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Different dendritic cells-based vaccine constructs influence HIV-1 antigen-specific immunological responses and cytokine generation in virion-exposed splenocytes

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ABSTRACT

In recent years, dendritic cells (DCs)-based vaccines have been developed to combat HIV-1 infection in preclinical and clinical trials. In this study, mice bone marrow cells-derived DCs were pulsed with the recombinant Nef, heat shock protein 27 (Hsp27) and Hsp27-Nef proteins, and also green fluorescent protein (GFP) as a positive control. Then, new platforms of DCs loaded with HIV-1 Nef and Hsp27-Nef proteins (*i.e.,* DC prime/DC boost, DNA prime/DC boost, and DC prime/protein boost) were used to evaluate immune responses in BALB/c mice. Finally, the potency of splenocytes exposed to single-cycle replicable (SCR) HIV-1 virions was investigated to secret cytokines *in vitro*. Our data indicated that the recombinant Nef (~30 kDa), Hsp27 (~27 kDa), GFP (~27 kDa), and Hsp27-Nef (~53 kDa) proteins were greatly generated in *E. coli*. Moreover, the modified DCs with the recombinant proteins were prepared in large scale. The results of mice immunization showed the highest levels of antibodies, cytokines, and Granzyme B in heterologous DC prime/protein boost regimen using Hsp27-Nef antigen ($DC_{Hsp27\text{-}Nef}$ prime/ protein $_{Hsp27\text{-}Nef}$ boost regimen). The levels of IFN-γ and IL-10 cytokines in splenocytes isolated from mice immunized with $DC_{Hsp27\text{-}Nef}$ prime/ protein $_{Hsp27\text{-}Nef}$ boost regimen were higher than those in other regimens after exposure to SCR virions. These findings demonstrated the importance of Hsp27 as an adjuvant and heterologous DC prime/ protein boost regimen in improvement of immune responses. Indeed, DC $_{Hsp27\text{-}Nef}$ prime/ protein $_{Hsp27\text{-}Nef}$ boost regimen can be utilized as a promising candidate for HIV-1 vaccine development.

1. Introduction

Human immunodeficiency virus (HIV) infection is a serious health issue in different parts of the world [1]. While continuous trials have been performed for development of an effective HIV vaccine, but there is no approved vaccine for control of this infection at present. Several dendritic cells (DCs)-based vaccine candidates have been developed in preclinical and clinical trials. Indeed, dendritic cells are a specialized family of professional antigen presenting cells (APCs) with unique ability to initiate and maintain immune responses when pulsed with antigens. These DCs-based vaccines represent a suitable approach to optimize the induction of immune responses [2,3] including DCs loaded with chemically inactivated autologous viruses [4,5], DCs transfected with RNA isolated from autologous virus [6], DCs transfected with viral genes, and DCs pulsed with viral antigens (*e.g.,* peptide, protein) [6–8]. A major challenge for designing effective therapeutic DC-based vaccines is the selection of immunogenic antigens acting as targets for immunological responses [9]. In this way, among different HIV-1 proteins, Nef was known as an effective antigen candidate in vaccine development [10]. Furthermore, adjuvants play a key role for induction of DC maturation and activation, and intensify the immune responses such as cytokines, toll-like receptor (TLR) agonists, CD40 ligands [11,12]. Heat shock protein 27 (Hsp27) along with other Hsps (*e.g.,* Hsp70) were also known as potent adjuvants for stimulation of immune system against

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cancer and infectious diseases [10,13]. Additionally, the interaction of Hsp27 with TLRs triggers downstream signaling cascades to activate nuclear factor-kappa B leading to cell maturation and secretion of Th1-type cytokines [14–16]. On the other hand, the use of homologous and heterologous prime-boost immunization strategies could induce both humoral and cellular immune responses [17]. In this study, we used these findings to generate the modified DCs with Nef, Hsp27 and Hsp27-Nef proteins. The efficiency of Hsp27 as an adjuvant was evaluated to enhance HIV-1 Nef antigen-specific immunity in mice. Moreover, our study addresses whether mice splenocytes (with or without antigen re-stimulation) could secrete cytokines after exposure to single-cycle replicable (SCR) HIV-1 virions *in vitro*.

In general, our specific objectives include production of DNA and protein constructs; preparation of the engineered DCs with antigens; generation of single-cycle replicable virions, determination of the best immunization strategy based on DCs (*i.e.,* homologous DC prime/DC boost, heterologous DNA prime/DC boost, and heterologous DC prime/protein boost regimens) through antibody, cytokine, and Granzyme B secretion; and analysis of antiviral effects *in vitro* using the exposure of mice lymphocytes to SCR virions through cytokines secretion. Briefly, our data showed the potency of heterologous DC prime/ protein boost strategy and the use of Hsp27 as an adjuvant in stimulation of effective immune responses and antiviral effects *in vitro* for HIV-1 vaccine development. Altogether, our results provide new insight into DC-based vaccine development. Fig. 1 shows general view of this study.

2. Materials and methods

2.1. Preparation of DNA constructs

The full length of HIV-1 Nef (from HIV-1 vector pNL4-3, Accession No: AF324493.2; ~648 bp), Mus musculus Hsp27 (Accession No: NM_013560; ~720 bp), and Hsp27-Nef fusion (~1368 bp) genes were previously cloned in pcDNA3.1 (-) eukaryotic vector (Invitrogen) by our group [10]. Herein, all DNA constructs were purified by endotoxin-free plasmid DNA purification kit (Qiagen). Then, the concentration and purity of DNA constructs were determined by NanoDrop spectrophotometer, and confirmed by digestion with restriction enzymes. Also, we used MPG as a cell penetrating peptide (CPP) for delivery of DNA constructs in mice immunization based on our previous study [18]. For this purpose, the formation of complexes between MPG peptide and DNA constructs (*i.e.,* MPG/pcDNA-Hsp27, MPG/pcDNA-Nef, and MPG/pcDNA-Hsp27-Nef complexes) was confirmed by gel retardation assay at certain ratio of N: P equal to 10:1. Indeed, the plasmid DNAs did not migrate into the agarose gel in this ratio.

2.2. Generation of the recombinant proteins

The *E. coli* Rosetta strains harboring the recombinant pET-*nef,* pET-*hsp27*, pET-*hsp27-nef*, and pET-*gfp* (*green fluorescent protein*) were previously prepared and stored at −70°C by our group [10,19]. The recombinant proteins were expressed by 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C in large scale under the optimized time conditions (4, 3, 16, and 4 h after induction for Hsp27, GFP, Nef, and Hsp27-Nef, respectively) as reported previously by our group [10,19]. Then, the recombinant Hsp27 (~27 kDa) and GFP (~27 kDa) proteins were purified by affinity chromatography using a Ni-NTA agarose column under native conditions (Imidazole buffer, pH = 8), and the recombinant Nef (~30 kDa) and Hsp27-Nef (~53 kDa) proteins were purified by affinity chromatography under denaturing conditions (Urea buffer, pH = 4.5) as reported previously [10,19]. The purified proteins were dialyzed and assessed by NanoDrop spectrophotometry. The recombinant proteins had a concentration range between 1.0 and 1.5 mg/ml. The endotoxin contamination was monitored by LAL assay (QCL-1000, Lonza). It was<0.5 EU/mg proteins.

2.3. Generation and characterization of dendritic cells

Dendritic cells were prepared from bone marrow cells of male BALB/c mice (Pasteur Institute of Iran) in the presence of 20 ng GM-CSF

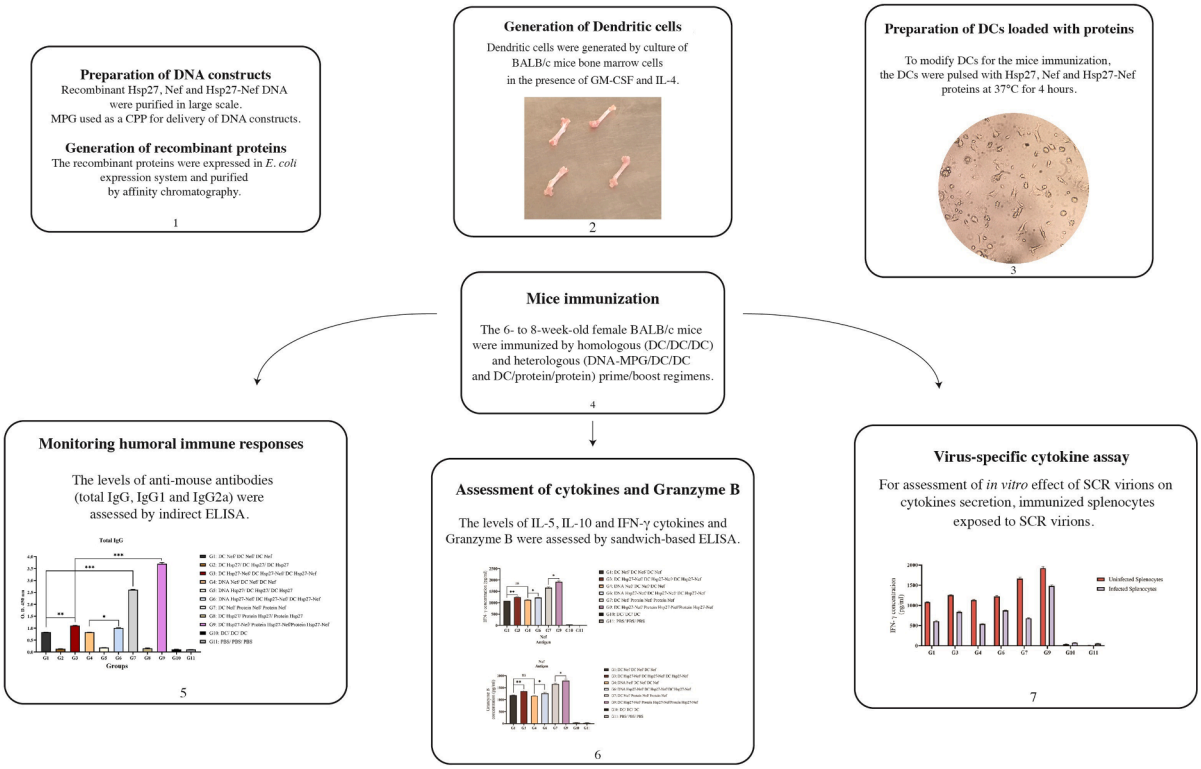

Fig. 1. Evaluation of immune responses induced by DCs loaded with the recombinant Nef, Hsp27 and Hsp27-Nef proteins in different modalities.

(Peprotech) and 10 ng IL-4 (Peprotech). The steps of DC isolation are as follows. At first, femurs and tibias were isolated from mice and kept in RPMI 1640 medium on ice. Next, muscles were cleaned from the bones and the cleaned bones were immersed for 2 min in 70 % ethanol. Both ends of each bone were cut off, and bone marrow cells were isolated by flashing out each of the shafts with 1 ml RPMI 1640 using a syringe. After that, erythrocytes were lysed using ACK buffer. Then, the remaining cells were cultured at a density of 5.0×10^6 cells/cm^2 in RPMI 1640 (Gibco) supplemented with 20 % FBS (Gibco) and 3 % penicillin/streptomycin (Gibco). Every-two days, the cells were fed with RPMI 1640 supplemented with 10 % FBS, 20 ng GM-CSF, and 10 ng IL-4. Finally, non-adherent and loosely-adherent cells as immature DCs were harvested on day 5. Characterization of dendritic cells was performed by assessment of surface molecules expression using flow cytometry. For flow cytometry, 1.0×10^6 cells were washed twice with cold phosphate-buffered saline 1X (PBS1X, Sigma). The cells were incubated with mAbs against CD86, MHCII, CD11c and CD40 markers (eBioscience) for 40 min on ice in dark place. The cells were washed twice with PBS1X. After that, FACS analysis was performed on a FACScan Flow Cytometer (Becton Dickinson).

2.4. Preparation of DCs pulsed with the recombinant proteins

To modify DCs with the recombinant proteins for mice immunization, the DCs isolated from bone marrow cells (1.0×10^6 cells) were seeded in 24-well culture plate, and pulsed with Hsp27 (DC_{Hsp27}), Nef (DC_{Nef}) and Hsp27-Nef ($DC_{Hsp27\text{-}Nef}$) proteins (concentration: 10 μg/ml) at 37 °C for 4 h. After washing the cells, they were cultured for another 48 h in the presence of 20 ng GM-CSF and 10 ng IL-4. Finally, DCs were harvested, washed in PBS1X, and counted to determine the cell number and viability on day 7. Detection of protein uptake in DCs was performed by western blotting using anti-His-tag antibody conjugated to horseradish peroxidase (1:10000 v/v, Abcam), and 3, 3′-diaminobenzidine (DAB) substrate. For investigation of the potency of immature DCs for protein uptake, GFP was used as a reporter protein. The presence of protein in DCs was observed by fluorescence microscopy.

2.5. Production of SCR virions

To produce the single-cycle replicable human immunodeficiency virus-1 (SCR HIV-1), HEK-293 T cells were co-transfected with three pmzNL4.3, psPAX2 and pMD2G plasmids using TurboFect reagent (Fermentas). Culture supernatants of the transfected cells were harvested at 24, 48, and 72 h post-transfection, clarified using 0.2 μm filter, and then virions were collected in 45,000 g for 120 mins. The pellet of virions was resuspended in RPMI 1640 by gentle mixing overnight at 4 °C, quantified by p24 ELISA assay kit (rETRO-TEK), and stored at −70 °C until use [20].

2.6. Mice immunization

The 5- to 7-week-old female BALB/c mice were purchased from Pasteur Institute of Iran. Groups of five animals were subcutaneously immunized three times with a two-week interval (Days 0, 14 & 28) using homologous DC prime/ DC boost, heterologous DNA prime/ DC boost, and heterologous DC prime/ protein boost modalities as shown in Table 1. Mice were immunized with the DNA (5 μg)/ MPG nanoparticles (the N: P ratio was 10: 1), 5 μg of the recombinant proteins, and 1.0×10^6 modified DCs in PBS1X. Unmodified DCs and PBS were used as control groups. The whole process was done based on approval protocols and care of laboratory animals in the Animal Experimentation Regulations of Islamic Azad University (national guideline) for scientific purposes (Ethics code: IR.IAU.PS. REC.1400.105; Approval date: 2021–08-08).

Table 1
Mice immunization using different prime/boost strategies.

Group	Modality	First injection (priming)	Second injection (booster 1)	Third injection (booster 2)
1	DC/DC/DC	DC_{Nef}	DC_{Nef}	DC_{Nef}
2	DC/DC/DC	DC_{Hsp27}	DC_{Hsp27}	DC_{Hsp27}
3	DC/DC/DC	$DC_{Hsp27\text{-}Nef}$	$DC_{Hsp27\text{-}Nef}$	$DC_{Hsp27\text{-}Nef}$
4	DNA/DC/DC	DNA_{Nef}/MPG	DC_{Nef}	DC_{Nef}
5	DNA/DC/DC	DNA_{Hsp27}/MPG	DC_{Hsp27}	DC_{Hsp27}
6	DNA/DC/DC	$DNA_{Hsp27\text{-}Nef}$/ MPG	$DC_{Hsp27\text{-}Nef}$	$DC_{Hsp27\text{-}Nef}$
7	DC/protein/ protein	DC_{Nef}	$Protein_{Nef}$	$Protein_{Nef}$
8	DC/protein/ protein	DC_{Hsp27}	$Protein_{Hsp27}$	$Protein_{Hsp27}$
9	DC/protein/ protein	$DC_{Hsp27\text{-}Nef}$	$Protein_{Hsp27\text{-}Nef}$	$Protein_{Hsp27\text{-}Nef}$
10	Control	DC	DC	DC
11	Control	PBS	PBS	PBS

Abbreviation: Hsp27: Heat shock protein 27; DC: Dendritic cell.

2.7. Monitoring humoral immune responses

Mice sera were prepared and pooled for each group three weeks after the last immunization. The levels of goat anti-mouse antibodies conjugated to horseradish peroxidase (total IgG, IgG1 and IgG2a; Sigma; 1:10000 v/v) were assessed by indirect enzyme-linked immunosorbent assay (ELISA). The coated antigens were the recombinant Nef and Hsp27-Nef proteins (5 μg/ ml) diluted in PBS1X. Moreover, mice sera were diluted in 1 % bovine serum albumin (BSA)/PBS-Tween (1:100 v/v). TMB (Tetramethylbenzidine) was used as a substrate for horseradish peroxidase (HRP) conjugated to antibodies. The optical density (OD) was assessed at the wavelength of 450 nm.

2.8. Antigen-specific cytokine assay in vitro

Mice splenocytes were prepared three weeks after the last immunization, and seeded in 24-well plates. Splenocytes were re-stimulated with the recombinant Nef, Hsp27-Nef proteins (5 μg/ml), and concanavalin A (5 μg/ml, positive control) for 72 h at 37 °C. After that, the supernatants were used to evaluate the levels of IL-5, IL-10 and IFN-γ cytokines using sandwich-based ELISA (Mabtech).

2.9. Virion-specific cytokine assay in vitro

Evaluating the effects of SCR virions on secretion of cytokines was performed in two approaches. In the first approach, the splenocytes of the immunized mice were cultured in 24-well plates (2.0×10^6 cells/ml), and infected with SCR virions for 4 h. After that, the splenocytes were washed twice with PBS1X to remove unbounded virions. In the second approach, the splenocytes of three groups (G3, G6 and G9) were infected with SCR virions for 4 h. After that, the splenocytes were washed twice with PBS1X to remove unbounded virus, and re-stimulated with the recombinant Hsp27-Nef protein (5 μg/ ml). In both approaches, the secretion of IFN-γ, IL-5 and IL-10 cytokines was evaluated after 72 h. The SCR virions were used equivalent to 50 μg of p24 antigen.

2.10. Granzyme B secretion

SP2/0 cells (as a target cell: T) were used to assess cytotoxic T lymphocyte (CTL) activity *in vitro*. SP2/0 cells were seeded in triplicate into U-bottomed, 96-well plates (2.0×10^4 cells/ well) along with the recombinant Nef and Hsp27-Nef proteins (~10 μg/ ml) for a day. The splenocytes (E: Effector cells) were co-cultured with target cells at E: T ratio of 100:1, and incubated for 6 h. After 6 h incubation, the cells were centrifuged at 250 × g for 5 min at 4 °C. The supernatants were

harvested to assess Granzyme B using ELISA kit (eBioscience).

2.11. Statistical analysis

The differences between the test and control groups were determined by one-way ANOVA (Prism 5.0, GraphPad Software). Each test was done in duplicate. The results were shown as mean (as column) ± standard deviation (SD as bar) for each group. The $p < 0.05$ was statistically considered significant (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). The studies were performed in two independent experiments.

3. Results

3.1. Uptake of proteins by DCs

The murine DCs were successfully provided from bone marrow precursors of BALB/c mice and harvested for use after 5-day culture in medium containing GM-CSF and IL-4 cytokines. The DCs were detected by surface expression of the DC associated marker CD11c, MHC II, and co-stimulatory molecules (CD86 and CD40) before pulsing with the recombinant proteins using flow cytometry. The expression levels of CD86, CD11c, CD40 and MHCII in immature DCs were about 55 %, 74 %, 40 % and 60 %, respectively. For preparation of the modified DCs, immature DCs were incubated with the recombinant Hsp27, Nef and Hsp27-Nef proteins at 37°C for 4 h, and were subsequently cultured for 48 h. The morphological change of immature DCs to mature DCs was confirmed by light microscopy indicating protein uptake. Moreover, the results of GFP uptake by DCs showed its presence at four hours after DC pulsing. Detection of green fluorescence in the cells was decreased after four hours, likely due to the protein entry to degradation and processing pathways [21]. After determination of a proper uptake time, DCs were pulsed with the recombinant Nef, Hsp27 and Hsp27-Nef proteins. Western blot analysis showed a clear band of ~ 27 kDa for the recombinant Hsp27 protein, but no band for the recombinant Nef and Hsp27-Nef proteins at 48 h after DC pulsing (Fig. 2). Its reason is likely due to the entrance of Nef and Hsp27-Nef proteins to DCs through the internalizing receptors and proteasomal activation (Adaptive immune system). In contrast, Hsp27 alone engages signaling receptors (Innate immune system) [13].

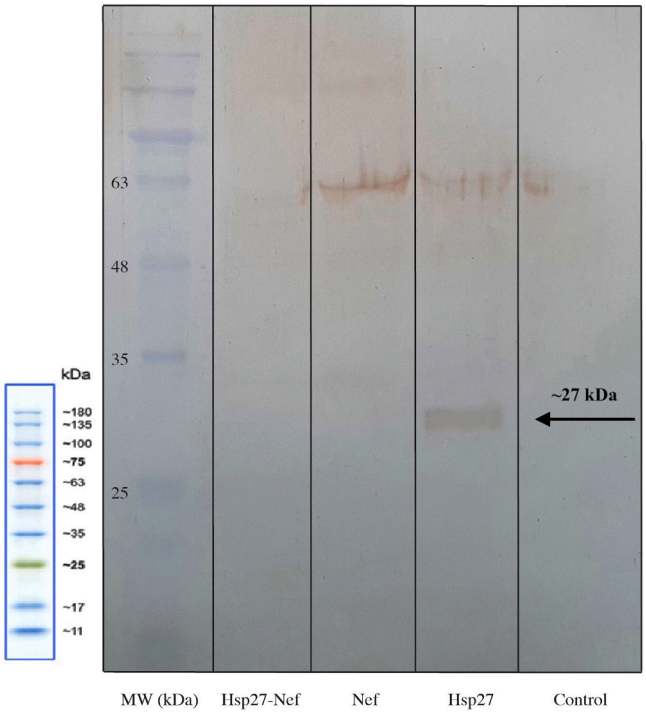

Fig. 2. Detection of protein uptake in DCs using western blotting: A clear band of ~ 27 kDa for Hsp27 protein was observed; but no band was detected for Nef and Hsp27-Nef proteins. MW is molecular weight marker (prestained protein ladder, 11–180 kDa, Fermentas).

3.2. Evaluation of antibody responses

The results of total IgG and other related isotypes showed that immunization of mice with heterologous DC prime/protein boost regimen (G9) could induce higher antibody responses than homologous DC prime/ DC boost regimen (G3) and heterologous DNA prime/DC boost regimen (G6, $p < 0.001$) against both Nef and Hsp27-Nef antigens **(**Fig. 3**).** Also, the levels of total IgG and other related isotypes didn't show any significant difference between homologous DC prime/ DC boost regimen (G3) and heterologous DNA prime/ DC boost regimen (G6, $p > 0.05$) against Hsp27-Nef antigen **(**Fig. 3**B).** On the other hand, groups receiving Hsp27-Nef antigen showed higher levels of total IgG and IgG2a than groups receiving Nef antigen ($p < 0.05$) against Nef antigen. These results confirmed the impact of Hsp27 in increasing Nef immunogenicity (Fig. 3**A**).

3.3. Cytokine secretion in splenocytes restimulated with antigens

The results of IL-5, IL-10 and IFN-γ secretion indicated that mice immunization with different modalities could efficiently increase the secretion of cytokines compared to controls ($p < 0.05$; Fig. 4). The results of IL-5 and IL-10 secretion showed that the lowest levels of IL-5 and IL-10 cytokines was observed in groups receiving DC $_{\text{Hsp27-Nef}}$ prime/ DC $_{\text{Hsp27-Nef}}$ boost (G3) and DNA $_{\text{Hsp27-Nef}}$ prime/DC $_{\text{Hsp27-Nef}}$ boost regimens (G6) against Nef antigen **(**Fig. 4**A).** Also, secretion of these cytokines in DC $_{\text{Hsp27-Nef}}$ prime/protein $_{\text{Hsp27-Nef}}$ boost regimen (G9) is significantly higher than DC $_{\text{Hsp27-Nef}}$ prime/ DC $_{\text{Hsp27-Nef}}$ boost (G3) and DNA $_{\text{Hsp27-Nef}}$ prime/DC $_{\text{Hsp27-Nef}}$ boost regimens (G6) against Hsp27-Nef antigen ($p < 0.001$, Fig. 4**B**). The secretion of IFN-γ in groups receiving Hsp27-Nef antigen with different modalities was significantly higher than groups receiving Nef antigen ($p < 0.05$). Indeed, Hsp27 as an adjuvant enhanced Nef-specific IFN-γ secretion. Moreover, secretion of IFN-γ in group receiving DC $_{\text{Hsp27-Nef}}$ prime/protein $_{\text{Hsp27-Nef}}$ boost regimen (G9) is significantly higher than that in groups receiving DC $_{\text{Hsp27-Nef}}$ prime/ DC $_{\text{Hsp27-Nef}}$ boost (G3) and DNA $_{\text{Hsp27-Nef}}$ prime/DC $_{\text{Hsp27-Nef}}$ boost regimens (G6; $p < 0.01$, Fig. 4). It should be mentioned that groups receiving Hsp27 showed no reaction against Nef antigen. Indeed, the levels of IL-5, IL-10 and IFN-γ secretion were similar to or less than those in control groups. Thus, we did not show these groups for further clarification of main groups.

3.4. Cytokine secretion in splenocytes exposed to virions

In the first approach, the splenocytes were exposed to SCR virions *in vitro*. The secretion of cytokines in splenocytes exposed to SCR virions (*i.e.,* infected splenocytes/ lymphocytes) showed that all immunized groups could significantly secrete IFN-γ and IL-10 cytokines in comparison with control groups ($p < 0.05$). Groups immunized with Hsp27-Nef antigen (G3, G6 and G9) showed significant difference in secretion of IFN-γ and IL-10 as compared to groups immunized with Nef antigen (G1, G4 and G7) in the same regimens ($p < 0.05$, Fig. 5**A**). Moreover, splenocytes of mice immunized with DC $_{\text{Hsp27-Nef}}$ prime/protein $_{\text{Hsp27-Nef}}$ boost regimen (G9) showed substantial difference in secretion of IFN-γ and IL-10 when exposed to SCR virions ($p < 0.001$). In contrast, the level of IL-5 secretion didn't show any significant difference between different groups ($p > 0.05$). On the other hand, the levels of IFN-γ, IL-10 and IL-5 secretion in splenocytes exposed to virions were lower than those in uninfected splenocytes ($p < 0.05$). However, the ratios of IFN-γ/IL-10 and IFN-γ/ IL-5 in infected splenocytes were significantly higher in test groups than in control groups **(**Fig. 5**A).**

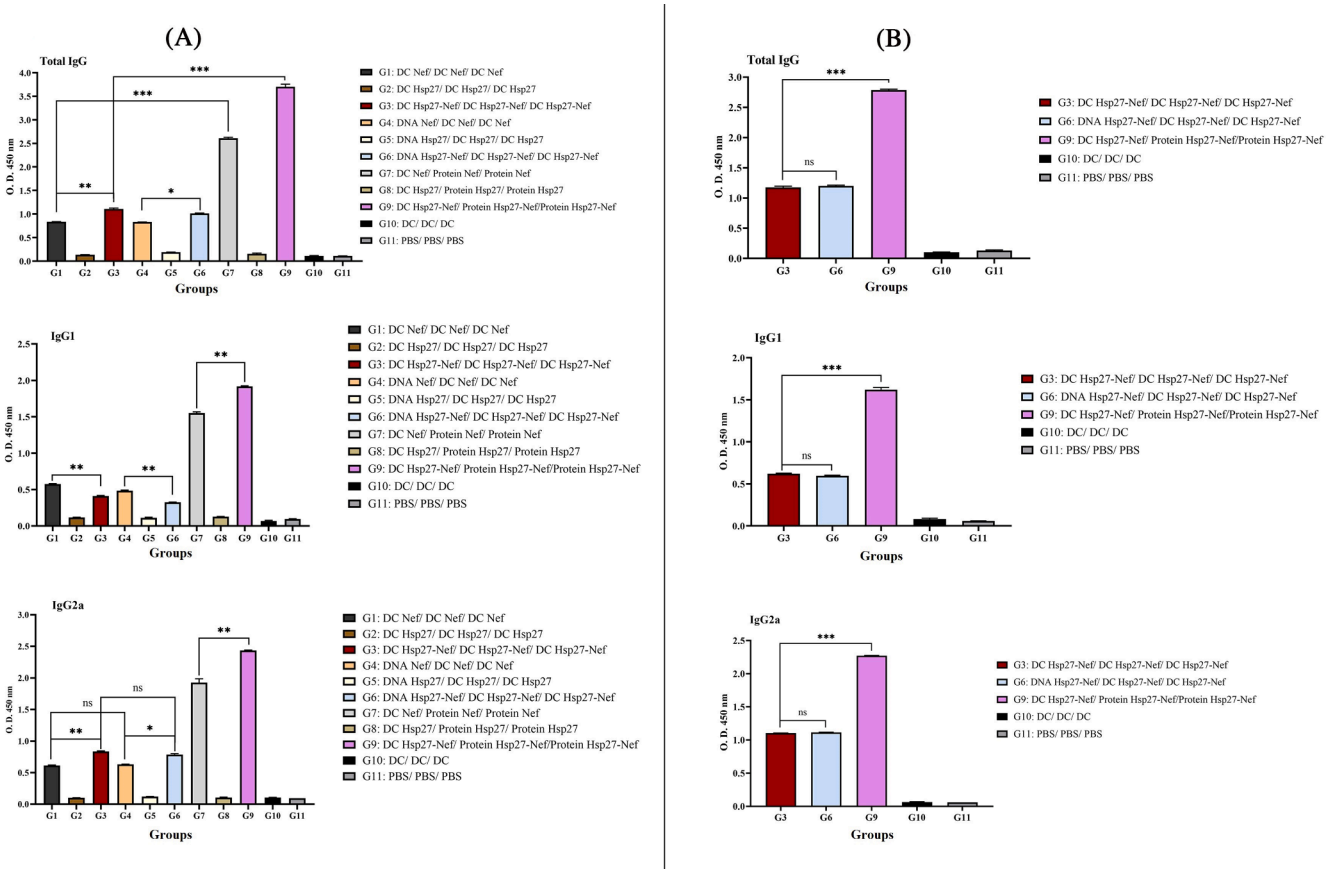

Fig. 3. Evaluation of antibody responses in different regimens: The analysis of total IgG and other related isotypes were performed using indirect ELISA against Nef antigen **(A),** and Hsp27-Nef antigen **(B)**. All analyses were performed in duplicate for each sample shown as mean absorbance at 450 nm ± SD (ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

In the second approach, the splenocytes of groups immunized with Hsp27-Nef antigen in different modalities (G3, G6 & G9) were exposed to virions and also restimulated with Hsp27-Nef antigen. The levels of IFN-γ and IL-10 cytokines in the infected splenocytes and restimulated with Hsp27-Nef antigen (*i.e.,* second approach) were higher than the infected splenocytes (*i.e.,* first approach; Fig. 5**B**). However, the secretion of IL-5 didn't show any significant difference between the infected and restimulated splenocytes of these groups (the second approach) similar to the infected splenocytes (the first approach, $p > 0.05$; Fig. 5**B**).

3.5. Granzyme B secretion

For *in vitro* CTL activity, the Granzyme B secretion was assessed using sandwich-based ELISA. Our data showed that the level of Granzyme B in group immunized with DC $_{Nef}$ prime/ protein $_{Nef}$ boost regimen was significantly higher than that in groups immunized with DC $_{Nef}$ prime/ DC $_{Nef}$ boost and DNA $_{Nef}$ prime/DC $_{Nef}$ boost regimens ($p < 0.05$; Fig. 6**A**). Moreover, the secretion of Granzyme B in groups immunized with Hsp27-Nef antigen in different modalities was higher than groups immunized with Nef antigen in the same modalities ($p < 0.05$; Fig. 6**A**). These results showed that Hsp27 could increase Nef-specific Granzyme B secretion. Additionally, groups immunized with DC prime/ DC boost regimens did not show any statistically significant differences with groups immunized with DNA prime/DC boost regimens in Granzyme B secretion with respect to Nef or Hsp27-Nef antigen ($p > 0.05$, Fig. 6). It should be mentioned that groups receiving Hsp27 showed no Granzyme B secretion against Nef antigen. Indeed, the levels of Granzyme B secretion were similar to or less than those in control groups. Thus, we did not show these groups for further clarification of main groups.

4. Discussion

In this study, we generated DCs modified with the main target antigen (HIV-1 Nef) alone and fused to a natural adjuvant (Hsp27). Three immunization strategies including homologous DC prime/ DC boost regimen, heterologous DNA prime/ DC boost regimen, and heterologous DC prime/ protein boost regimen were investigated in mice, and immunological responses were analyzed to determine the best immunization regimen. The role of Hsp27 was studied to increase Nef antigen-specific immune responses. For the first time, we analyzed antiviral effects of regimens after *in vitro* exposure of lymphocytes to virions through cytokine assay. Dendritic cells-based vaccines present the relevant antigens to immune system for induction of protection against disorders such as cancer, autoimmunity, and allergy [22]. Dendritic cells are only APCs that can activate unsensitized initial T cells [23]. Immature dendritic cells (imDCs) have strong antigen capture and processing capacity, and mature dendritic cells (mDCs) stimulate the initial T cells involved in immune response [23,24]. Based on the published reports, DCs could increase the efficiency of prophylactic HIV vaccine by generating pro-inflammatory cytokines, improving T cell responses, and recruiting effector cells to target tissues. Furthermore, DCs are targets for therapeutic HIV vaccines due to their ability to reverse latency, present antigen, and enhance T- and B-cell immunity. However, a major barrier of DC-based vaccines is the activation state of the DC. Moreover, another barrier is the selection of target antigens that will activate both $CD8^+$ and $CD4^+$ T cells in a potent, immune-specific manner [25,26].

Recently, various approaches of delivering tumor antigens into DCs *in vitro* and *in vivo* have been developed including microbial components loaded with tumor antigens, antigen transfer mediated by lentivirus vector, Hsp-antigen complex-mediated cross-presentation, and virus-

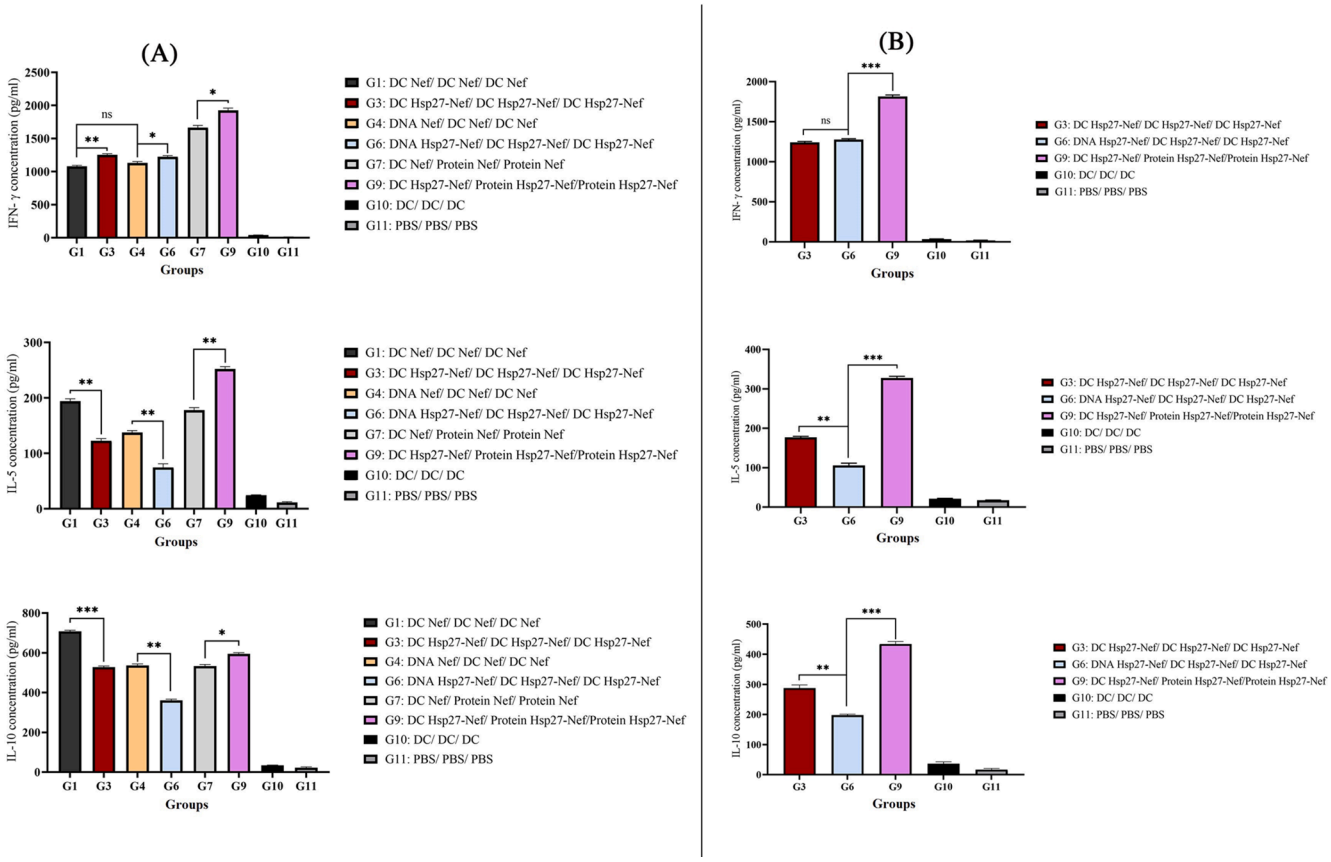

Fig. 4. Evaluation of cytokines secretion by the lymphocytes re-stimulated with Nef antigen **(A)** and Hsp27-Nef antigen **(B)** *in vitro* using sandwich-based ELISA: All analyses were performed in triplicate for each sample shown as mean absorbance at 450 nm ± SD (ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

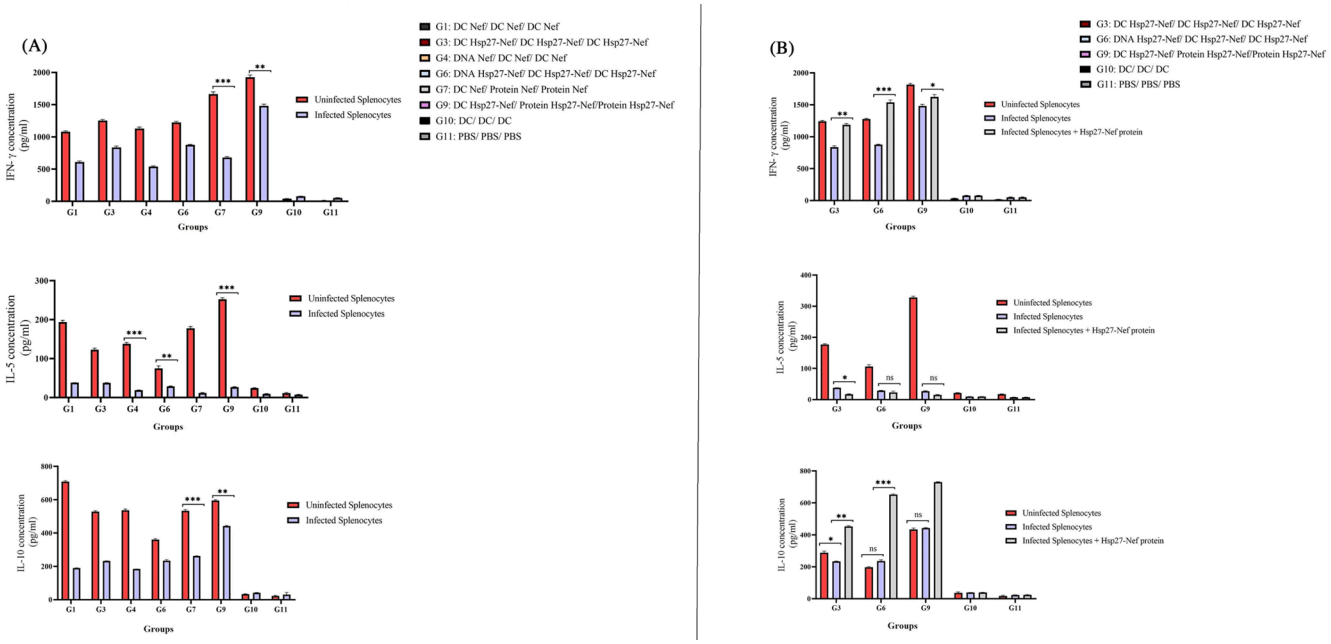

Fig. 5. Evaluation of cytokines secretion by SCR HIV-1-infected lymphocytes: **(A)** Secretion of cytokines by SCR HIV-1-infected lymphocytes without re-stimulation with antigens compared to uninfected lymphocytes re-stimulated with Nef antigen; **(B)** Secretion of cytokines by SCR HIV-1-infected lymphocytes without re-stimulation with antigens compared to uninfected lymphocytes re-stimulated with Hsp27-Nef antigen, and also SCR- infected lymphocytes re-stimulated with Hsp27-Nef antigen; All analyses were performed in duplicate for each sample shown as mean absorbance at 450 nm ± SD (ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

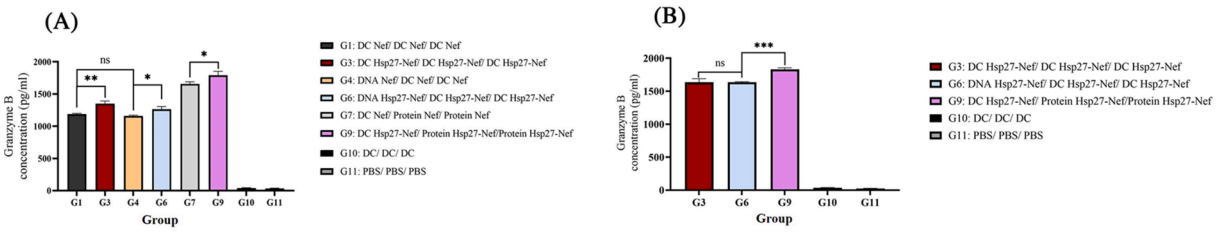

Fig. 6. Evaluation of Granzyme B secretion in the splenocytes re-stimulated with Nef antigen (A) and Hsp27-Nef antigen (B) using ELISA: All analyses were performed in triplicate for each sample shown as mean absorbance at 450 nm ± SD (ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

like particles (VLP) delivery system for proteins [27–29]. In this study, the immunogenicity of different DC platforms loaded with Nef and Hsp27-Nef proteins was investigated in BALB/c mice. Generally, mice immunization with DCs pulsed with Hsp27-Nef protein in homologous and heterologous prime-boost strategies induced both humoral and cellular immune responses. As known, adaptive immunity plays a protective role against an infectious agent that is mediated by B- and T-lymphocytes. For stimulation of adaptive immunity, Hsps bind to antigenic peptides and deliver them to the APCs for *T*-cell presentation through the MHC molecules. Indeed, immunization with Hsp-peptide complexes could elicit potent antitumor, antiviral and antiparasitic effects along with certain immune mechanisms [13,30]. The interaction of Hsp-peptide complexes with APCs (*i.e.,* macrophages or DCs) led to the presentation of antigenic peptides to $CD8^+$ and $CD4^+$ T lymphocytes. The cross-presentation of peptides bound to Hsps was also shown to be receptor-mediated and induce the secretion of different cytokines (*e.g.,* IL-2, IFN-γ, *etc.*). Moreover, Hsps could stimulate DC maturation through the upregulation of surface markers such as MHC-I and -II molecules, and CD86 and CD80 costimulatory molecules. On the other hand, the activation and maturation of DCs by Hsps not only stimulate the innate immune system but also activate effector natural killer (NK) cells [13,30].

Gene transfection using chemical reagents, electroporation or adenovirus was applied to assess the processing and presentation of antigens by DCs [31–33]. Moreover, protein uptake can be investigated by some methods such as immuno-electron microscopy, as well. In this study, for the first time, detection of protein uptake by DCs was performed using an easy and cost-effective method, western blot analysis, *in vitro*.

Our results showed a clear band of ~ 27 kDa for Hsp27 protein, but no band for Nef and Hsp27-Nef proteins after DC pulsing. It is likely due to binding of Nef and Hsp27-Nef proteins to the internalizing receptors of DCs leading to endocytosis pathway. The proteasome-processed peptides can be directly presented on the MHC-II or followed by cross-presentation on the MHC class I through Golgi and activate adaptive immune responses. But, Hsp27 similar to other Hsps engages signaling receptors leading to start signaling pathways and escape from degradation in lysosome and activate innate immune responses [13]. Additionally, the efficiency of Hsp27 as an adjuvant in DC-based immunization was evaluated to enhance HIV-1 Nef antigen-specific immunity in mice. We found significant difference between immunized groups with Hsp27-Nef and Nef in various modalities for stimulation of immune responses. Our data indicated that the levels of total IgG, IgG2a, IFN-γ and Granzyme B secretion in groups immunized with Hsp27-Nef were significantly higher than those in groups immunized with Nef. These results showed that Hsp27 as an adjuvant could improve HIV-1 Nef-specific B- and *T*-cell immune responses. These data confirmed our previous results about the effective role of Hsp27 as an adjuvant. Indeed, the Hsp27-Nef fusion protein could significantly elicit the Nef-specific immune responses as compared to the mixture of Nef protein + Hsp27 and Nef protein + Freund's adjuvant [10]. In other reports, Hsp molecules were utilized in DNA- or protein (peptide)-based vaccines, as antigens or adjuvants, against cancer and infectious diseases, as well [13].

On the other hand, the prime-boost regimens were shown to be an effective approach to induce both humoral and cellular immune responses [17]. Some studies indicated that heterologous prime-boost strategy is more effective than homologous prime-boost strategy [34]. Our results showed that immunization of mice with heterologous DC prime/protein boost regimens (G7 and G9) could significantly induce higher total IgG and other related isotypes, IFN-γ, and Granzyme B secretion than other regimens. The reports showed that heterologous HIV-1 MPER-V3 fusion peptide-loaded DC prime/ MPER-V3 fusion peptide boost regimen [35], and heterologous human papillomavirus (HPV) E7 + Hsp27 proteins-pulsed DC prime/ E7 + Hsp27 proteins boost regimen [36] induced higher levels of IgG2a, IgG2b, IFN-γ and Granzyme B than homologous DC prime/DC boost regimen [35,36].

Nchinda *et al.* demonstrated that mice vaccination with DC-targeted gag protein prime followed by gag DNA boost provides improved and long-lived $CD4^+$ and $CD8^+$ T cell immunity [37]. Also, Apostolico *et al.* indicated that DNA prime/ DC boost immunization strategy induced higher polyfunctional proliferative and cytokine-producing T cell responses than homologous DNA immunization or heterologous DC prime/ DNA boost in mice [38]. These data confirmed that heterologous prime-boost strategy is more effective than homologous prime-boost strategy in eliciting immune responses. Moreover, the order of immunization strategy in heterologous prime-boost strategy (*e.g.,* DNA prime/ DC boost regimen versus DC prime/DNA boost regimen) is important for induction of effective immunity.

Cell penetrating peptides (CPP) are small peptides which facilitate delivery of different cargos (*e.g.,* DNA) into the cell. Among CPPs, MPG was used to deliver plasmid DNA efficiently *in vivo* [39]. In this study, MPG peptide was utilized for delivery of pcDNA-Hsp27, pcDNA-Nef and pcDNA-Hsp27-Nef *in vivo* with the N: P ratio of 10: 1 based on our previous study [18]. The results of mice immunization showed that DC prime/ DC boost regimens (G1 and G3) did not have significant difference with DNA prime/ DC boost regimens (G4 and G6) in the secretion of IFN-γ, total IgG and its isotypes, and Granzyme B. However, the levels of IL-5 and IL-10 cytokines were significantly lower in DNA prime/ DC boost regimens than those in DC prime/ DC boost regimens. Thus, priming with the DNA/MPG nanoparticles was effective in stimulation of immune responses.

In the present study, the potency of mice lymphocytes exposed to single-cycle replicable (SCR) virions was assessed to secret cytokines *in vitro*. SCR virions are the replication-competent safe viruses which can be used for vaccine development studies [20]. For this purpose, we considered two approaches. In the first approach, splenocytes of immunized mice were infected with SCR virions for 4 h. After that, the production of IFN-γ, IL-5 and IL-10 cytokines was assessed after 72 h. Our results showed that all immunized groups could secrete cytokines in comparison with controls. Groups receiving Hsp27-Nef (G3, G6 and G9) showed significant difference in secretion of IFN-γ and IL-10 than groups receiving Nef (G1, G4 and G7). Despite the level of IL-5 secretion that didn't show any significant difference between groups, the splenocytes of immunized mice with Hsp27-Nef in DC prime/protein boost regimens showed substantial difference in secretion of IFN-γ and IL-10 when

exposed to SCR virions. In the second approach, splenocytes of groups receiving Hsp27-Nef (G3, G6 and G9) were infected with SCR virions for 4 h. After that, the splenocytes were re-stimulated with the recombinant Hsp27-Nef protein. Our data indicated that the levels of IFN-γ and IL-10 secretion from the infected splenocytes (/lymphocytes) were higher than those from the infected splenocytes just exposed to SCR virions (first approach).

Some studies showed impairment in the production of cytokines in HIV-infected individuals [40–42]. For instance, cytokine production was evaluated in the supernatant of *in vitro* activated PBMC from HIV-infected patients. The data showed that production of IL-2, IFN-γ, IL-4, IL-5, and IL-10 cytokines were significantly decreased in patients. This result is possibly due to the generalized impaired response of T cells from HIV-infected individuals to activation signals *in vitro* [42]. Thus, finding an effective vaccine platform is important to maintain *T*-cell activity and the levels of cytokines in patients. Although in our study, the infected lymphocytes showed lower secretion of IFN-γ, IL-10 and an impairment in IL-5 secretion, but re-stimulation of infected lymphocytes with antigen was increased IFN-γ and IL-10 secretion again. Moreover, the ratios of IFN-γ to IL-5, and IFN-γ to IL-10 were high in infected lymphocytes with or without re-stimulation with antigen similar to uninfected lymphocytes re-stimulated with antigen. Indeed, direction of immune responses was toward Th1 response. These results demonstrated the potency of mice immunization with different modalities especially DC $_{\text{Hsp27-Nef}}$ prime/ protein $_{\text{Hsp27-Nef}}$ boost regimen in virus exposure. The DC $_{\text{Hsp27-Nef}}$ prime/ protein $_{\text{Hsp27-Nef}}$ boost regimen could activate *T*-cells and maintain their activity against viral infection *in vitro*.

In conclusion, DCs pulsed with Nef and Hsp27-Nef proteins in heterologous DC prime/ protein boost regimens could significantly enhance the Nef-specific B- and *T*-cell responses as compared to other regimens (*i.e.,* heterologous DNA prime/ DC boost, and homologous DC prime/ DC boost regimens). Also, group receiving DC $_{\text{Hsp27-Nef}}$ prime/ protein $_{\text{Hsp27-Nef}}$ boost regimen showed significant immune responses as compared to group receiving DC $_{\text{Nef}}$ prime/ protein $_{\text{Nef}}$ regimen. Indeed, the highest secretion of total IgG, IFN-γ and Granzyme B was observed in group immunized with DC $_{\text{Hsp27-Nef}}$ prime/ protein $_{\text{Hsp27-Nef}}$ boost regimen. Moreover, the highest levels of IFN-γ and IL-10 secretion were detected in the infected lymphocytes isolated from mice immunized with DC $_{\text{Hsp27-Nef}}$ prime/ protein $_{\text{Hsp27-Nef}}$ boost regimen. Generally, our data showed the potency of heterologous DC prime/ protein boost strategy and the use of Hsp27 as an adjuvant in stimulation of effective immune responses further toward Th1 response and CTL activity. Thus, this modality can be used to develop a therapeutic HIV-1 vaccine candidate. Furthermore, this study helps improving DC-based vaccines as well as conferring an *in vitro* method for evaluation of antiviral effects. However, it is required conducting other *in vitro* approaches (*e.g.,* co-culture of infected DCs with lymphocytes), and also conferring effective protection in HIV-1-infected mouse model and large animal models (*e.g.,* macaque) in near future. It is useful for evaluating the stability of antibody secretion in longer times (*e.g.,* 3 or 6 months after the last immunization), as well.

CRediT authorship contribution statement

Alireza Milani: Investigation, Project administration, Formal analysis, Writing – original draft, Writing – review & editing. **Elnaz Agi:** Investigation, Writing – review & editing. **Mohammad Hassan Pouriayevali:** Investigation, Formal analysis, Writing – review & editing. **Mahdieh Motamedi-Rad:** Investigation, Writing – review & editing. **Fatemeh Motevalli:** Investigation, Writing – review & editing. **Azam Bolhassani:** Conceptualization, Validation, Investigation, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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