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Small heat shock protein 27: An effective adjuvant for enhancement of HIV-1 Nef antigen-specific immunity

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ABSTRACT

Novel vaccine modalities have been designed to improve the efficiency of vaccines against HIV infections. In this way, the HIV-1 Nef protein has been known as an attractive antigenic candidate in therapeutic vaccine development. Moreover, the endogenous adjuvants such as heat shock proteins (HSPs) and high mobility group box 1 protein (HMGB1) have been suggested effectively to induce antigen-specific humoral and cellular immune responses. In this study, different Nef DNA and protein constructs were produced in eukaryotic and prokaryotic expression systems, and their immunostimulatory properties were evaluated using small heat shock protein 27 (Hsp27) and the HMGB1-derived peptide (Hp91) in a mouse model. Generally, our results indicated that the Hsp27-Nef fusion DNA or protein could significantly elicit higher humoral and cellular immune responses than Nef DNA or protein, respectively. Analysis of the immune responses demonstrated that the Hsp27-Nef fusion protein, and also the mixture of Nef and Hp91 significantly enhanced the Nef-specific T cell responses. Indeed, these regimens induced high levels of IgG2a and IFN-γ directed toward Th1 responses and also Granzyme B secretion as compared to other immunization strategies. The immunostimulatory properties of Freund's adjuvant were significantly less than Hsp27 and Hp91 peptide in various immunization strategies. These findings showed that the use of Hsp27 and Hp91 in protein strategy could improve HIV-1 Nef-specific B- and T-cell immune responses, and also represent a promising HIV-1 vaccine candidate in future.

1. Introduction

Acquired immune deficiency syndrome (AIDS) caused by HIV infection was known as the most destructive disorders in history of human health [1]. A gradual loss of $CD4^+$ T cells, dysfunction of $CD8^+$ T cells and also a decreased number of natural killer cells (NK-cells) are some impairments observed in HIV-infected patients [2,3]. Therefore, it is critical to develop novel vaccine strategies eliciting both humoral and cellular immune responses against HIV infection. The HIV-1 genome contains nine genes encoding three main proteins (Gag, Pol, Env), two regulatory proteins (Tat, Rev), and four accessory proteins (Nef, Vpr, Vpu, Vif) [4,5]. Among different HIV-1 proteins, Nef is an early-expressed protein (~27–30 kDa) which plays an important role in the down-regulation of CD4 and MHC class I as a virulence factor for AIDS pathogenesis. Thus, Nef protein can be considered as a possible attractive target in therapeutic HIV vaccine development [6,7]. The most important limitation of DNA- and protein-based vaccines is their low immunogenicity. One approach to improve their immunogenicity is co-delivery of antigen with molecular adjuvants such as danger-associated molecular patterns (DAMPs). For instance, high mobility group box 1 (HMGB1) protein is a highly conserved protein which plays a critical role in enhancing antigen-specific immune responses through interaction with toll-like receptors 2 and 4 [8,9]. Moreover, an HMGB1-derived peptide, Hp91 could induce potent immunity against viral infections as an endogenous adjuvant in preclinical studies [10]. On the other hand, heat shock proteins (HSPs) are conserved molecular chaperons that participate in stimulating antigen-specific immunity to viral *pathogens* as a promising vaccine adjuvant [11]. Among HSPs, small heat shock proteins are *low molecular weight* chaperones (MW: 12–43 kDa) which possess similar properties including a conserved α-crystallin domain, tendency to form large homo- or hetero-oligomers, and chaperone activity [12,13]. The studies showed that small heat shock protein 27 (Hsp27) interacts with toll-like receptors 2–4, and induces the activation of NF-κB and subsequently the release of pro-inflammatory cytokines such as IL-6 [13–16]. Except the use of adjuvants, heterologous prime-boost vaccination strategy is another way to develop both humoral and cellular immunity of DNA vaccines against a specific antigen [17,18]. With regard to the importance of the

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endogenous adjuvants and the heterologous prime/boost immunization strategy, we evaluated the efficiency of Hsp27 and Hp91 as a vaccine adjuvant to enhance HIV-1 Nef antigen-specific immunity in both DNA- and protein-based immunization strategies.

2. Methods

2.1. Construction of the recombinant eukaryotic expression vectors

2.1.1. In vitro study

At first, the full length of Mus musculus Hsp27 gene (Accession No: NM_013560) was synthesized in a prokaryotic expression vector (pQE30-Hsp27, Biomatik Co., Canada). Then, for generation of pEGFP-Hsp27, the Hsp27 fragment was subcloned into the pEGFP-N3 eukaryotic expression vector in *Nhe*I/*Sal*I cloning sites. Also, HIV-1 Nef sequence (from HIV-1 vector pNL4-3, Accession No: AF324493.2) was subcloned from pUC19-Nef into the pEGFP-N1 in *Nhe*I/*Pst*I sites. To make pEGFP-Hsp27-Nef, both pUC19-Nef and pQE30-Hsp27 vectors were digested by *Bam*HI/*Pst*I and *Bgl*II/*Pst*I enzymes, respectively. Then, the Nef fragment was ligated to the linearized pQE30-Hsp27 using T4 DNA ligase. Finally, the Hsp27-Nef fusion was subcloned from pQE-Hsp27-Nef into the pEGFP-N1 using *Nhe*I/*Pst*I restriction enzymes.

2.1.2. In vivo study

For generation of pcDNA-Hsp27, the Hsp27 fragment was subcloned from pQE30-Hsp27 into the pcDNA3.1 (−) using *Nhe*I/*Afl*II (or *Bspt*I) restriction enzymes. In parallel, to generate pcDNA-Nef-Hsp27, the Hsp27 fragment was digested from pQE30-Hsp27 with *Bam*HI/*Afl*II enzymes and ligated to the linearized pcDNA3.1-Nef [19] using T4 DNA ligase. Finally, all DNA constructs containing Hsp27, Nef and Nef-Hsp27 fusion were purified by an Endo-free plasmid Mega kit (Qiagen). The concentration and purity of DNA constructs were determined by NanoDrop spectrophotometer.

2.2. Construction of the recombinant prokaryotic expression vectors

The pET-23a and pQE-30 bacterial vectors were used to express *histidine-tagged recombinant proteins.* To generate pET-Hsp27, the Hsp27 fragment was subcloned from pQE30-Hsp27 into the pET-23a in *Nhe*I/*Sal*I cloning site. Moreover, to construct the expression vector harboring HIV-1 Nef and Hsp27-Nef fusion, the Nef DNA was amplified by PCR from pUC19-Nef using primers designed to generate *Eco*RI and *Sal*I restriction sites at the 5′ and 3′ ends of the amplified fragments, respectively.

Forward primer: 5′-CGGAATTCATGGGTGGCSSGTGGTC-3′ (*Eco*RI)

Reverse primer: 5′-GGATTCGTCGACGCAGTTCTTGAAGTAC-3′ (*Sal*I)

The amplified Nef DNA was then cloned into the unique *Eco*RI/*Sal*I cloning sites of the pET-23a, pET-Hsp27, and pQE-30, individually. The generation of pQE-Hsp27-Nef was also described in Section 2.1.1.

2.3. Expression of the recombinant proteins

2.3.1. Protein expression in a mammalian cell line

For *in vitro* protein expression, human embryonic kidney 293T cells (HEK-293T, Pasteur Institute of Iran) were grown in complete DMEM and then transfected with pEGFP-Hsp27, pEGFP-Nef, pEGFP-Hsp27-Nef, and pEGFP-N1 as a positive control using TurboFect reagent. After 48 h, the transfection efficiency was evaluated by fluorescent microscopy and flow cytometry.

2.3.2. Protein expression in bacterial expression systems

The *E. coli* BL21 and Rosetta strains were transformed with the recombinant pET-Nef, pET-Hsp27 and pET-Hsp27-Nef plasmids. In addition, the *E. coli* M15 strain was transformed with the recombinant pQE-Hsp27, pQE-Nef and pQE-Hsp27-Nef vectors. The transformants were selected on Luria-Bertani (LB) agar plate and grown to an optical density of 0.7-0.8 at 600 nm in Ty2 x medium. Induction of protein expression with *1 mM* IPTG was optimized at different times of incubation (*i.e.,* 2, 3, 4 and 16 h) and temperature scales (*i.e.,* 25° C and 37 °C) after induction. The cell pellets was harvested and analyzed by 12% SDS-PAGE and western blotting.

2.4. Protein purification and assessment

The recombinant proteins were purified by affinity chromatography using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column under native conditions (*i.e.,* 300 mM imidazole buffer, pH = 8) for Hsp27 protein and/or denaturing conditions (*i.e.,* 8 M urea buffer and pH = 4.5) for Nef and Hsp27-Nef fusion proteins according to the manufacturer's instructions (Qiagen). Then, the purified proteins were dialyzed against PBS1X. The endotoxin contamination was less than 0.5 EU/mg protein as monitored by LAL assay. Finally, their concentrations were measured by Bradford protein assay kit and NanoDrop spectrophotometry, and stored at −70 °C, until used.

2.5. Vaccination procedure

Six to eight week old female BALB/c mice (n = 4 per group) were purchased from the breeding stock maintained at Pasteur Institute of Iran. Sixteen groups were selected and immunized subcutaneously at

Table 1
Different immunization strategies using HIV-1 Nef antigen.

Group	Modality	First injection	Second injection	Third injection
G1	DNA/DNA/DNA	pcDNA-Nef	pcDNA-Nef	pcDNA-Nef
G2	DNA/DNA/DNA	pcDNA-Hsp27	pcDNA-Hsp27	pcDNA-Hsp27
G3	DNA/DNA/DNA	pcDNA-Nef + pcDNA-Hsp27	pcDNA-Nef + pcDNA-Hsp27	pcDNA-Nef + pcDNA-Hsp27
G4	DNA/DNA/DNA	pcDNA-Nef-Hsp27	pcDNA-Nef-Hsp27	pcDNA-Nef-Hsp27
G5	Protein/Protein/Protein	[a]rNef + complete freund's adjuvant (CFA)	rNef + incomplete freund's adjuvant (IFA)	rNef + incomplete freund's adjuvant (IFA)
G6	Protein/Protein/Protein	rHsp27	rHsp27	rHsp27
G7	Protein/Protein/Protein	rNef + rHsp27	rNef + rHsp27	rNef + rHsp27
G8	Protein/Protein/Protein	rHsp27-Nef	rHsp27-Nef	rHsp27-Nef
G9	DNA/Protein/Protein	pcDNA-Nef	rNef+ IFA	rNef+ IFA
G10	DNA/Protein/Protein	pcDNA-Hsp27	rHsp27	rHsp27
G11	DNA/Protein/Protein	pcDNA-Nef + pcDNA-Hsp27	rNef + rHsp27	rNef + rHsp27
G12	DNA/Protein/Protein	pcDNA-Nef-Hsp27	rHsp27-Nef	rHsp27-Nef
G13	Protein/Protein/Protein	rNef + Hp91	rNef + Hp91	rNef + Hp91
G14	control	PBS	PBS	PBS
G15	Control	CFA	IFA	IFA
G16	Empty vector (control)	pcDNA3.1	pcDNA3.1	pcDNA3.1

[a] r: recombinant.

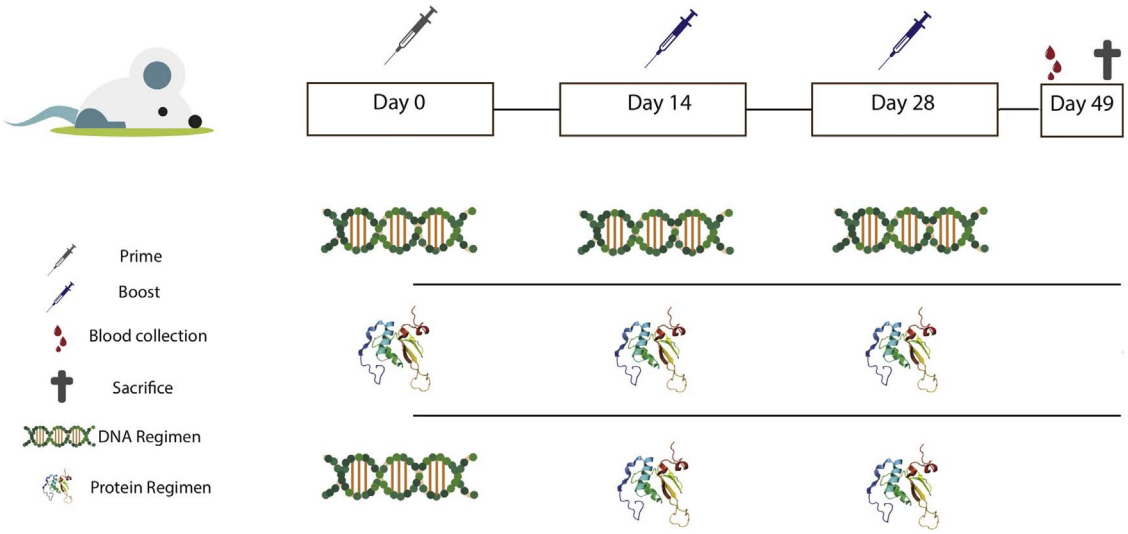

Fig. 1. Schematic representation of different immunization modalities such as DNA prime/DNA boost, protein prime/protein boost, and DNA prime/protein boost strategies.

the footpad with three different modalities including DNA/DNA, protein/protein, and DNA/protein strategies (Table 1). Mice were immunized on days 0, 14, and 28 with 50 μg of the DNA constructs and 10 μg of the recombinant proteins in PBS1X. The complete (CFA) and incomplete (IFA) Freund's adjuvants along with rNef antigen were used at ratio of 50:50 *v/v* for comparison with Hsp27 and Hp91 adjuvants. The immunization program was shown in Fig. 1.

2.6. Evaluation of antibody levels

Three weeks after third injection, pooled sera were prepared from the whole blood samples of each group (n = 4). The levels of Nef-specific antibodies (total IgG, IgG1, IgG2a, IgG2b; Southern biotechnology Association) in the sera were determined using indirect ELISA as previously described [20]. The coated antigens were the recombinant Nef and Hsp27-Nef fusion proteins (~10 μg/ ml) diluted in PBS1X.

2.7. In vitro cytokine secretion

Three weeks after the last immunization, two mice from each group (n = 2 per group) were sacrificed and the spleens were removed. The red blood cell depleted pooled and/or single splenocytes (2×10^6 cells/ml) were cultured in U-bottomed, 96-well plates for 72 h in the presence of 10 μg/ml of rNef or rHsp27-Nef, RPMI 5% (negative control), and 5 μg/ml of concanavalin A (ConA, positive control) in complete culture medium. The rest of the splenocytes were simultaneously used to assess Granzyme B release as the next section. The presence of IFN-γ and IL-4 in supernatants was measured using a DuoSet sandwich-based ELISA system (R & D) according to the manufacturer's instructions. The detection limit was 2 pg/ ml for IFN-γ and 7 pg/ ml for IL-4.

2.8. Granzyme B (GrB) ELISA assay

P815 target cells (T) were seeded in triplicate into U-bottomed, 96-well plates (2×10^4 cells/well) incubated with Nef antigen (~30 μg/ ml) for 24 h. The splenocytes (Effector cells: E) previously provided in Section 2.7 were counted using trypan blue and added to the target cells at E: T ratio of 100:1 in which maximal release of Granzyme B was observed. The target and effector cells were co-cultured in complete RPMI-1640 supplemented with 10% heat-inactivated FCS at 37 °C and 5% CO_2 under humidified conditions. The wells containing effector cells were considered for measurement of possible spontaneous release of Granzyme B. After 6 h incubation, microplates were centrifuged at 250*g* for 5 min at 4° C and the supernatants were harvested. The concentration of Granzyme B in these samples was measured by ELISA (eBioscience) according to the manufacturer's instruction.

2.9. Statistical analysis

The differences between the control and test groups were assessed using one-way ANOVA and also Student's *t*-test (Graph-pad Prism, GraphPad Software, USA). A p-value < 0.05 was statistically considered significant.

3. Results

3.1. Confirmation of the DNA constructs

The Hsp27, HIV-1 Nef and Hsp27-Nef genes were correctly cloned in eukaryotic and prokaryotic expression vectors (**see Supplementary data**). The presence of Hsp27, HIV-1 Nef and Hsp27-Nef genes was confirmed using digestion as a clear band of ~720 bp, ~648 bp, and ~1368 bp migrated in agarose gel, respectively, and also sequencing. The recombinant endotoxin-free plasmids (*i.e.,* pcDNA-Hsp27, pcDNA-Nef-Hsp27 and pcDNA-Nef) had a concentration range between 1.8 and 2.3 mg/ml. The 260: 280 UV absorption ratios ranged from 1.8 to 2.0.

3.2. Expression and purification of the recombinant proteins

At first, the expression of Hsp27, Nef and Hsp27-Nef proteins was confirmed by flow cytometry using GFP reporter marker in HEK–293 T cells. The percentage of Hsp27-GFP, Nef-GFP and Hsp27-Nef-GFP expression was 72.19 ± 0.60, 47.66 ± 3.58, 63.76 ± 1.49 respectively using TurboFect system in two independent experiments. The percentage of GFP-positive cells was 89.13 ± 0.83 after transfection with pEGFP-N1 as a positive control. As observed, Hsp27 could significantly increase HIV-1 Nef DNA delivery in the cells as compared to Nef DNA alone ($p < 0.05$). On the other hand, the expression of Hsp27, Nef and Hsp27-Nef proteins was evaluated in two bacterial systems including pQE-30/M15, and pET-23a/Rosetta or pET-23a/BL21 systems. Our data showed that all recombinant proteins were generated at 37° C in pET expression system. In contrast, no protein expression was observed in pQE30/M15 system. Moreover, the recombinant HIV-1 Nef protein could be expressed in both *E. coli* BL21 and Rosetta strains at 16 h after induction. In contrast, the recombinant Hsp27 and Hsp27-Nef proteins were expressed in the Rosetta strain at 4 h after induction. The

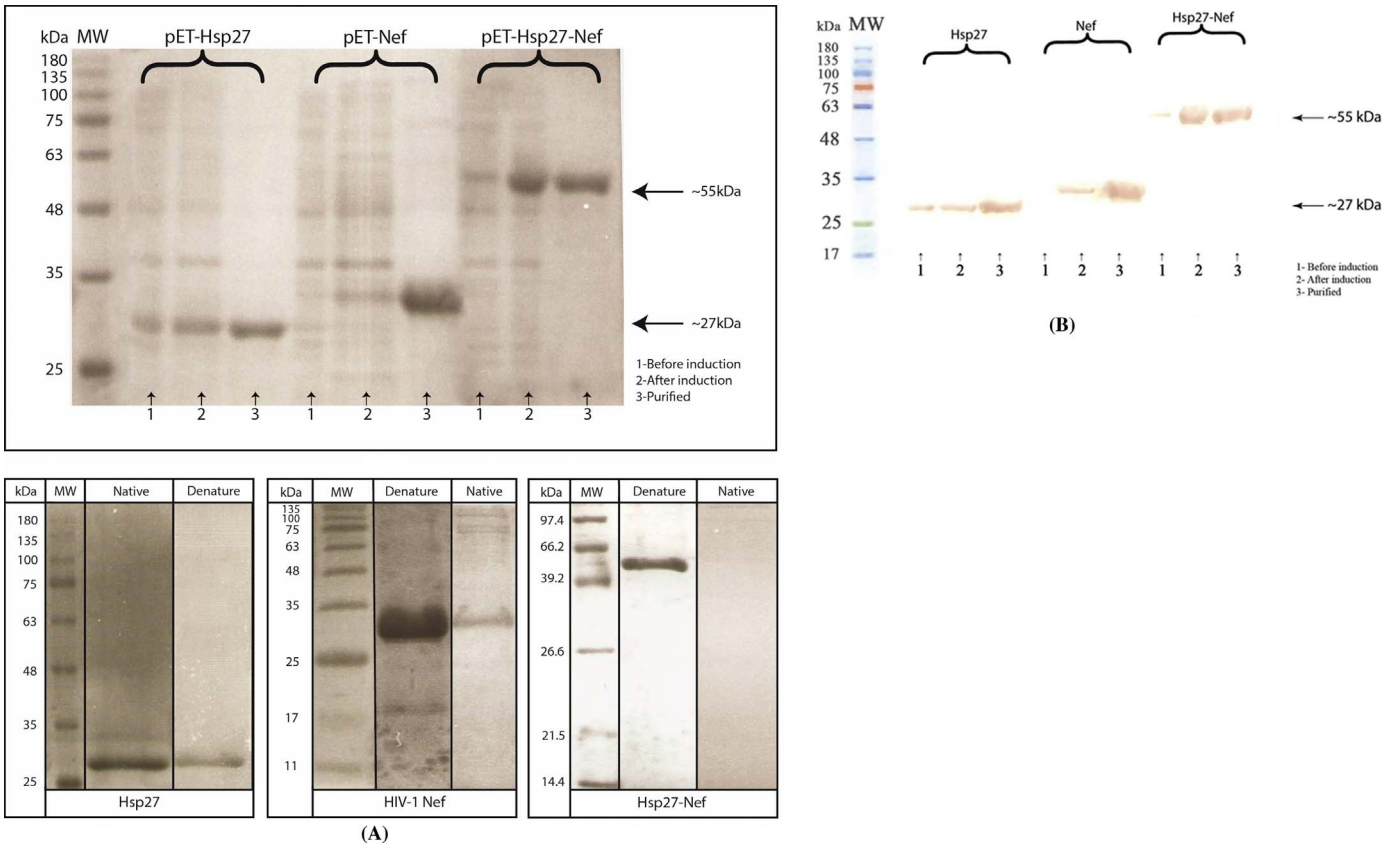

Fig. 2. A) Expression and purification of Hsp27, Nef and Hsp27-Nef proteins in *E. coli* expression system. The protein purification was performed by affinity chromatography under native and denaturing conditions; B) Identification of the recombinant Hsp27, Nef and Hsp27-Nef proteins by western blot analysis using anti-His, and anti-Nef antibodies. MW is molecular weight marker (prestained protein ladder, 10–170 kDa, Fermentas).

results indicated that Hsp27 can be successfully purified under native conditions, but Nef and Hsp27-Nef proteins showed higher purification under denaturing conditions suggesting the structural changes of Hsp27 fused to Nef protein. The purified Nef, Hsp27 and Hsp27-Nef proteins migrated as clear bands of ~30 kDa [21], ~27 kDa and ~53 kDa in SDS-PAGE, respectively as indicated in Fig. 2**A**. Furthermore, the recombinant Hsp27, Nef and Hsp27-Nef proteins were detectable using anti-His and anti-Nef antibodies in western blotting (Fig. 2**B**). The recombinant proteins had a concentration range between 0.7 and 0.9 mg/ml.

3.3. Evaluation of antibody responses

To compare antibody responses elicited in different groups, the serum levels of total IgG and the related subclasses against the recombinant (r) Nef and rHsp27-Nef proteins were detected using indirect ELISA (Fig. 3**)**. Our data showed that the levels of total IgG and all isotypes in the sera of mice immunized with rNef protein (G5, G7 & G8) in different formulations were significantly higher than those in the groups immunized with DNA constructs harboring Nef gene (G1, G3 & G4) and DNA prime/protein boost (G9, G11 & G12), respectively ($p < 0.05$, Fig. 3). Moreover, the levels of total IgG and IgG2a in the sera of mice immunized with Hsp27-Nef fusion protein (G8) were significantly higher than groups immunized with HIV-1 Nef mixed with Freund's adjuvant (G5) and/or Hsp27 (G7) as shown in Fig. 3**A and E**. On the other hand, Hp91 could elicit more effective Nef-specific total IgG and IgG1 responses than Hsp27 in mouse model ($p < 0.05$, Fig. 3**A and C)**. In contrast, Nef-specific IgG2a and IgG2b responses were similar in groups immunized with Hsp27-Nef protein and Hp91 mixed with Nef ($p > 0.05$, Fig. 3**E and G)**. In addition, the levels of Hsp27-Nef fusion-specific total IgG and IgG2a in the group immunized with Nef-Hsp27 DNA/Hsp27-Nef protein were importantly higher than those in the group immunized with Hsp27-Nef protein/protein ($p < 0.05$, Fig. 3**B and F)**. In contrast, the levels of Hsp27-Nef fusion-specific IgG1 and IgG2b were higher in the group receiving Hsp27-Nef protein/protein compared to the group receiving Nef-Hsp27 DNA/Hsp27-Nef protein ($p < 0.05$, Fig. 3**D and H).** The highest level of IgG2a was detected in the sera of mice immunized with Nef protein + Hp91 peptide regimen (G13, $p < 0.05$, Fig. 3**E**). However, there is no significant difference in the level of IgG1 between groups immunized with Hsp27 + Nef (G7) and Hsp27-Nef (G8) in protein regimens ($p > 0.05$, Fig. 3**C**). **Supplementary 3** also shows the determination of serum dilution (1:50) and antibody dilution (1:10000) in a test group (G8). As observed the antibody detection in serially diluted sera revealed that the IgG1 levels against Nef coated antigen start to reduce from 1:500, although the IgG2a level decreased from 1:200 serum dilution; thus, we considered 1:50 dilution in all experiments. In addition, the ratio of IgG1, IgG2a, IgG2b and total IgG was set up in experiments. We used 1:1000, 1:2000, 1: 5000, 1:10000, 1:15000 and 1:20000 dilutions for all antibodies. The OD_{492} was reduced between 1:15000 and 1:20000; thus, we selected 1:10000 for all antibodies.

3.4. Cytokine assay

The cytokine results for the pooled splenocytes of two mice in each group showed that the group immunized with Nef-Hsp27 DNA (G4) is more effective than other DNA immunizations (G1-G3) in eliciting IFN-γ responses ($p < 0.05$, Fig. 4**A**). Moreover, both protein/protein and DNA/protein-based immunizations with Nef-Hsp27 constructs (G8 and G12) could increase the secretion of IFN-γ ($p > 0.05$) as compared to other groups immunized with different regimens ($p < 0.05$, Fig. 4**A**). Moreover, the level of Hsp27-Nef-specific IFN-γ secretion in the group

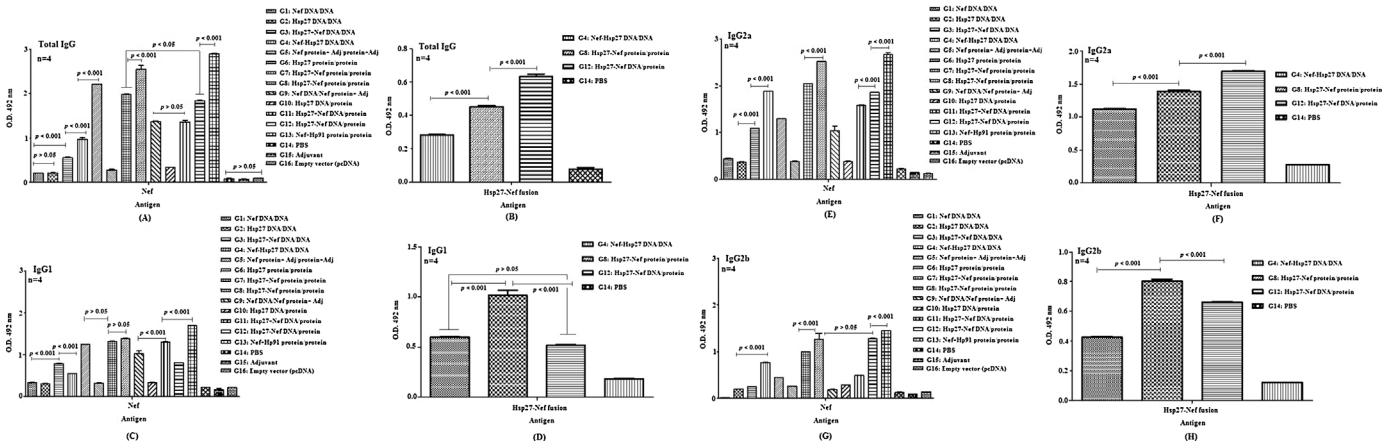

Fig. 3. Evaluation of antibody responses (total IgG, IgG1, IgG2a and IgG2b; 1:10000 dilutions) against Nef and Hsp27-Nef antigens in different regimens: Mice sera were prepared from the whole blood samples of each group (n = 4) three weeks after the last immunization. All analyses were performed in duplicate for each sample. The results from the 1:50 sera dilutions are shown as mean absorbance at 492 nm ± SD. The results were shown for groups from left (G1) to right (G16).

injected with Nef-Hsp27 DNA prime/protein boost was similar to that in the group immunized with Hsp27-Nef protein regimen ($p > 0.05$) and higher than that in the group immunized with Hsp27-Nef DNA regimen ($p < 0.05$, Fig. 4**B**). The level of IFN-γ was significantly lower in mice immunized with Nef + Freund's adjuvant in both protein/protein and DNA/protein modalities compared to the group injected with Hsp27-Nef fusion in the same regimens ($p < 0.05$, Fig. 4**A**). On the other hand, mice immunization with Nef protein + Hp91 showed significant differences in IFN-γ secretion with other groups ($p < 0.05$). All mice immunization with different modalities effectively enhanced the levels of IFN-γ compared to control groups ($p < 0.05$, Fig. 4**A**). It should be noted that the results obtained from the pooled splenocytes of two mice were similar to those from each mouse in one group **(supplementary data 4).** The test groups showed no significant IL-4 response in comparison with control groups ($p > 0.05$). Indeed, the levels of IL-4 were ~15–20 pg/ml for all groups except for the group immunized with Nef protein + Hp91 adjuvant that could produce approximately 65 pg/ml of IL4 compared to other groups ($p < 0.05$).

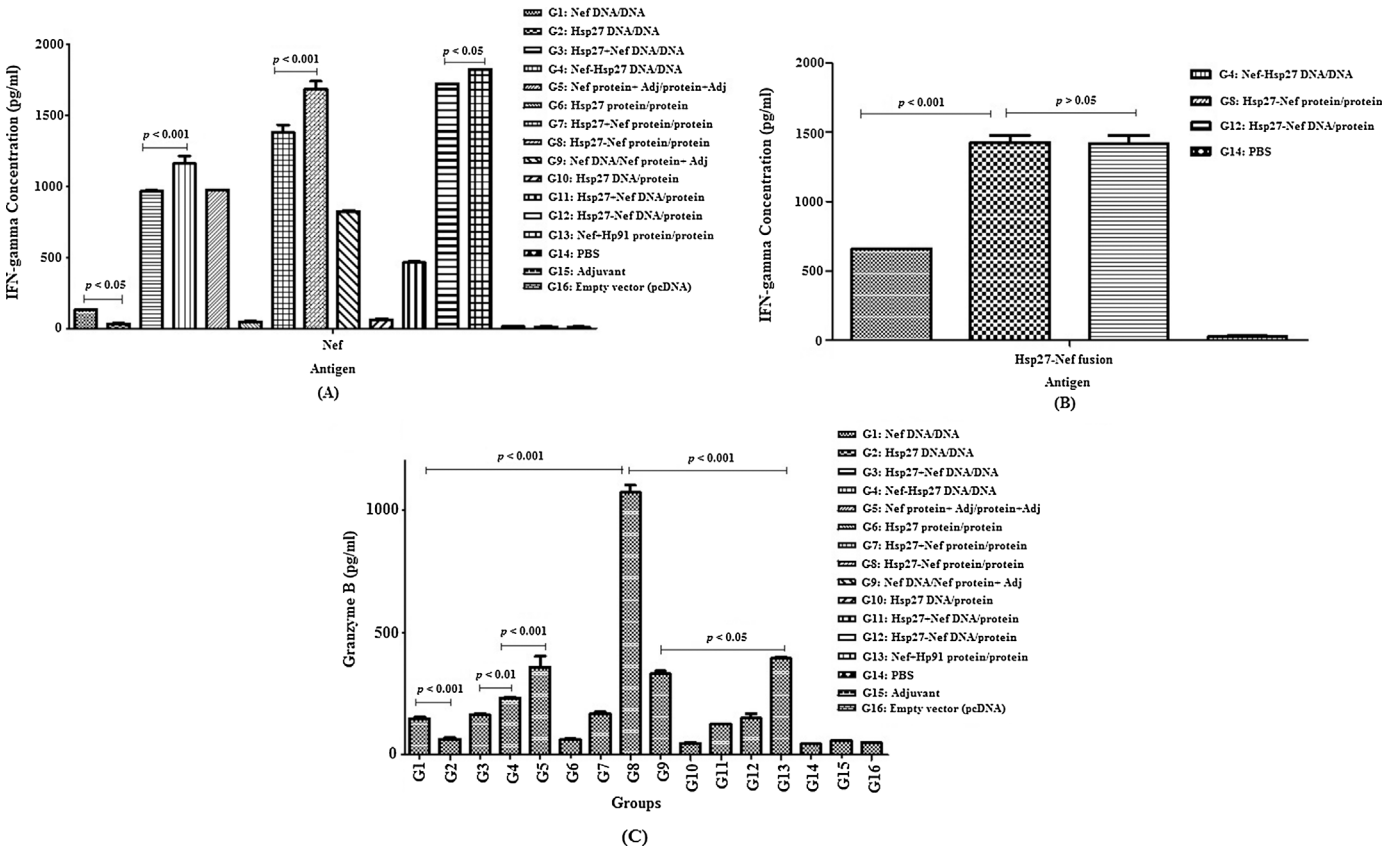

Fig. 4. IFN-γ levels in immunized groups with various formulations (A and B): Pooled splenocyte cultures were prepared from two mice in each group (n = 2 per group) and re-stimulated with rNef and rHsp27-Nef *in vitro*. The levels of IFN-γ were determined in the supernatant with ELISA as mean absorbance at 405 nm ± SD for each set of samples. All analyses were performed in duplicate for each sample. The detection limit was 2 pg/ml for IFN-γ. (C) Granzyme B concentration measured by ELISA using pooled splenocytes from two mice in each group (n = 2 per group): Mice injected with Hsp27-Nef protein/protein secreted significantly higher concentration of granzyme B than all other groups. All analyses were performed in triplicate for each sample. The results represent mean values calculated from triplicate samples as well as the standard deviation (SD) as error bars.

3.5. Granzyme B secretion

Three weeks after the last immunization, splenocytes from each immunized group were co-cultured with p815 target cells in E: T ratio of 100:1 for 6 h at 37 °C, and the supernatants were harvested. Granzyme B secretion in each sample was measured by ELISA (Fig. 4**C**). The data showed that the group immunized with Nef-Hsp27 fusion protein (G8) produced significantly higher concentration of Granzyme B than all other groups ($p < 0.001$). On the other hand, mice immunized with Nef protein formulated with Freund's adjuvant in both protein/protein and DNA/protein modalities (G5 & G9), and also mixed with Hp91 peptide (G13) showed higher levels of Granzyme B than other groups ($p < 0.05$). However, Hp91 peptide and Freund's adjuvant were less effective than Hsp27 fused to Nef (G8) in eliciting Granzyme B secretion ($p < 0.05$). Moreover, the Granzyme B secretion was significantly higher in group immunized with Nef-Hsp27 DNA compared to other groups with the same regimen ($p < 0.05$, Fig. 4**C**).

4. Discussion

Up to now, various strategies were employed to develop the immunogenicity of antigens in both DNA- and protein-based vaccines against HIV-1 infection [22–25]. The goal of this study was to compare the levels of humoral and cellular immune responses elicited by different vaccination regimens using Nef antigen and three adjuvants (*i.e.,* Hsp27, Hp91 and Freund's adjuvant). Several studies indicated the importance of heat shock proteins such as Hsp65, Hsp70 and Hsp110 as an adjuvant for enhancement of humoral and cellular immune responses against HER2/*neu*, Mucin 1 and E7 antigens in breast cancer, melanoma, and cervical cancer models, respectively [26–29]. Herein, we used the small heat shock protein 27 as an adjuvant in both HIV-1 Nef DNA- and protein-based immunizations. Our data showed that immunization with Hsp27-Nef fusion protein increases Nef-specific antibodies, and secretion of IFN-γ and Granzyme B as compared to other groups. Similar to our results, Krupka et al. reported that the levels of HIV-1 p24-specific total IgG and IFN-γ secretion elicited by immunization with Hsp70-p24 fusion protein were significantly higher than those in mice immunized with p24 and the mixture of p24 and Hsp70 [11]. Other experimental studies demonstrated that the combination of Grp170 (a molecular chaperon from HSP70 family) with prostate stem cell antigen (PSCA) could enhance the secretion of IFN-γ and killing activity of target cells *in vitro* at different effector-to-target cell ratios [30]. In this line, a report indicated that MAGE-3 melanoma antigen-specific lysis of CTLs in mice vaccinated with Hsp70-MAGE-3 fusion protein was significantly higher than that in mice vaccinated with MAGE-3 and also MAGE-3 mixed with Hsp70 [31]. Moreover, the group vaccinated with Mage-a1-Hsp70 fusion protein promoted more CTL lysis, antibody and IFN-γ production compared to groups injected with Mage-a1 (aa 118–219) and Mage-a1 + Hsp70 [32]. Our data also showed that the linkage of Hsp27 to Nef was necessary to enhance the Nef-specific cellular immune responses. On the other hand, the ability of Hp91 peptide as an adjuvant was revealed in infectious diseases caused by intracellular bacteria or viruses [33]. A recent study showed that co-immunization of HPV16 E7 protein with Hp91 peptide significantly augmented the secretion of IFN-γ and IgG2a and protected 100% of mice against a TC-1 tumor challenge [34]. Our findings also confirmed the potency of Hp91 peptide for increasing Nef-specific IFN-γ, total IgG, IgG1 and IgG2a production. In contrast, the secretion of Granzyme B in mice injected with Hsp27-Nef fusion protein was higher than that in mice receiving Nef + Hp91 against Nef antigen. Moreover, an important result was higher potency of Hsp27 fused to Nef than Nef emulsified with Freund's adjuvant in generation of IFN-γ, Granzyme B, and total IgG, IgG2a and IgG2b antibodies.

Many studies have shown that the use of adjuvants and heterologous prime-boost strategies have the potential to improve DNA-based immunization [35]. Our data showed that groups receiving Hsp27 + Nef DNA and Hsp27-Nef fusion DNA could significantly produce IFN-γ, Granzyme B, and also antibodies as compared to groups receiving Nef and/or Hsp27 DNA suggesting the potency of Hsp27 as an adjuvant. This result was similar to the effective role of Glycoprotein 96 (Gp96) along with HPV16 E7 in DNA-based immunizations compared to E7 or Gp96 DNA, alone [20]. Also, the importance of Gp96 as an adjuvant was confirmed to increase immune responses induced by pcDNA-Nef or pcDNA-Tat-Nef regimens [19]. Immunization with a DNA vaccine encoding HPV16 E7 CTL epitopes linked to Hsp70 (Hsp70-E7) also increased significantly IFN-γ and CTL responses similar to the full length E7 gene compared to Hsp70 control group, but IL-4 assay did not show any statistically significant differences in these groups. However, E7-specific immune responses were not strong enough to be used as a therapeutic vaccine. Indeed, the design of a *polytope* DNA vaccine is required to improve immunity [36]. On the other hand, the recent reports indicated the efficiency of HIV-heterologous prime-boost strategies for stimulation of antibody and cellular responses [37,38]. For example, a multiple-antigen polyvalent DNA prime followed by boosting with a recombinant HIV-1 envelope protein was used in a phase I clinical trial [39]. Moreover, priming with MPER-V3 DNA + MPG cell penetrating peptide followed by MPER + V3 peptides induced strong T cell responses toward a Th1-type [40]. Our data demonstrated that the group immunized with Nef-Hsp27 DNA/protein increased dramatically the levels of total IgG, IgG1 and IgG2b antibodies, and IFN-γ in comparison with Nef-Hsp27 DNA/DNA immunization. In contrast, the group immunized with Nef-Hsp27 DNA/protein elicited significantly lower levels of granzyme B than Nef-Hsp27 DNA/DNA immunization. Our observations were consistent with the same studies in prime-boost strategy. For instance, the researchers showed that the efficiency of HIV-1 Nef DNA prime/protein boost immunization was enhanced by the co-delivery of a plasmid encoding IL-18 cytokine. Indeed, this modality could increase total IgG, IgG1 and IgG2b antibodies, and induce protective responses [41]. On the other hand, the levels of Hsp27-Nef-specific total IgG and IgG2a in the group immunized with Hsp27-Nef DNA/protein were higher than those in groups immunized with Hsp27-Nef DNA/DNA and Hsp27-Nef protein/protein. This result was reversed for production of IgG1 and IgG2b antibodies. However, the secretion of IFN-γ showed no difference between groups immunized with Hsp27-Nef DNA/protein and Hsp27-Nef protein immunizations against Hsp27-Nef antigen. This similarity may be related to IgG2a and IgG2b responses in groups immunized with Hsp27-Nef DNA/protein and Hsp27-Nef protein/protein, respectively. In general, our results showed that the group immunized with Hsp27-Nef fusion protein has the highest levels of IFN-γ and Granzyme B as compared to other groups. Calarota et al. showed independence of Granzyme B secretion and IFN-γ production during Acute Simian Immunodeficiency virus infection [42]. Shafer-Weaver et al. also indicated that the release of Granzyme B by cytolytic lymphocytes upon effector-target interaction may be a more specific indicator of CTL and NK cytotoxic ability than IFN-γ secretion [43]. On the other hand, Lin et al. reported that Granzyme B secretion by human memory CD4T cells is less strictly regulated compared to memory CD8T cells [44].

Briefly, our data indicated that the recombinant Hsp27-Nef protein can elicit the effective Nef-specific immune responses as compared to the mixture of Nef protein + Hsp27 and Nef protein + Freund's adjuvant. This modality could significantly generate IFN-γ, Granzyme B and antibodies in comparison with DNA/DNA and DNA/protein immunizations. These findings showed the role of Hsp27 as an effective adjuvant similar to other HSPs such as HSP70, Calreticulin, and Gp96 [45,46]. Moreover, the group receiving Nef protein + Hp91 peptide showed Nef-specific antibody and IFN-γ responses higher than the group receiving Hsp27-Nef protein. In contrast, Granzyme B secretion was lower in this group than the group receiving Hsp27-Nef protein.

5. Conclusion

Generally, the recombinant Hsp27-Nef protein could induce the effective Nef-specific immune responses compared to other groups. Furthermore, both Hsp27 and Hp91 could induce Th1 immune responses against Nef antigen. Thus, further studies are required to determine their effects after virus challenge in large animals such as Macaque in Future.

Conflict of interest

The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.imlet.2017.09.005.

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