer). Red blood cells were lysed using Tris-ammonium chloride lysis buffer (0.16 M NH₄Cl and 0.17 M Tris-HCl) after removal of debris. Finally splenocytes were resuspended in complete RPMI-1640 medium and cell viability was determined by trypan blue dye (0.4% w/v) exclusion. All cell culture reagents were purchased from SIGMA (ALDRICH, USA) unless otherwise specified. All experiments were done in triplicates.

Figure 4: IFN- γ results in immunized mice.

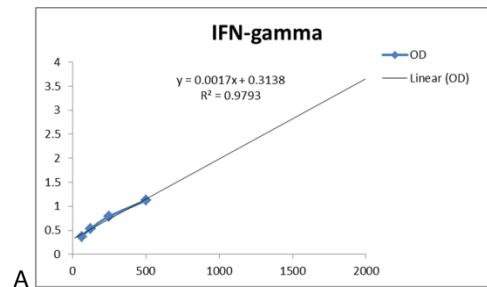


Figure 4: Analysis of Top4 peptide responses. (A) Linear graph of Top4 T cell responses evaluated by IFN- γ ELISA. Two weeks after the last boost, mice were sacrificed and their spleens harvested. Concanavalin A was used as high control.

IL-4 and IFN- γ cytokines ELISA measurement.

Two weeks after the final immunization, a total number of 4×10^6 splenocytes were seeded in a 24-well plate using a complete RPMI-1640 medium, stimulated in vitro with 10 μ g/ml of recombinant protein and incubated at 37°C in 5% CO₂. Three days post antigen recall, supernatants were collected and centrifuged at 300 x g for 10 min and stored at -70°C for cytokine analysis. IFN- γ and IL-4 cytokines were quantified using Quantikine ELISA Kit (R&D Systems, USA) according to the manufacturer's instructions.

Statistical analysis

All experiments were performed in triplicates and the data was expressed as means \pm SD of each experiment. The significance of the differences among various groups was carried out by one-way ANOVA and Tukey HSD test was used to compare the differences between the mean values of experimental groups using SPSS v18 software. P values < 0.05 were considered statistically significant. In some parts statistical analyses were performed using GraphPad Prism 5 Software (San Diego, USA).

RESULTS

Protein expression and purification:

Induction of *E. coli* BL21 (DE3) cells harboring pET-HIVtop4 plasmid resulted in the expression of a protein of approximately 24 kDa which was detected in bacterial lysate (Fig. 1). The fusion protein was purified by Ni-NTA affinity chromatography and confirmed by anti-His monoclonal antibody by Western blotting(42). The mean size distributions of nanoparticles were determined by dynamic light scattering using Zetasizer. Formulated nanoparticles showed the potential of 10.3 mV and conductivity of 0.0449 mS/cm. Loading efficiency was 70%.

SDS-PAGE Results of Top4

The expression and sizes of Top4 protein were further determined by SDS-PAGE and WB as described. A single protein band of, 24 kDa MW was detected (Fig.1).

Specific IgG1 and IgG2a Isotypes determination

Immunization of mice with the candidate vaccine adjuvanted with CPG and Alum increased IgG1 and IgG2a isotypes (Fig.1, Fig.2) compared to the control groups ($P < 0.05$). In the IgG1 investigation of immunized groups, candidate vaccine with Alum adjuvant had the most induction in comparison with all other groups ($P < 0.05$), which is indicator of increase in humoral immunization (Th2). The CPG adjuvanted mice had the second highest increase in comparison to other groups and control group ($P < 0.05$).also polytope vaccine without adjuvant had significant increase in comparison with control group ($P < 0.05$). But polytopes which were loaded or mixed with TMC had not significant increase in comparison with control group ($P > 0.05$).

IgG2a results indicated high increases in cellular responses (Th1), polytope vaccines adjuvanted with

CPG had the highest increase in IgG2a levels which shows high cellular responses (Fig.3). IgG2a levels in Top4 loaded with TMC group, top4 loaded with TMC adjuvanted with Alum, polytope mixed with TMC, and single polytope vaccine had all significant increases ($P < 0.05$).

ELISA results of IL-4 and IFN- γ cytokines production.

Result of IFN- γ cytokine assay showed that immunization with candidate vaccine adjuvanted with CPG and Alum increased IL-4 cytokine compared with the control groups ($P < 0.05$). Immunization of mice with the Polytope loaded with TMC with and without adjuvant increased IL-4 cytokine in comparison to control group (Fig. 4). These results are indicator of high immune responses of cellular immune system of mice.

Interleukin 4 results indicated no significant increase in any of immunized groups in comparison with control group ($p > 0.05$). IL4 responses are indicator of humoral immunity (Th2). Figure 5 demonstrates IL4 responses of immunized groups.

DISCUSSION

The results presented here demonstrate that TMC nanoparticles hold great promise as a delivery system for polytope subunit vaccination. Top4 Antigen was successfully associated with TMC nanoparticles and SDS-PAGE and Western blotting indicated that the structure of the incorporated antigen remained intact. Moreover, the antigens used in our study were shown to remain largely associated with the TMC nanoparticles for at least 3 hours under physiological conditions. Immunizations with antigen-loaded TMC nanoparticles resulted in strong systemic immune responses.

Increased Levels of IgG2a and INF-Y results had significant increases in antigen loaded TMC groups which was indicator of the role of TMC in increasing cellular immune responses. This clearly demonstrates that TMC particles have an intrinsic immunostimulating effect, which is according to previous results (15, 43, and 44). The particulate nature of TMC presumably contributes to improve uptake and processing of the encapsulated antigen by APC as well as a more efficient delivery to peripheral lymph nodes (43, 45-48).The impact of the TMC nanoparticles on the antibody subtype profile was investigated in vaccinated mice.

In this study, a polyepitope candidate vaccine for HIV-1 had designed which was based on proteins

coded by *tat*, *pol*, *gag* and *env* genes that were conserved and could bind to a range of human and mouse MHCs and also to T-cell, B-cell, T-helper and T-cytotoxic receptors. Another consideration in selecting these proteins were their key roles in the viral cells cycle such as construction of the virus core, attachment to CD4+ receptors on T-lymphocytes, replication of the virus and increasing viral transcription (28,49, 50).

Therefore, immunological responses against these combined epitopes in a vaccine candidate which could attack the virus from several points might result in a more effective vaccine. After immunization of the mice with the fusion protein, cellular and humoral immune responses were evaluated. Results of the lymphocyte proliferation assays showed that the vaccine candidate with TMC nanoparticles could induce proliferative responses by both two adjuvants, namely Alum, and CPG; however, the strongest proliferative responses were observed when CPG adjuvant was used.

Responses of proliferative tests are indicator of the cellular immunity (51) and considering the importance of the cellular immune responses in controlling viral infections, this could be considered as one of the advantages of this candidate vaccine.

In the Reed et al. studies it was indicated that administration of a subunit polytope vaccine consisted of genes coding for Gag, Tat, Rev and Nef proteins led to a significant increase in the cellular immune responses (52) which demonstrates effect of the subunit vaccine in controlling the viral replication.

Also previous studies had indicated that a polytope recombinant protein based on Nef and P24 of HIV-1 was able to inoculate lymphocyte proliferation (53, 54).

The outcomes of IFN- γ cytokine demonstrated that CPG adjuvant induced IFN- γ . The IFN- γ cytokine is a symbol of Th1 cellular immune responses and a stimulating factor of TCD8 + cells that have an important role in control and elimination of virus caused infections (55). Various studies have illustrated the role of IFN- γ in the control of virus basis infections (56).

In another study Ullum et al. have tested the IFN- γ levels in healthy, HIV-infected and AIDS patients and indicated a significant correlation in IFN- γ reduction with progression to AIDS (57).

Emu et al. have also demonstrated that IFN- γ levels were highly correlated with HIV control in patients with HIV-1 infection (58). Our data indicated that our candidate vaccine has been able to increase IFN- γ levels and has been able to induce a branch of the immune responses which has a key role in controlling viral infections.

The results of IL-4 assessment as a Th2 cytokine did not show stimulation of IL-4 production with or without TMC Nano particles and adjuvants. It has been shown that the production of this cytokine has a significant effect on the function of B cells and induction of the humoral immunity (59). It is concluded that our candidate carrier nanoparticle (TMC) had only increased the induction of cellular immunity and it could not increase humoral immunity. This may be because of the conformational properties of top 4 antigens when are loaded on TMC nanoparticles. It is hypothesized that neutralizing antibodies cannot distinguish the polytope in the loaded position and maybe some parts of the antigen becomes entrapped in the Nano particles.

The antibody levels obtained indicated that our multiepitope fusion protein has been able to induce antibody responses with both adjuvants used. Humoral responses are highly important in neutralizing viruses and for prevention of viral infections (60).

Given that an increase in IgG1 indicates Th2 induction and IgG2a is an indicative of Th1 (61), it can be concluded that our candidate vaccine could stimulate both Th1 responses.

These results are in agreement with other studies indicating that poly epitope vaccines can induce vaccine specific IgG levels which are essential for controlling viral infections. Moreover, both adjuvants could induce different levels of humoral and cellular immune responses antigen loaded nanoparticles that indicate the effect of adjuvants on the vaccine immunogenicity.

CONCLUSION

These findings demonstrate that N-Trimethyl chitosan nanoparticles may be a potent delivery system for HIV polytope candidate vaccine delivery.

The overall results of our study indicated that the candidate vaccine which we had prepared as a recombinant protein could significantly stimulate the cellular and humoral immune responses showing

Th1 shift and an increase in IFN- γ . However, further studies are required in order to define the exact effects of our candidate vaccine on the viral infection, its biological properties and its suitability for use in pre- and clinical trials.

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CONFLICT OF INTEREST

The authors do not have any conflict of interests.

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