

Clustered epitopes within a new poly-epitopic HIV-1 DNA vaccine shows immunogenicity in BALB/c mice

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Received: 12 August 2013 / Accepted: 29 April 2014 / Published online: 20 May 2014
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Abstract Despite a huge number of studies towards vaccine development against human immunodeficiency virus-1, no effective vaccine has been approved yet. Thus, new vaccines should be provided with new formulations. Herein, a new DNA vaccine candidate encoding conserved and immunogenic epitopes from HIV-1 antigens of *tat*, *pol*, *gag* and *env* is designed and constructed. After bioinformatics analyses to find the best epitopes and their tandem, nucleotide sequence corresponding to the designed multi-epitope was synthesized and cloned into pcDNA3.1+ vector. Expression of pcDNA3.1-*tat/pol/gag/env* plasmid was evaluated in HEK293T cells by RT-PCR and western-blotting. Seven groups of BALB/c mice were intramuscularly immunized three times either with 50, 100, 200 µg of plasmid in 2-week intervals or with similar doses of insert-free plasmid. Two weeks after the last injection,

proliferation of T cells and secretion of IL4 and IFN-γ cytokines were evaluated using Brdu and ELISA methods, respectively. Results showed the proper expression of the plasmid in protein and mRNA levels. Moreover, the designed multi-epitope plasmid was capable of induction of both proliferation responses as well as IFN-γ and IL-4 cytokine production in a considerable level compared to the control groups. Overall, our primary data warranted further detailed studies on the potency of this vaccine.

Keywords HIV-1 · Epitope · DNA vaccine · Proliferation assay · Cytokine assay

Introduction

Although by using vaccine technology many diseases have been controlled or eradicated, but after three decades of efforts, an effective vaccine against HIV-1 has not yet been made [1]. HIV-1 is capable of causing long-term latent diseases. Viruses show many point mutations per day and these mutations lead to the development of various types of genetically different viruses during the course of infection. This sequence variability is mostly seen in the coding regions of envelope proteins such as gp120 and gp41 [2]. The frequent mutations observed in HIV's genome are due to the high error rate of its reverse transcriptase (RT) enzyme and cause the emergence of new virus types resistant to therapeutic drugs or with higher virulence [3]. The new generation DNA vaccines are still in trial stage, but their many benefits including stimulating both arms of the immune system and production of vaccine antigen with the native structure have made them a good candidate for future prospects [4–6]. In the recent years, the multi-epitope (polytope) DNA vaccine strategy has received much attention. In this strategy,

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protective and immunogenic epitopes are arranged together in a small gene structure and are used as a vaccine [7, 8]. One of the greatest advantages of this strategy is focusing the immune response on critical/conserved epitopes and hence, providing the possibility of making a simple and universal vaccine [9, 10]. Selection of target epitopes for a candidate polytope vaccine must be based on some criteria such as epitope immunogenicity, epitope conservancy in different variants and similar binding-affinity of incorporated epitopes for special MHC molecules. It is presumed that co-utilizing of structural protein genes, which are common to all retroviruses, including *gag*, *pol*, *env* in addition to regulatory protein genes including *tat*, *rev*, *nef*, *vpr*, *vif* and *vpu* within the structure of DNA vaccines increase the vaccine efficacy [11]. HIV-1 *gag* gene is responsible for expressing cellular and nuclear proteins in precursor (pre-protein) form. For instance, p55 protein is a pre-protein that breaks down to p15, p18 and p24. Among them, p24 is an important nuclear antigen that is detected in HIV positive person's sera during the early stages of infection, prior to the antibody rise [12]. *env* gene codes for gp160 protein, which breaks into gp120 and gp41. gp120 is a large mace-like antigen that attaches to co-receptor CD4 in the host cell, and gp41, which is a stem protein, is the connection between the virus and the host cell. Antibodies against gp120 appear at the beginning of infection with HIV-1 and are present until the end of the disease [12]. *pol* gene codes for the vital enzymes of RT, integrase (INT) and protease (PR) that are responsible for turning the single stranded RNA into a double stranded DNA, connecting the viral double stranded DNA to the host genome, and cutting the precursor polypeptides into pieces of functional proteins, respectively. These proteins are important in the pathogenesis of virus [12]. *tat* gene synthesizes the TAT protein, which activates all virus genes, or in other words, increases the virus's gene transcription [12]. Altogether, targeting of these proteins in the format of a multi-epitopic vaccine modality may have the capacity to inhibit virus replication in several stages of viral cell cycle. To address that, herein, we sought to design and construct a multi-epitope DNA vaccine carrying a selection of conserved and immunogenic domains from TAT, Pol, Gag and Env of HIV-1. Furthermore, we primarily evaluated the immunopotency of this construct in immunized BALB/c mice.

Materials and methods

Designing and construction of pcDNA3.1-*tat/pol/gag/env* plasmid

In order to select the immunogenic and conserved epitopes, by searching through the *LosAlamos HIV immunology database* (<http://www.hiv.lanl.gov/content/immunology/>

[index.html](#)) six amino acid fragments identified as the regions enriched of HIV-1 CTL epitopes were selected from HIV-1 Tat, Pol, Gag and Env antigens (Table 1). The six separate fragments were conjoined as a full length polytopic tandem of *tat/pol/gag/env* while three spacer sequences of AAY and AAA were incorporated at their junctions to first reduce the creation of junctional epitopes at the juxtaposition of two neighboring epitopes and second, optimize the cleavage of proteasome complex (Fig. 1). The mentioned criteria were respectively predicted by means of Rankpep (<http://bio.dfci.harvard.edu/RANKPEP>) and PaProc (<http://paproc.de>) software. Another criterion for assembling the fragments was to reduce the accumulation of hydrophobic residues in one region in order to optimize the expression rate of the protein. This factor was assessed by protein analysis program in CLC Main Workbench 5.5 software (CLCbio, USA). The nucleotide sequence corresponding to HIV-1 *tat/pol/gag/env* fragment was obtained by amino acid reverse translation based on mammalian cells codon table (www.kazusa.org.jp/codon/), synthesized and cloned into *EcoRI/XhoI* restriction sites of pBMH cloning vector by Biomatik company (Canada). The recombinant synthetic gene containing the kozak/ATG sequence at 5' and six histidine amino acid codons (CAT) in frame of ATG at 3' was pulled out of pBMH via *EcoRI/XhoI* double-digestion and sub-cloned into the same enzymatic sites of pcDNA3.1 + vector (Invitrogen, USA) that directed the expression of C-terminally 6× His-tagged *tat/pol/gag/env* polytope under the control of hCMV promoter. pcDNA3.1-*tat/pol/gag/env* plasmid confirmed by restriction enzyme analysis and sequencing reactions, was finally extracted in a large-scale using endotoxin-free anion exchange chromatography giga columns (Qiagen, Germany).

In vitro transfection and plasmid expression analysis

HEK293T cell line, maintained in RPMI 1640 medium supplemented with 10 % FBS, 2 mM L-glutamine, 100 µg/ml streptomycin and 100 IU/ml penicillin, was transfected with pcDNA3.1-*tat/pol/gag/env* using Lipofectamin 2000 (Invitrogen, USA), according to the manufacturer's protocol. Briefly 2 µg of Lipofectamin reagent-mixed plasmid was added to 4×10^5 cells cultured in antibiotic-free medium when they reached to 40–60 % confluency and 18 h later the medium was refreshed. Seventy-two hours post-transfection the expression was checked at both mRNA transcription and protein translation levels using RT-PCT and Western-blotting assays, respectively. Total RNA was extracted from 2×10^6 transfected cells using TRIzol reagent (Invitrogen, USA). Reverse transcription (RT) and PCR steps were respectively carried out using avian myeloblastosis virus RT and pfu DNA polymerase

Table 1 Complete definition of selected epitopes (retrieved from LosAlamos HIV-1 Molecular Immunology Database)

Epitope name	Derived from	Residue numbers ^a	Amino acid sequence ^b
T44	TAT	44–61	GISYGRKKRRQRRRAHQN
T1	TAT	1–20	MEPVDPRLEPWKHPGSQPKT
P150	POL (RT)	150–190	PQGWKGSPIAFQSSMTKILEPFRKQNPDIIVYQYMDLDLYVG
G158	GAG (p24)	158–186	KEPFRDYVDRFYKTLRAEQASQEVKNWMT
E577	ENV (gp41)	577–610	QARILAVERYLKDQQLGIWGCSEGLICTTAVPW
E296	ENV (gp120)	296–323	CTRPNNNTRKRIRIQRGPGRAFVTIGKI

^a Based on individual protein numbering

^b Based on HXB2 reference sequence (GenBank accession no. K03455)

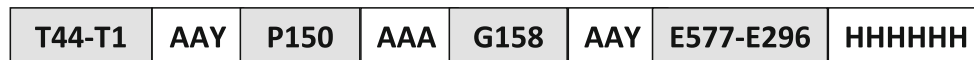


Fig. 1 Order of epitopes in *tat/pol/gag/env* DNA vaccine. The multi epitopic sequence comprising T44, T1, P150, G158, E577 and E296 HIV-1 epitopes was designed based on computer predictions. To avoid creation of junctional neo-epitopes and optimize the cleavage of

proteasome AAY and AAA spacer sequences were considered between some epitopes. Six histidine (H) residues were also added to the C terminal side to provide purification/in vitro detection of the final product using anti-His antibody

enzymes (both from Fermentas, Lithuania) and primer pairs (forward: 5'-CACCAGAATGCCGCCTACATG-3', Reverse: 5'-CGAATACGTTTGC GCGTGTG-3') specifically designed for *tat/pol/gag/env* gene. For expression assay at protein level, lysate of transfected cells (2×10^6) prepared with sonication (five 30 s pulses with 10 s intervals on ice) was initially used for 12 % SDS-PAGE and subsequently electro-transferred onto PVDF paper, based on standard protocols [13]. After blocking with 3 % bovine-serum albumin (BSA), protein bands were detected using treatment of membrane with mouse anti-His monoclonal antibody (1/3,000 dilution, Qiagen, Germany) for 2 h at room temperature, three times washing steps, incubation with HRP-conjugated goat anti-mouse antibody (1/10,000 dilution, Sigma, USA) for 2 h, washing steps and final incubation with DAB substrate (Sigma, USA).

Mice and immunization protocol

Female BALB/c mice (6–8 weeks old) were obtained from Lab. Animal Center (Pasteur Institute, Karaj, Iran) and handled in accordance with the National animal care and use protocol. Mice were divided into three test (T) and three control (C) groups (6 mice/group) and respectively received pcDNA3.1-*tat/pol/gag/env* and pcDNA3.1+ plasmids in various doses of 200 µg (T200 and C200), 100 µg (T100 and C100) and 50 µg (T50 and C50). Another control group was injected with PBS. All injections were done intramuscularly (i.m.) at tibia muscle and the injection sites were immediately pulsed using tweezer-type electrodes (CUY650, Sonidel Limited®, Ireland) to administer eight 60 V pulses each of 20 ms duration with a

200 ms interval using a BTX ECM830 generator (Harvard Apparatus, USA). All animals were boosted on days 14 and 28 with the same immunogen/conditions. Two weeks after the last immunization, immunologic parameters were evaluated.

Lymphocyte proliferation assay

Spleen was taken from each individual mouse, dissected and resuspended in sterile cold PBS containing 2 % FBS. RBCs were lysed with lysis buffer, and single-cell suspension was adjusted to 3×10^6 cells/ml in RPMI 1640 (Gibco, Germany) supplemented with 10 % FBS, 4 mM L-glutamine, 1 mM sodium pyrovalate, 50 µM 2ME, 100 µg/ml streptomycin and 100 IU/ml penicillin. One hundred micro liter of the cell suspension was dispensed into 96-well flat-bottom culture plates and stimulated with 10 µg/ml of recombinant *tat/pol/gag/env* protein (expressed in E. coli cells) as antigen recall. Phytohemagglutinin-A (5 µg/ml, Gibco) was used as a positive control. Unstimulated wells were used as the negative controls and complete culture medium was used as blank. All experiments were done in triplicate. After 72 h of culture, 100 µl of 5-bromo-2-deoxy-uridine (BrdU) labeling solution was added into each well and incubation continued for 18 h. The plates were then centrifuged and after removing culture medium wells were dried and fixed with 100 µl of fixation/permeabilization buffer. Subsequently, 100 µl of anti-BrdU antibody was added to each well and the plates were washed four times and tetramethylbenzidine (TMB) substrate was added. The reaction was stopped by adding 100 µl of 2 N H₂SO₄. Optical density (OD) for each well

was measured at 450 nm. Stimulation Index (SI) was calculated according to the formula: OD of stimulated wells/OD of un-stimulated wells.

Cytokine assay

A total number of 3×10^6 cells from single-cell suspension of each mouse spleen were placed onto the wells of a 24-well plate, stimulated in vitro with 10 µg/ml of recombinant *tat/pol/gag/env* protein and incubated at 37 °C in 5 % CO₂. Seventy two hours post antigen recall, supernatants were collected and centrifuged at 300 g for 10 min and stored at -70 °C for cytokine analysis. IFN-γ and IL-4 cytokines were quantified using commercial ELISA Kits (Quantikine, R&D Systems, USA) according to the manufacturer's instructions. The quantity of each cytokine was reported as pg/ml according to the plotted standard curve.

Statistical analysis

All experiments were performed in triplicate, and the data was expressed as mean ± S.D of each experiment. Statistical analyses were carried out by Mann–Whitney U test. In all of the cases, *P* values <0.05 were considered to be statistically significant.

Results

pcDNA3.1-*tat/pol/gag/env* vector characterization

Mammalian cell codon-optimized sequence of *tat/pol/gag/env* was designed, synthesized and cloned into a classical DNA vaccine vector in order to express the optimized assembly of six HIV-1 CTL epitopes that were retrieved from LosAlamos HIV-1 CTL epitope map based on their immune domination, originating from important HIV-1 antigens and partial conservancy (Table 1, Fig. 1). Enzymatic digestion of recombinant vector with *EcoRI/XhoI* revealed a 600 bp DNA band in electrophoresis, which was confirmed by sequencing reactions (data not shown). To evaluate the expression of recombinant vector we used RT-PCR for detection of *tat/pol/gag/env* specific mRNA in transfected HEK293T cells. Here again, a 600 bp fragment corresponding to the RT-PCR product of *tat/pol/gag/env* gene was detected (Fig. 2a, lane 1). Of note, no similar band was observed in control wells labeled as untransfected (sole HEK293T cell, lane 2) and negative RT (lacking RT process, lane 3), which verified the method specificity. Western blot analysis using anti-His monoclonal antibody further confirmed the expression of *tat/pol/gag/env* gene in protein level (Fig. 2b). Appearance of a single protein band

with approximately 20 kDa in transfected cells (lane 2) and its absence in untransfected ones (lane 1) showed the specificity again.

Immunogenicity of pcDNA3.1-*tat/pol/gag/env* DNA vaccine

To assess the immune-potency of designed multi-epitope DNA vaccine, BALB/c mice intramuscularly received three injections of the vaccine with simultaneous electroporation pulses. Moreover, as the immunogenicity of DNA vaccines is generally thought to be dose-dependent [14], we sought to evaluate the effect of 50, 100 and 200 µg DNA injections, as well. In this protocol each group of animals was compared with a control counterpart (mock) that only received the empty plasmid (pcDNA3.1+) in a similar dosage. Analysis of lymphocyte proliferation using Brdu method indicated that in contrast to the mock and PBS controls that did not promote the lymphocyte proliferation (SI = 1) other animals vaccinated with pcDNA3.1-*tat/pol/gag/env* showed strong proliferation with a SI between 1.8 and 2.7 (Fig. 3a). Of note, the differences of proliferative activity between test (T) and control (C) groups were statistically significant (*P* ≤ 0.05). The highest proliferation response was observed in (T100) animals, which were vaccinated with 100 µg of DNA immunogen. This increase was statistically significant compared to the T50 (*P* = 0.02) though not significant in comparison with T200 group.

In another attempt to evaluate the pattern of cytokine secretion due to the multi-epitope DNA vaccination, culture of individual mouse splenocytes was in vitro re-stimulated with recombinant *tat/pol/gag/env* protein, expressed and purified in *E. coli* cells (data not shown). Collected supernatants were monitored for the amount of IFN-γ and IL-4 to determine the type (T helper 1 vs. T helper 2) of induced immune responses. The results showed that immunization of mice with HIV-1 *tat/pol/gag/env* DNA vaccine (200, 100 and 50 µg immunization groups) induced high level of IFN-γ (Fig. 3b) in comparison to the control groups (*P* ≤ 0.0001). While no significant differences were observed between 200 and 100 µg of immunization (T200 and T100 groups) in the induction of IFN-γ cytokine (*P* = 0.0951), however, using 200 and 100 µg of DNA immunogen significantly increased the IFN-γ secretion level compared to the dosage of 50 µg (*P* = 0.0001). Results of IL-4 quantification also showed that immunization of experimental groups with DNA vaccine (200, 100 and 50 µg immunization groups) induced high level of IL-4 (Fig. 3c), as compared to the control groups (*P* ≤ 0.0001). However, no statistically significant differences were observed between all three formulations (*P* > 0.826). Overall, these data evidenced for the

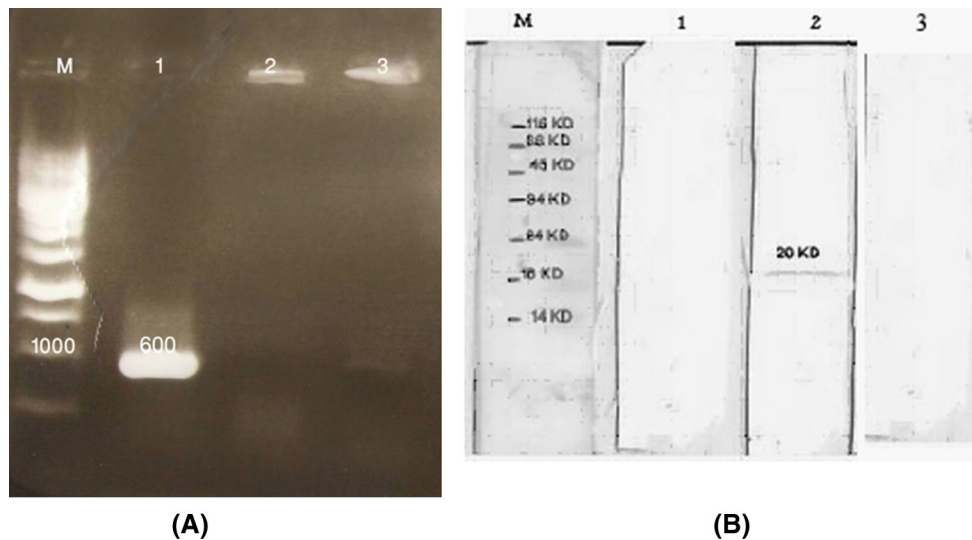


Fig. 2 Expression analysis of pcDNA3.1-*tat/pol/gag/env* plasmid. **a** RT-PCR using specific primers showed the presence of 600 bp band corresponding to the transcription of *tat/pol/gag/env* sequence in transfected HEK293T cell line (lane 1) while it was absent in untransfected cells (lane 2) and transfected cells lacking the RT

process (lane 3). **b** Western-blotting with anti-His antibody confirmed the expression of 20 kDa protein band corresponding to C-terminally Hist-tagged *tat/pol/gag/env* protein in transfected HEK293T cells (lane 2) while it was absent in untransfected control cells (lane 1)

immunogenicity of pcDNA3.1-*tat/pol/gag/env* DNA vaccine, at least towards the induction of lymphocyte proliferation and cytokine secretion and further showed the superiority of 100 $\mu\text{g} \times 3$ immunization protocol over the two others.

Discussion

Construction of novel vaccine candidates for HIV-1 may result in achievement of more efficient and immunogenic vaccines. In the present study a new poly-topic DNA vaccine against HIV-1 was designed and primarily evaluated for its immunogenicity in BALB/c mice. Different sequences of HIV-1 virus have been used as candidate targets for inclusion in vaccines and the immunogenicity of many of HIV-1 protein sequences are well known. Furthermore, comprehensive studies in infected populations have further revealed the immunogenic epitopes of HIV in individuals with different MHC alleles. Based on a precise and detailed survey through the *LosAlamos HIV Molecular Immunology Database* and studying the CTL epitope map of HIV-1 antigens we succeeded to select six short regions from TAT (residues 1–20 and 44–61), ENV (residues 296–323 and 577–610), POL (residues 150–190) and GAG (residues 158–186) proteins of HIV-1 that carried CTL epitopes with a wide range of binding affinity for different MHC alleles of human, mouse and monkey. Moreover, the sequences were tried to be selected based on their partial conservancy in different subtypes. Furthermore, these

selections were based on several previous studies reporting the immunogenicity of sequences in a majority of HIV-1 positive patients, which confirmed that the selected regions were highly immunogenic and conserved [15, 16]. Additionally, all of the mentioned proteins have very important roles in the process of HIV-1 pathogenesis and inhibiting some of them such as TAT and POL will greatly reduce the viral pathogenesis process [11, 17–24]. Numerous studies have shown that targeting GAG protein is very effective in controlling the progression of AIDS [25]. ENV protein which includes GP120 and GP141 subunits, has a key role in the attachment of virus to T lymphocytes and the initiation of infection, hence, as it is shown the induction of immune responses against ENV may be very important in HIV neutralizing [26–29]. In the present study, the mentioned sequences were studied precisely by bioinformatics analysis and were placed side by side in form of a gene in order to introduce a new vaccine candidate for HIV-1. Here, bioinformatics analysis was performed to avoid the creation of unwanted epitopes at the junction of adjacent sequences. To this end, whenever it was necessary AAY and AAA amino acid spacers were logically included at the junction of selected epitopes to facilitate the proteasomal cleavage [30, 31] and hinder the neo-epitope formation. DNA vaccines have been known for nearly two decades; these vaccines initially brought high hopes, but soon the initial results of the clinical phase were presented which showed that unlike the results obtained from the animal phase, these vaccines had failed to effectively stimulate human immune systems [32]. Using the strategy to inject

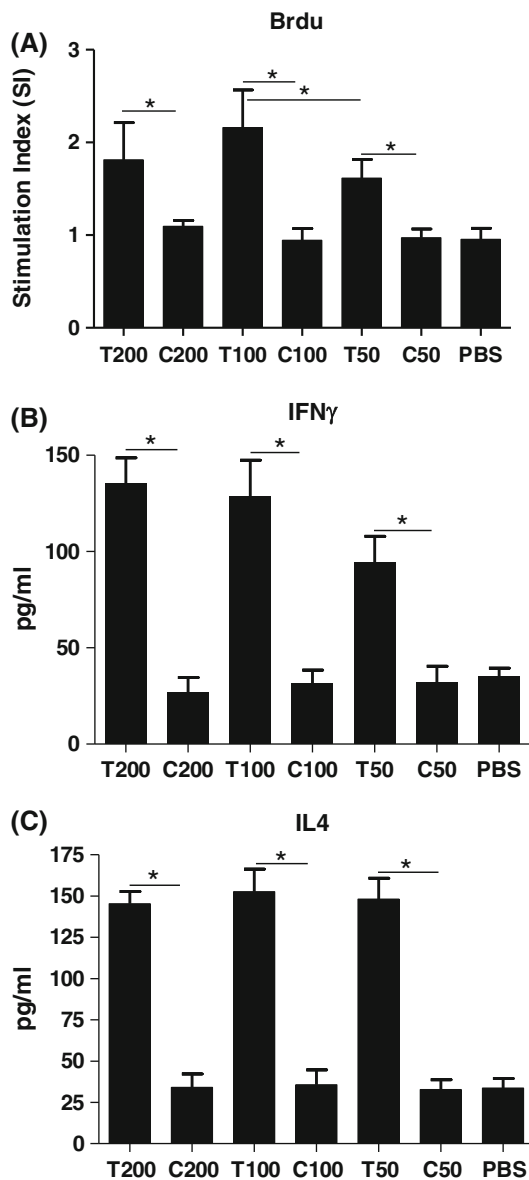


Fig. 3 Analysis of immune response in BALB/c mice intramuscularly injected with pcDNA3.1-*tat/pol/gag/env* DNA vaccine. Animals (six/group) were injected three times either with 50, 100 and 200 μ g of DNA vaccine (T50, T100 and T200, respectively) or with the same doses of control pcDNA3.1 plasmid (C50, C100 and C200, respectively) or PBS. After *in vitro* antigen recall with *tat/pol/gag/env* protein expressed in *E. coli* the rate of lymphocytes proliferation was assessed based on Brdu method and was expressed as mean of SI \pm standard deviation (SD) in six individual mice of each group (a). Obtained SI in test groups was significantly higher than that of corresponding control groups ($P < 0.029$). Also, T100 showed a significantly higher SI in comparison with T50 ($P = 0.02$). IFN- γ (b) and IL4 (c) ELISA analysis of experimental groups after *in vitro* stimulation of splenocytes with *tat/pol/gag/env* protein. Both IFN- γ and IL4 secretion in DNA vaccinated groups was significantly higher than that's of control groups ($P < 0.0001$)

booster dose peptides of the DNA vaccines was a method which was able to affect the quality and quantity of immune responses, so that the result of using this strategy

even enhanced humoral and cellular immune responses in the human trial phase. This caused DNA vaccines to be considered again. Provided that the function of DNA vaccines is very similar to attenuated viral vaccines but without their risks, they are still being discussed as serious vaccine candidates. [33, 34]. However, some approaches to improve their efficacy such as application of *in vivo* electroporation [14, 35] or validated dosage of DNA vaccine [14] are needed to be considered, too. Here, the plasmids were injected intramuscularly in three concentrations of 50, 100 and 200 μ g that were followed by *in vivo* electroporation. Many studies have shown that intramuscular injection can stimulate both humoral and cellular immune responses and perhaps this is why *i.m.* is the most used vaccine injection route in the world [6, 36]. According to the results of immune response analysis, injection of 50 μ g multi-epitopic DNA vaccine for three times with the mentioned protocol showed the lowest rates of T cell proliferation and cytokine secretion, while the two other doses (100 and 200 μ g) induced a more potent cellular immunity (Fig. 3). DNA vaccines are generally accepted to induce both humoral and cellular responses. Analysis of cytokine secretion in our study revealed the parallel secretion of IFN- γ (as the indicator of Th1 and cellular response) and IL4 (as the indicator of Th2 and humoral response) [37], confirming the active induction of both immune system arms. However, it was interesting to see that immunization of animals with 50 μ g DNA vaccine partially shifted the response towards the Th2 immunity, as evidenced with higher ratio of IL4/IFN- γ secretion (1.57) compared to other two groups (T200: 1.07 and T100: 1.18). Despite the fact that in this study, the candidate vaccine was administered alone and no immunologic adjuvants were used, it can be expected that by using immunologic adjuvants, immune response patterns could be changed according to utilized adjuvant. Also, with regard to the importance of cellular immunity in controlling intracellular and viral infections, the findings of this study propounded this vaccine as a good candidate for inducing cellular immune responses and by using the proper adjuvants, the immune responses could even be enhanced.

Overall, results of this study showed that pcDNA3.1-*tat/pol/gag/env* DNA vaccine candidate encoding a selection of critical HIV-1 epitopes and designed based on immunoinformatics predictions could initially induce the immune responses in BALB/c mice and hence, deserves for further *in vivo* studies to achieve more insights about its potency.

Acknowledgments This work was supported in part by a grant from Pasteur Institute of Iran. N.J. contributed to this study to fulfill the requirements for her M.Sc. thesis. Authors like to thank Dr. K. Azadmanesh and Dr. M. Sadat for their helpful and technical supports.

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