Immunogenicity of a New HIV-1 DNA Construct in a BALB/c Mouse Model

Mehdi Mahdavi[1], Massoumeh Ebtekar[1]*, Fereidoun Mahboudi[2], Hamidreza Khorram Khorshid[3,4], Fatemeh Rahbarizadeh[5], Kayhan Azadmanesh[6], Haydeh Darabi[7], Farzaneh Pourasgari[8], Zuhair Mohammad Hassan[1]

[1]Departments of Immunology and [5]Medical Biotechnology, Tarbiat Modares University, [2]Departments of Biotechnology, [6]Virology and [7]immunology Pasteur Institute of Iran, [3]Genetics Research Centre, University of Social Welfare and Rehabilitation Sciences, [4]Reproductive Biotechnology Research Centre, Avicenna Research Institute, [8]Department of Molecular Biology and Genetic Engineering, Stem Cell Technology Company, Tehran, Iran

ABSTRACT

Background: Cell mediated immunity, especially cytotoxic T cell responses against HIV-1 infection, plays a critical role in controlling viral replication and disease progression. DNA vaccine is a novel technology which is known to stimulate strong cellular immune responses. Many DNA vaccines have been tested for HIV infection but there is still no effective vaccine against this infection. Construction of a vaccine consisting of multiple conserved and immunogenic epitopes may increase vaccine efficacy. **Objective:** In the present study, a DNA vaccine candidate constructed from HIV-1 P24-Nef was evaluated and cellular immune responses were assessed in murine BALB/c model. **Methods:** HIV-1 P24-Nef gene was cloned in pCDNA3.1 expression vector. Mice were immunized with DNA construct and IL-4 and IFN-γ evaluation was performed using ELISPOT. Cytotoxicity response was evaluated with Granzyme B ELISPOT assay and lymphocyte proliferation was evaluated with LTT assay. **Results:** Analysis of immune responses showed that, compared to control groups, the candidate vaccine induced production of higher levels of both IL-4 and IFN-γ ($p<0.05$). Cytotoxicity and lymphocyte proliferation responses of mice vaccinated with the candidate vaccine were significantly increased compared to control groups ($p<0.05$). **Conclusion:** HIV-1 P24-Nef DNA construct displayed strong immunogenicity in a murine model.

Keywords: HIV-1 DNA Vaccine, IFN-γ, BALB/c Mouse

INTRODUCTION

HIV infection is one of the most fatal infections in the world and studies show that an effective vaccine could help to control AIDS pandemic. At present many reports indicate that cellular immune responses, especially CTL responses, have an important role in

*Corresponding author: Dr. Massoumeh Ebtekar, Department of Immunology, School of Medical Sciences, Tarbiat Modares University, PO. Box 14115-331, Tehran, Iran. Tel: (+) 98 21 82883891, Fax: (+) 98 21 82884555, e-mail: ebtekarm@modares.ac.ir

controlling AIDS progression (1). Studies in HIV positive patients reveal that an HIV-1 specific CTL response inversely correlates with disease progression (2,3). Recently, considerable studies have focused on the development of a vaccine that elicits strong CTL responses against HIV-1 (4).

The DNA vaccine is a relatively novel technology and various reports suggest that it could stimulate both humoral and cellular immune responses in animal models (5-7). After DNA vaccination, the expression vector is transferred into cells, translated and the recombinant protein is expressed, processed and finally presented via the MHC-I pathway and this may explain why DNA vaccine stimulates CTL responses (8).

Many candidate DNA vaccines for HIV-1 have been tested in basic research or clinical trials and only a few have been shown to induce favorable immune responses (9,10). The HIV-1 virion has a high mutation rate in key epitopes which gives it the opportunity to evade the CTL immune response (10). One method to increase the efficacy and breadth of the immune response against HIV-1 is utilizing multiple highly conserved and immunogenic epitopes for vaccine design (11). Studies on HIV-1 P24 and Nef revealed that certain parts of these proteins have immunogenic and conserved epitopes that stimulate immune response (12,13). Evaluations of P24 as a vaccine candidate demonstrated that CTL responses against this protein have a dominant role in controlling HIV-1 infection, therefore promoting this epitope as a suitable candidate for the development of an effective vaccine (12,14,15).

Nef is a regulatory protein and has a key role in viral replication, pathogenicity and evasion of immune responses (16). Comprehensive analyses of HIV sequences revealed that this protein has multiple conserved and immunogenic epitopes which are recognized by cytotoxic and T helper lymphocytes (17,18). Due to its expression during early viral replication, high immunogenicity and key role in HIV pathogenicity, Nef may be a good choice for HIV vaccine design in order to induce strong $CD8^+$ CTL responses (17-19).

In the present study, based on the finding that multiple epitope constructs may induce better protectivity, we designed a fusion DNA vaccine using HIV P24 and Nef epitopes as HIV candidate vaccine for immunoassay in murine BALB/c model. We used the most conserved and immunogenic sequences in these proteins (P24 $aa_{159\text{-}173}$ and Nef $aa_{102\text{-}117}$) for our work. After construction of an expression vector for HIV-1 P24-Nef gene, animals were vaccinated and immune responses to this candidate vaccine were evaluated in a murine model.

MATERIALS AND METHODS

Construction of Recombinant HIV-1 P24-Nef Expression Vector. HIV-1 p24-Nef fusion DNA fragment was synthesized by GL Biochem Company (Shanghai, China) in PUC18 cloning vector. The recombinant cloning vector was digested with *Hind*III/ *Bam*HI and sub-cloned into the same enzymatic sites in pCDNA3.1Hyg+ expression vector (Invitrogen, France). Expression of fusion peptide was under hCMV promoter control, followed by a polyadenylation signal sequence. A recombinant plasmid containing p24-Nef DNA fragment fused with pEGFPC-1 gene was also constructed (Clontech, CA). The DNA construct was confirmed with enzymatic digestion, PCR and sequencing.

Cell Lines. P815 cell line (mouse mastocytoma, H2d) were used as target cells and 293T cell line (eukaryotic host for gene expression assay) obtained from Pasteur Institute of Iran was cultured in RPMI 1640 supplemented with 10 % FBS, 2 mM L-glutamine, 25 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 µm 2ME, 100 µg/ml streptomycin, 100 IU/ml penicillin and incubated at 37˚C in 5% CO2 with 95% humidity.

In Vitro Transfection and Expression of HIV-1 P24-Nef Constructs. The expression of pCDNA3.1/P24-Nef was tested by RT-PCR and fluorescence. Recombinant expression vectors were transfected to 293T cell line with Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, the 293T cell line was cultured in antibiotic free medium in 24-well plates. After reaching to 40 to 60% confluency, 800 ng of plasmid was transfected to 293T cell line with Lipofectamin 2000. After 18 h, culture medium was replaced with complete medium. Protein expression assay was monitored by Green Fluorescence Protein (GFP) expression as a reporter gene 72 h post transfection with a fluorescence microscope (Nikon, Japan).

RT-PCR Analysis. RT-PCR was performed 72 h post transfection analyzing the expression at mRNA level. Total RNA was extracted from 1×10^{6} transfected cells with RNA extraction solution (RNX-Plus, Cinnagen, Iran). Then cDNA was synthesized and used for PCR amplification. PCR was carried out with 300 ng of cDNA in a total volume of 25 µl of the reaction mixture comprising: 1x PCR buffer, 0.5 µM of the primers (Forward: 5'-CCAAGCTTGCCACCATG-3') and (Reverse: 5'-CGGATCCGCGTTATGTG-3'), 1.5 mM MgCl2, 250µM of dNTPs, 1.5 U of Taq polymerase (Cinnagen, Iran). Human Hypoxanthine Phosphoribosyl Transferase (HPRT1) as a house-keeping gene was used as endogenous control and the Primers (Forward: 5'-CCTGGCGTCGTGATTAGT G-3', Reverse: 5'- TCAGTCCTGTCCATAATTAGTCC-3') were added to the PCR reaction. After an initial denaturation step at 94°C for 5 min, PCR amplification was done for 30 cycles (94°C for 30s, 59°C for 30s, 72°C for 30s). The program was followed by a 7 min extension at 72°C and then the PCR product was analyzed on a 3 % agarose gel by ethidium bromide staining.

Peptide. HIV-1 P24-Nef fusion peptide from P24 ($aa_{159-173}$) and Nef ($aa_{102-117}$) was synthesized according to the solid-phase method described by GL Biochem Company (Shanghai, China) with a purity of >95%.

Mice. Six- to eight-week old female inbred BALB/c mice were purchased from the Pasteur Institute (Karaj, Iran) and were housed for one week before the experiment. All animal care and use were conducted according to the approved protocols and in accordance with the recommendations for the proper use of laboratory animals under the supervision of the Ethical Committee of Tarbiat Modares University.

Purification of HIV-1 P24-Nef DNA Construct. HIV-1 P24-Nef DNA construct was transformed in E.coli Top10 F' as host using CaCl2 method. Bacterium was cultured in a large scale and plasmid purification was done using Qiagen endofree anion exchange chromatography using Giga column (Qiagen, Germany). All purified plasmids were stored at -20 ˚C until further use.

Immunization of Mice with HIV-1 p24-Nef DNA Construct. Mice were divided into three groups, each consisting of four mice immunized intramuscularly (i.m.) on day 0 with 100 µg of HIV-1 p24-Nef DNA construct in PBS. As control groups, four mice were injected with 100 µg of plasmid backbone and four mice were injected with sterile PBS under the same protocol. All mice were boosted on day 28 with the same materials.

LTT Assay. Four weeks after last immunization, the spleens of immunized mice were removed under sterile conditions and suspended in PBS containing 2% FBS. RBCs were lysed with lysis buffer. Single-cell suspension was prepared in RPMI 1640 (Gibco) and adjusted to 4×10^6 cells per milliliters in RPMI 1640 supplemented with 5% FBS, 4 mM L-glutamine, 25 mM HEPES, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 µm 2ME, 100 µg/ml streptomycin and 100 IU/ml penicillin. 100 µl of the suspension was stimulated with 10µg/ml of fusion peptide in 96 well plates.
Phytohemagglutinin-A (5 µg/ml; Gibco) was used as a positive control and the volume was adjusted to 0.2 ml and all experiments were done in triplicate. After incubating for 72 h at 37˚C in 5% CO2 in a humidified incubator, cells were pulsed with 0.7 µCi of [^{3}H] Thymidine (Amersham, UK) per well and the incubation was continued for another 18 h and then the cells were harvested and the radioactivity was measured by a beta counter (Pharmacia, Sweden). Stimulation Index (SI) was calculated according to the following formula: cpm of the wells stimulated with the antigen / cpm of the wells containing only the cells with the medium.
Granzyme B ELISPOT. Cytotoxicity assay was performed using Granzyme B ELISPOT kit according to the manufacturer's instruction (R&D systems, USA). Briefly, PVDF 96-well plates (Millipore) were coated with Granzyme B capture antibody overnight at 4 ˚C. The plates were washed 4 times with PBS-T20 and blocked with PBS containing 1% BSA and 5% sucrose for 2 h at room temperature. Mouse mastocytoma P815 (H-2d) as target cells, were pulsed overnight with 20µg/ml of fusion peptide. $2x10^4$ target cells in a volume of 75 µl per well were incubated with 75 µl of freshly isolated single-cell splenocyte suspensions as effector cells at 50:1 or 100:1 effector:target (E:T) ratios for 4 h in complete RPMI 1640 at 37° C and 5% CO2.
The wells containing un-pulsed P815 cells with splenocytes were used as a specificity control (20). Spleen cells, P815 and RPMI 1640 were used as negative controls and recombinant mouse Granzyme B (eBioscience, UK) was used as a positive control.
The plates were washed five times and incubated for 18 h at 4˚C with 100 µl of 1/60 diluted anti-mouse GrB detection antibody in PBS containing 1% BSA. Afterwards, the plates were washed and 100 µl of 1/1000 diluted streptavidin-conjugated alkaline phosphatase was added to each well. After a final wash with the washing buffer, spots were developed by adding 100 µl of BCIP/NBT substrate to each well and incubating for 60 min at room temperature in the dark. The plates were then rinsed three times with distilled water and dried at 4˚C. Spots were counted by stereo microscope (Nikon, Japan).
IL-4 and IFN-γ ELISPOT Assays. In order to detect the frequency of IL-4 and IFN-γ producing cells, ELISPOT assay was performed according to the manufacturer's (Mabtech, Stockholm, Sweden) protocol. Briefly, a total number of 1×10^6 spleen cells were plated on each well of a 96-well PVDF plate using complete RPMI 1640 medium. The cells were stimulated in vitro with 10 µg/ml of the peptide. PHA, as positive control and un-stimulated splenocyte and RPMI 1640 as negative control were used. After 24 h of stimulating the cells, plates were washed five times with washing buffer and then 100 µl of 1 µg/ml mAb directed to mouse IL-4 or IFN-γ in PBS containing 0.5% FBS were added to the wells and incubated for 2 h at room temperature. The plates were then washed five times with washing buffer and incubated for 1 h at room temperature with 100 µl of 1/1000 diluted sterptavidine-conjugated alkaline phosphatase. After a final wash with the washing buffer, spots were developed by adding 100 µl of BCIP/NBT substrate to each well and incubating for 45 min at room temperature in the dark. The

plates were then rinsed three times with distilled water and dried at 4˚C. Spots were counted by stereo microscope (Nikon, Japan).

Statistical Analysis. The data were expressed as mean ± SE. All statistical analyses were done by one-way ANOVA followed by Tukey's test. In all of the cases, $p<0.05$ were considered to be statistically significant.

RESULTS

Cloning and Expression of Recombinant Vector. In the present study, pCDNA3.1 was used as an expression vector and HIV-1 P24-Nef DNA fragment was cloned into this vector. The authenticity of clones was confirmed by PCR using specific forward and reverse primers for DNA fragment. Enzymatic digestion of positive recombinant vector with *Hind*III/*Bam*HI revealed a 159 bp DNA band in electrophoresis (Figure 1). Sequencing of positive recombinant vector (Figure 2) confirmed the sequence of DNA fragment in the expression vector. RT-PCR revealed a 165 bp in electrophoresis (Figure 3). Analysis of expression with GFP showed positive cells 72 h after transfection (Figure 4).

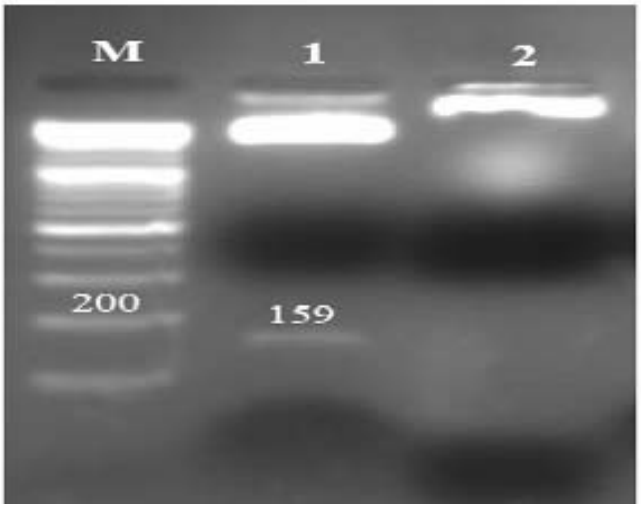

Figure 1. Double digestion of pCDNA3.1/ P24-Nef construct. Lane M, 100 bp DNA ladder, Lane 2, plasmid digested with *Hind*III/*Bam*HI restriction endoneucleases, Lane 3, undigested plasmid.

CCAAGCTTGCCACCATGGCCCACCACCACCACCACCACTATG AACCCTTT

AGAGACTATGTAGACCGATTCTATAAAACTCTAAGAGCCGCCTATCACTCCC

AAAGAAGACAAGATATCCTTGATCTGTGGATCTACCACACATAACG**CCGGAT**

CCGCGTTATGTG

Figure 2. Nucleotide sequence of the P24-Nef fusion gene segment inserted into pCDNA3.1 expression vector. The bold nucleotides show primers.

Figure 3. RT-PCR analysis of the 293 T cells after transfection with pCDNA3.1/P24-Nef vector. Lane M, 1-kb DNA ladder, Lane 1, 293 T cell transfected with pCNDA3.1 mock vector as a negative control, Lane 3, HPRT1 house-keeping gene as an endogenous control.

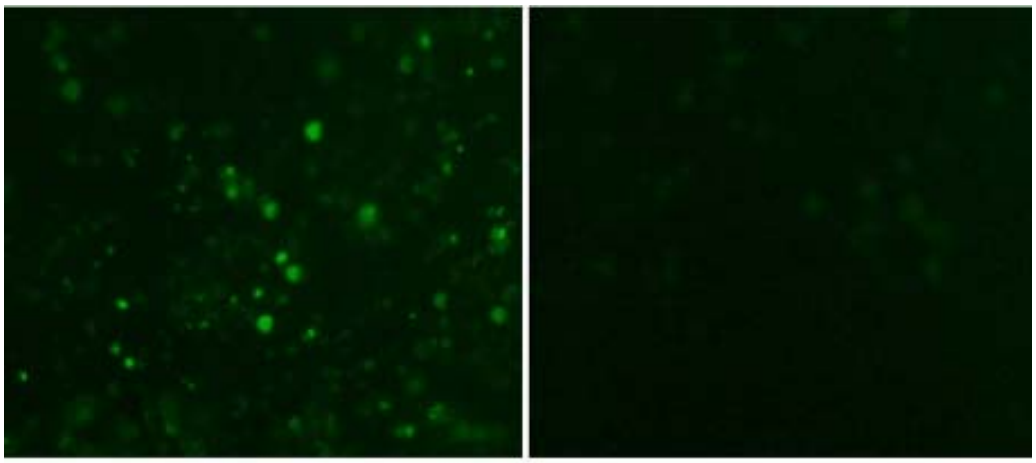

Figure 4. Expression of HIV-1 P24-Nef protein in eukaryotic system with Green Fluorescence Protein as reporter gene. P24-Nef DNA fragment was fused with GFP gene and transfected to 293 T cell line. After 72 h of transfection, cells transfected with recombinant construct showed positive results (A) and cells transfected with backbone showed negative results (B).

Lymphocyte Proliferation Assay. To determine lymphocyte response against DNA vaccine candidate, splenocytes of immunized mice were harvested and re-stimulated in vitro for 72 h with 10 μg/ml of P24-Nef fusion peptide and proliferation was detected with ^{3}H thymidine radioactivity. As shown in Figure 5, mice immunized with P24-Nef DNA construct significantly ($p<0.0001$) induced lymphocyte proliferation response in comparison to the control groups and there was no significant difference between control groups ($p>0.05$).

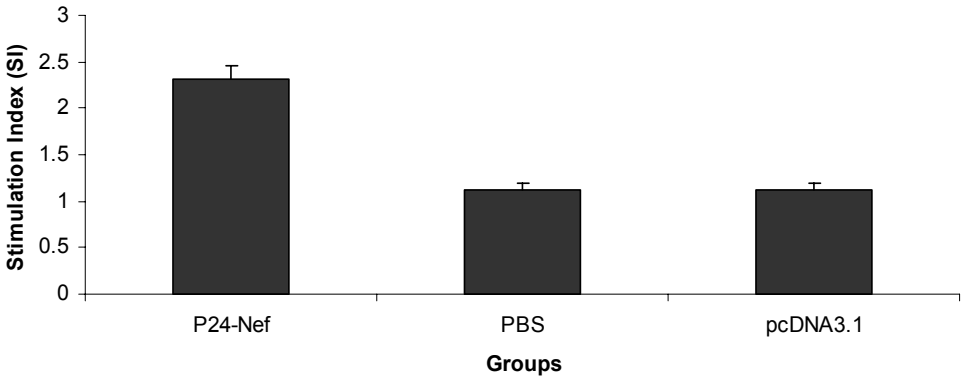

Figure 5. Lymphocyte proliferation responses after in vitro stimulation with HIV p24-Nef peptide. Immunization of mice with DNA construct significantly increased lymphocyte proliferation compared to control groups. Values are mean ± SE for 4 experiments. For experimental protocol see Methods.

Cytotoxicity of Candidate Vaccine. For detection of cytotoxic activity, Granzyme B ELISPOT method was used. Analysis of GrB producing lymphocytes revealed that the DNA immunization of mice has increased cytotoxic activity compared to the control groups. In both 50:1 and 100:1 of E:T ratio, cytotoxic activity of mice immunized with P24-Nef DNA construct was significantly increased ($p<0.0001$) compared to the control groups (Figure 6) and there was no significant difference between the control groups ($p>0.05$).

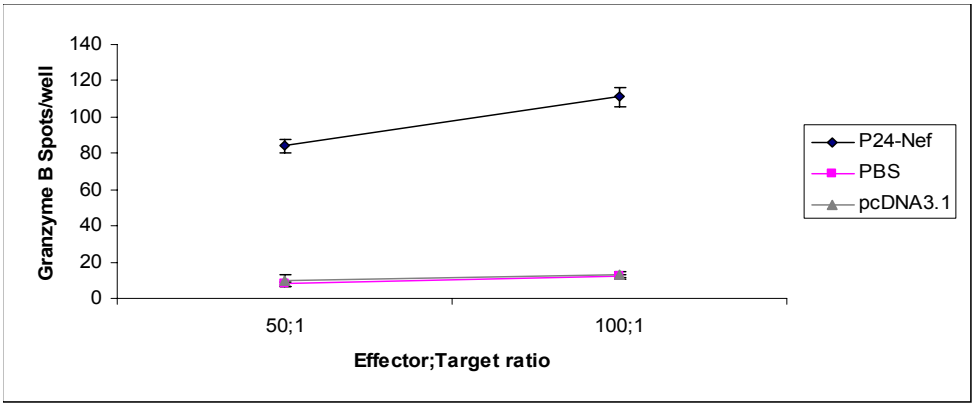

Figure 6. Cytotoxic activity in experimental groups. Immunization of mice with DNA construct significantly increased lymphocyte cytotoxicity compared to control groups. Values are mean ± SE for 4 experiments. For experimental protocol see Methods.

Cytokine ELISPOT Assay. Analysis of cytokine profile was done using ELISPOT method. IFN-γ and IL-4 secreting cells were evaluated (Figure 7) four weeks after the final boosting with DNA construct and the re-stimulation of splenocytes with P24-Nef peptide in vitro. As shown in Figure 8, immunization of mice significantly ($p<0.0001$) increased IFN-γ producing lymphocytes compared to the control groups and there was no significant difference between control groups ($p>0.05$). IL-4 producing lymphocytes were evaluated to serve as a Th2 cytokine marker. Analysis of IL-4 producing cells revealed that after immunization of mice with DNA construct, IL-4 producing lymphocytes increased significantly ($p<0.0001$) compared to control groups (Figure 9) and there was no significant difference between control groups ($p>0.05$).

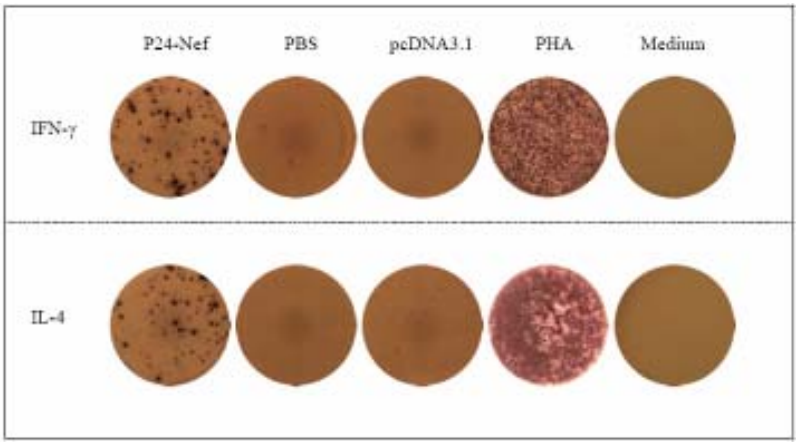

Figure 7. ELISPOT analysis of cytokine pattern in experimental groups. Individual cytokine ELISPOT are shown in experimental groups. All experiments were done on freshly isolated lymphocytes from spleen and the cells were stimulated for 24 h with 2 µg of peptide per well.

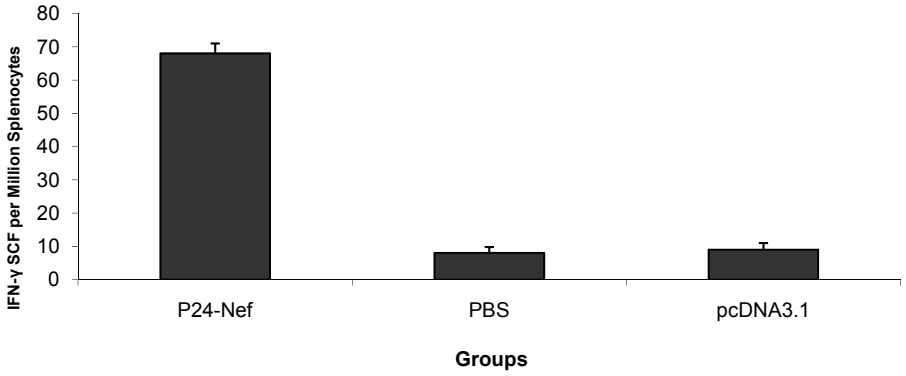

Figure 8. IFN-γ ELISPOT analysis of mice after in vitro stimulation with HIV p24-Nef peptide. Immunization of mice with candidate DNA vaccine significantly increased IFN-γ producing lymphocytes compared to control groups. Values are the mean ± SE for 4 experiments. For experimental protocol see Methods.

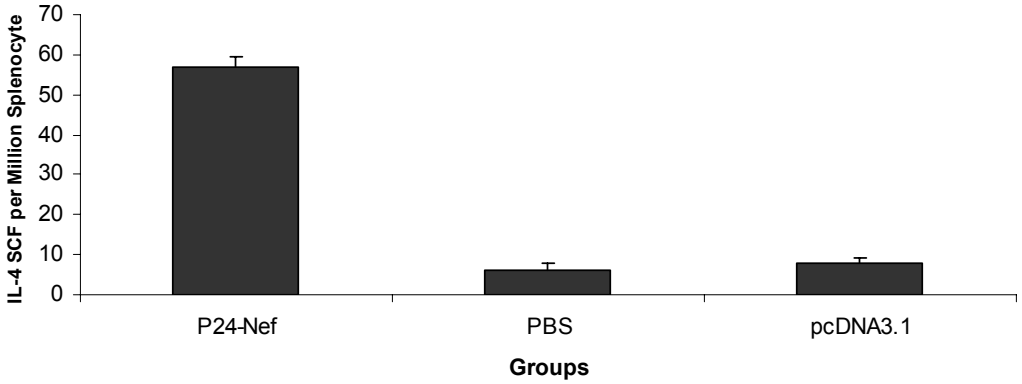

Figure 9. IL-4 ELISPOT analysis of mice after in vitro stimulation with HIV p24-Nef peptide. Immunization of mice with DNA construct significantly increased IFN-γ producing lymphocyte compared to control groups. Values are the mean ± SE for 4 experiments. For experimental protocol see Methods.

DISCUSSION

DNA immunization is a novel technology and studies have revealed that this method could elicit both humoral and cellular immune responses (21). Many DNA vaccines have been tested in human trials and animal models but only a few have been shown to stimulate a strong protective response as expected from a successful vaccine (22). In this study a new DNA vaccine candidate based on HIV P24 and Nef epitopes was constructed. This is the first work in which only P24 and Nef have been used in a DNA construct. Other investigators have used P24 and Nef in conjunction with other epitopes from gp120, tat and rev. Studies have shown that the inclusion of multiple conserved and immunogenic epitopes in DNA constructs results in broadening of the immune response and this strategy is known to increase vaccine efficacy (11,23,24). Conserved and immunogenic epitopes from P24 ($aa_{159-173}$) and Nef ($aa_{102-117}$) were used for the construction of our vaccine candidate. Studies directed at these sequences revealed a good deal of flexibility in binding a wide range of MHC molecules in humans, mice and non-human primates (24,25). These proteins elicit strong immune responses against HIV-1 infection and prevent HIV progression and decrease viral replication (12,26-28). For the linkage of P24 and Nef epitopes, AAY sequences were used in between. According to other works, this sequence helps the proteasome to digest the peptide at this site and prevent pseudo-epitope formation (29,30).

Analysis of immune response against our construct through different approaches has revealed that it could be considered as a potential vaccine candidate for further studies in non-human primate models. Evaluation of proliferative response of lymphocytes against P24-Nef fusion peptide suggests that this vaccine candidate significantly increases lymphocyte proliferation as compared to control groups. Lymphocyte proliferation is an important parameter of cellular immunity (31) and the results indicate that this vaccine candidate could successfully elicit the cellular immune response which is an essential requirement of successful DNA vaccines (32). CTL response was also evaluated against candidate vaccine utilizing the Granzyme B ELISPOT method which is a highly sensitive single cell analysis method (33,34). Cellular immunity, specially CTL responses play a critical role in controlling viral infections such as HIV-1 (12). The data suggests that immunization of mice with HIV-1 P24-Nef construct significantly increases GrB producing lymphocytes (CTL activity) compared to control groups. Studies of natural infection in HIV-1 positive patients and simian immunodeficiency virus (SIV) infection in non-human primate models suggest that CD8+ T cell responses have a protective effect in HIV-1 infection and depletion of CTL population during SIV infection of macaques and result in an increase of viral load (11,25,28). However, recent emerging evidence places the role of CTL responses in a doubtful position. Researchers argue whether it is the lower viral load that allows better CD8 response in some patients or is it the CD8 T cell response that controls viremia (35,36). Other works have indicated that induction of Th1 cytokine pattern is directly related with the protective response to HIV-1 infection (37). Studies on the cytokine profile after immunization of mice with HIV-1 P24-Nef DNA construct suggest that HIV P24-Nef candidate vaccine induces both IL-4 and IFN-γ producing lymphocytes as compared to the control groups, but IFN-γ production has been stronger, thereby indicating the dominance of a Th1 response. The shift to a Th1 immune response plays an important role in viral infections such as HIV-1 and this profile facilitates the induction of cellular immunity against viral pathogens (37-39)

Taken together, results of this work demonstrate that the candidate vaccine elicits strong cellular immune responses in the murine model. Further work on the protectivity and various dimensions of the immune response are necessary, in addition to studies on the efficacy and safety of this construct. Also the important role of humoral immune response in HIV infection and the possible consequences of hyper immune activation need to be undertaken as a future direction in both basic and clinical dimension. In our work, a prime-boost strategy for the induction of humoral immune response against candidate vaccine will be followed. In order to elucidate the role of cytokines as biological mediators that could enhance a favorable vaccine response, future studies will consider the role of memory T cells using cytokine genes as genetic adjuvants in our model. The goal of creating a successful HIV vaccine which fits the criteria of a protective, safe and affordable vaccine for all is still elusive, although ongoing research in this field provides promising results for vaccines that may diminish a major health threat still looming over the globe.

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