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RESEARCH ARTICLE

Comparison of Adjuvant Effects of Montanide ISA-720 and Heat Shock Protein 27 in Increasing Immunostimulatory Properties of HIV-1 Nef-Vif Fusion Protein Construct

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Abstract: ***Introduction*:** Effective T-cell-mediated immunity has emerged as an essential component of human immunodeficiency virus-1 (HIV-1) vaccination. Thus, inducing an immune response against HIV proteins such as Nef and Vif, two major accessory proteins with critical roles in HIV pathogenesis and immune evasion, may lead to an effective approach.

***Aim*:** Our goal is to evaluate and compare Montanide ISA-720 and heat shock protein 27 in increasing immunostimulatory properties of HIV-1 Nef-Vif fusion protein as a vaccine candidate.

***Methods*:** In this study, the *nef-vif* fusion gene with and without the *heat shock protein 27* (*hsp27*) gene was cloned in the prokaryotic pET24a (+) vector. Then, the recombinant Nef-Vif and Hsp27-Nef-Vif proteins were generated in the *E. coli* system. Finally, their immunostimulatory properties were evaluated in mice. Indeed, the potency of Hsp27 as an endogenous natural adjuvant was investigated to enhance HIV-1 Nef-Vif antigen-specific immunity compared to Montanide ISA-720 as a commercial adjuvant in protein-based immunization strategy.

***Results*:** Our results approved the role of Hsp27 as an effective adjuvant in the stimulation of B- and T-cell immunity. The linkage of Hsp27 to antigen could elicit higher levels of IgG1, IgG2a, IFN-γ, IL-5 and Granzyme B than antigen mixed with Montanide ISA-720. Moreover, the ratios of IFN-γ/IL-5 and IgG2a/IgG1 were significantly increased in groups receiving Nef-Vif protein + Montanide ISA-720 and Hsp27-Nef-Vif protein indicating the direction of the immune response pathway toward strong Th1 response. These ratios were higher in the group receiving Hsp27-Nef-Vif protein than in the group receiving Nef-Vif protein + Montanide ISA-720.

***Conclusion*:** Our findings suggest that Hsp27 can be used as an effective adjuvant to enhance antigen-specific immune responses in HIV-1 infectious models for therapeutic vaccine development.

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1. INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) continues to be a major global public health issue [1, 2]. HIV belongs to the genus Lentivirus within the family Retroviridae. HIV genome contains two identical single-stranded positive-sense RNA copies enclosed within the core of the virus particle. The genome comprises at least nine genes (*gag, pol, env, tat, rev, vif, vpr, vpu and nef).* Among them, *vif, vpr, vpu* and *nef* genes encode accessory proteins with critical roles in HIV pathogenesis and immune evasion [3, 4]. The well-conserved nature of these genes implies that their protein products play a critical role in viral pathogenies and may be important targets for vaccine development. Vif protein targets a family of host immune factors known as APOBEC3 (or A3) mutator enzymes. A3 converts cytosine to uracil in single-stranded DNA replication intermediates. The antiviral activity of APOBEC3 is strongly inhibited by Vif, allowing the virus to replicate [5, 6]. Nef protein has a negative effect on the presentation of $CD4^+$ molecules on the cell surface. So, the membrane of the infected cell becomes deficient in CD4. The loss of CD4 decelerates the cellular immune reaction against infected cells [7, 8]. According to the importance of Vif and Nef proteins in the HIV life cycle, applying them as an HIV vaccine candidate seems to be logical [9-12].

Some studies showed that using fusion accessory proteins of HIV may enhance immune responses and protection [13-16]. However, the most important limitation of protein-based vaccines is their low immunogenicity. One approach to improve their immunogenicity is the co-delivery of cargos

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with adjuvants. Montanide ISA-720 adjuvant is an example of water-in-oil emulsion for human vaccines such as malaria and HIV vaccines. Montanide ISA720 is a safe and well-tolerated adjuvant with high efficacy, and can greatly enhance cellular (Th1 type) as well as a humoral immune response [17-19]. Moreover, the concept of adjuvants was expanded to include soluble mediators and antigenic carriers that interact with surface molecules presented on dendritic cells (DCs) such as lipopolysaccharides (LPS), heat shock proteins (Hsps) and viral vectors that infect antigen-presenting cells (*e.g.,* lentiviruses). Among mentioned novel adjuvants, Hsps were known as potent inducers of innate and antigen-specific immunity against pathogens [20-23]. They are localized in the cytoplasm and cellular organelles and act as molecular chaperones or proteases [24]. Besides their role as immunogenic antigens, Hsps can also act as adjuvants to stimulate the immunogenicity of peptides and proteins that are either covalently or non-covalently coupled. Their role as natural adjuvants was exploited in vaccine design against cancers and infectious diseases [22]. The interaction of Hsp-peptide complexes or peptide-free Hsps with receptors on antigen-presenting cells promotes the maturation of DCs, and induces secretion of pro- and anti-inflammatory cytokines and chemokines [25-28].

Among Hsps, small heat shock protein 27 (Hsp27) is one of important adjuvants which interacts with toll-like receptors (TLRs) 2, 3 and 4, and induces the release of pro-inflammatory cytokines. The interaction of Hsp27 with TLRs triggers downstream signaling cascades to activate nuclear factor-kappa B (NF-κB), leading to cell maturation and the secretion of pro-inflammatory cytokines in innate cells [29-32].

With regard to the importance of the endogenous natural adjuvants such as heat shock proteins, we attempted to generate the recombinant Nef-Vif and Hsp27-Nef-Vif fusion proteins in *E. coli* system and evaluate their immuno-stimulatory properties in BALB/c mice. Indeed, the potency of Hsp27 as a natural adjuvant was investigated to enhance HIV-1 Nef-Vif antigen-specific immunity as compared to Montanide ISA-720 as a commercial adjuvant in protein-based immunization strategy.

Generally, the published articles often describe the use of heat shock proteins as an adjuvant for vaccine development against cancer and bacterial diseases [20, 22]. This study is one of the few to examine the potential role of Hsps as an adjuvant against viral diseases.

2. MATERIALS AND METHODS

2.1. Bacterial Strains and Plasmids

DH5α, and BL21 (DE3) or Rosetta strains of *Escherichia coli* (*E. coli*) were used for cloning and protein expression, respectively. The prokaryotic expression vector of pET24a (+) was used (Novagen Co.). The *nef* and *vif* gene sequences were obtained from the pNL4.3 vector (accession No: AF324493.2). The *nef* and human *hsp27* (NM_001540) genes were previously prepared in pUC19 and pEGFP-N3 vectors, respectively by our group (Department of Hepatitis and AIDS, Pasteur Institute of Iran).

2.2. Preparation of the Recombinant pET24a-nef-vif

At first, the *nef* gene was subcloned from pUC19-*nef* (previously prepared by our group) into the pET24a (+) vector using the *Nhe*I/*Bam*HI restriction enzymes. Then, in order to amplify the *vif* gene from pNL4.3 vector using polymerase chain reaction (PCR), specifically designed primers of *vif* (F: 5'- CGGGATCCGGATTATGGAAAAC AGATGGC-3' and R: 5'- CAGCGGCCGCTGACCGTTCA TTGTATGGCTC-3') containing a restriction enzyme site for *Bam*HI in forward primer and *Not*I in reverse primer (shown in underline) were used. Steps for PCR included initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30s, annealing at 63°C for 45s and extension at 72°C for 1 min, and final extension at 72°C for 4 min. The purified PCR product of *vif* gene was digested with *Bam*HI/*Not*I restriction enzymes and cloned in the same restriction sites of the recombinant pET24a-*nef* vector. The recombinant pET24a-*nef-vif* was confirmed by PCR, digestion, and sequencing. Its concentration and purity were determined by NanoDrop spectrophotometry. The schematic representation for the recombinant plasmid construction is shown in Figure (**S1**).

2.3. Preparation of the Recombinant pET24a-hsp27-nef-vif

To amplify the *hsp27* gene from pEGFP-N3-*hsp27* (formerly prepared by our group) using PCR, specifically designed primers of *hsp27* (F: 5'- AGATCCCATATGATG GCCGAGCGCCGC-3' and R: 5'- ATGGATCCGCTAGCC TTGGCT-CCAGACTGTTC-3') containing a restriction enzyme site for *Nde*I in forward primer and *Nhe*I in reverse primer (shown in underline) were used. Steps for PCR included initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30s, annealing at 63°C for 45s and extension at 72°C for 1 min, and final extension at 72°C for 4 min. The purified PCR product of the *hsp27* gene was digested with *Nde*I/*Nhe*I restriction enzymes and cloned in the same restriction sites of the recombinant pET24a-*nef-vif* vector. PCR, digestion, and sequencing confirmed the recombinant pET24a-hsp27-nef-vif. Its concentration and purity were determined by NanoDrop spectrophotometry. The schematic representation for the recombinant plasmid construction is shown in Figure (**S1**).

2.4. Generation of the Recombinant Nef-Vif and Hsp27-Nef-Vif Proteins

E. coli BL21 (DE3) and Rosetta strains were transformed with the recombinant pET24a-*nef-vif* and also the recombinant pET24a-*hsp27-nef-vif*, and cultured in Ty2x media (Sigma, Germany). The protein expression was induced by the addition of 0.5 and 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the mid-log phase culture of bacteria at 37°C and 30°C. For time optimization of protein expression, the sampling was conducted at 2, 3, 4 and 16 hours after the induction. SDS-PAGE harvested and analyzed the cell pellets, then transferred to nitrocellulose membranes (Sigma, USA) for western blotting. Anti-His tag antibody conjugated to peroxidase (Abcam, USA) was applied to confirm the proteins of interest. After the

identification of proteins, their purification was performed using HisPur Ni-NTA resin (Thermo Fisher Scientific, Germany)-based affinity chromatography under native conditions (the Qiagen protocol; Imidazole 300 mM and pH = 8) followed by the reverse staining method. The purified proteins were dialyzed against phosphate buffer saline (PBS) 1X using a dialysis membrane (10 kDa, Thermo Fisher Scientific, Germany), and their concentration and purity were assessed by the Bradford kit and NanoDrop spectrophotometer. According to the LAL assay, contamination with LPS was less than 0.4 EU/mg (QCL-1000, Lonza).

2.5. Mice Immunization

Three groups of inbred female BALB/c mice aged five to seven weeks (n = 5 per group) were purchased from breeding stock maintained at the Pasteur Institute of Iran. The whole process was done based on approval protocols and care of laboratory animals in the Animal Experimentation Regulations of Pasteur Institute of Iran (national guideline) for scientific purposes (Ethics code: IR.PII.REC.1400.037). The recombinant proteins were subcutaneously injected at the footpad (volume: ~ 40 µL) three times with a two-week interval (Days 0, 14 & 28). The overall immunization studies are summarized in Table **1**. The first group of mice (G1) received the recombinant Nef-Vif protein (5 µg) mixed with Montanide-ISA 720 adjuvant (the ratio of protein: adjuvant is 30: 70 *v/v*). The second group of mice (G2) received the recombinant Hsp27-Nef-Vif protein (5 µg). The third group of mice (G3) was considered as the control group administrated with PBS 1X.

2.6. Antibody Assay

The blood samples from mice were collected from retro-orbital after anesthesia with ketamine/ xylazine at one month after the third administration. Next, the levels of goat anti-mouse total IgG, IgG1, and IgG2a antibodies conjugated to horseradish peroxidase (1:10,000 *v/v*, Sigma) were measured in the pooled sera (1:100 *v/v*) of each group by indirect enzyme-linked immunosorbent assay (ELISA). The coated antigens were the recombinant Nef-Vif, Hsp27-Nef-Vif, Nef and Vif, (dissolved in PBS 1X) individually for each group of mice. It should be mentioned that the recombinant Nef and Vif proteins (~27 and ~23 kDa, respectively) were previously prepared in *E. coli* by our group [33]. 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 (a reference wavelength for TMB).

2.7. Cytokine Secretion

All mice from each group were sacrificed after anesthesia with ketamine and xylazine one month after the last administration. After removing spleens, the pooled splenocytes of mice without red blood cells (2×10^6 cells/ mL) were seeded in 48-well plates (Greiner) exposed to the recombinant Nef-Vif, Hsp27-Nef-Vif, Nef and Vif proteins (dissolved in PBS 1X) individually (5 µg/ mL), in complete RPMI medium for 72 h. Concanavalin A (5 µg/ mL; Sigma, USA) was considered as a positive control. The supernatants were used to assess IFN-γ and IL-5 (Mabtech Swedish Biotech Co.), using the sandwich-based ELISA method according to the manufacturer's instructions. It should be mentioned that the pooled splenocytes of each group were stimulated with the recombinant Hsp27 protein (previously provided by our group [34] for showing the immunostimulatory effects of Hsp27, alone.

2.8. Granzyme B Assay

The SP2.0 target cells (T: 3×10^4 cells/well) were incubated with the recombinant Nef-Vif, Hsp27-Nef-Vif, Nef and Vif proteins (dissolved in PBS 1X), individually (~5 µg/ mL) for 24 h. Then, the effector cells (E: the red blood cell-depleted pooled splenocytes) were added to the target cells at an E: T ratio of 100:1, and incubated for 6 hours at 37°C in a complete RPMI medium. Finally, after 6 hours of incubation, microplates were centrifuged at 250 g for 5 min at 4°C, and the supernatants were harvested to assess the concentration of Granzyme B using ELISA (eBioscience kit) according to the manufacturer's instructions. It should be mentioned that the SP2.0 target cells were also incubated with the recombinant Hsp27 protein (previously provided by our group [34] for showing the immunostimulatory effects of Hsp27 alone.

2.9. Statistical Analysis

Statistical analysis was done by Prism software (GraphPad) using one-way ANOVA. The p-value < 0.05 was statistically considered significant. The experiments were independently performed twice. Moreover, each test was done in duplicate for each sample.

3. RESULTS

3.1. Generation of the Recombinant pET24a-nef-vif and pET24a-hsp27-nef-vif

The *nef-vif* and *hsp27-nef-vif* fusion genes were successfully cloned in pET-24a (+) expression vector. The presence of *nef-vif* and *hsp27-nef-vif* fragments was confirmed by enzymatic digestion and PCR as clear bands of ~1200 bp and ~1900 bp on agarose gel, respectively (Figure **1**). The sequencing method confirmed these constructs, as well. The concentrations of pET24a-*nef-vif* and pET24a-*hsp27-nef-vif* purified by plasmid extraction Mini-kit for 10 mL bacterial culture medium were about 555 ng/ µL and 478 ng/ µL, respectively.

3.2. Expression and Purification of the Recombinant Proteins in *E. coli*

The recombinant Nef-Vif and Hsp27-Nef-Vif proteins were expressed in Rosetta and BL21 (DE3) strains (Figure **2**). The recombinant Nef-Vif and Hsp27-Nef-Vif proteins migrated as the clear bands of ~50 kDa and ~80 kDa in SDS-PAGE (Figures **2A** and **D**)**,** and then were identified using anti-His tag antibody in western blotting (Figures **2B** and **E**). It should be mentioned that the optimal time, IPTG concentration and temperature were 16 hours after IPTG induction, 1 mM IPTG and 37°C for both proteins. Both proteins were purified by affinity chromatography under native conditions followed by reverse staining method

Table 1. Mice immunization program.

Group	Modality	First Injection (Prime: Day 0)	Second Injection (Booster 1: Day 14)	Third Injection (Booster 2: Days 28)
G1	Protein/protein/protein	rNef-Vif + montanide	rNef-Vif + montanide	rNef-Vif + montanide
G2	Protein/protein/protein	rHsp27-Nef-Vif	rHsp27-Nef-Vif	rHsp27-Nef-Vif
G3	Control	PBS	PBS	PBS

Abbreviation: r: recombinant

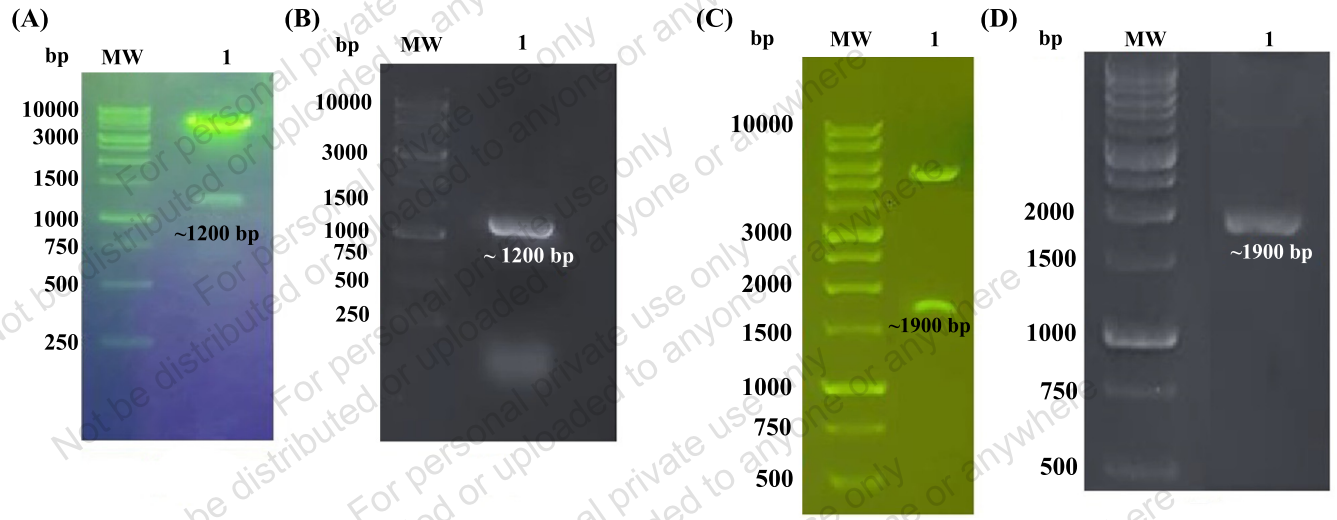

Figure 1. Confirmation of the *nef-vif* (**A** & **B**) and *hsp27-nef-vif* (**C** & **D**) gene constructs in pET-24a using enzymatic digestion and PCR on agarose gel electrophoresis: **A**) Lane 1: double digestion of pET-24a(+)-*nef-vif* using *Nhe*I/*Not*I restriction enzymes; **B**) Lane 1: PCR product of the *nef-vif* gene; **C**) Lane 1: double digestion of pET-24a(+)-*hsp27-nef-vif* using *Nde*I/*Not*I restriction enzymes; **D**) Lane 1: PCR product of the hsp27-*nef-vif* gene; DNA ladder (1 kb, Fermentas). The clear bands of ~1200 bp and ~1900 bp were observed on agarose gel for the *nef-vif* and *hsp27-nef-vif* genes, respectively indicating the generation of the recombinant pET-24a (+)-*nef-vif* and pET-24a (+)-*hsp27-nef-vif* in *E. coli*. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

(Figures **2C** and **F**). The concentrations *of Nef-Vif and Hsp27-Nef-Vif* proteins were between 0.3 and 0.4 mg/ mL.

3.3. Antibody Assay

To compare antibody responses elicited in different groups, the serum levels of total IgG and the related subclasses against the recombinant Nef-Vif, Hsp27-Nef-Vif, Nef and Vif proteins individually were detected using indirect ELISA. Titration of antibodies (anti-mouse total IgG, IgG1, IgG2a antibodies: 1:1000, 1:2000, 1:5000, 1:10000, 1:15000, 1:20000, 1:25000 *v/v*) was performed to obtain the best optical density for the experiment. The data of linear plots indicated that the optical density increased from the ratio of 1:1000 *v/v* to 1:10000 *v/v* and became constant up to the 1: 25000 *v/v*. Thus, we considered the ratio of 1:10000 *v/v* and repeated the experiment in this ratio. Our data showed that the levels of total IgG, IgG1 and IgG2a in the group receiving the recombinant Hsp27-Nef-Vif protein (G2) were significantly higher than those in the group receiving the recombinant Nef-Vif protein + Montanide ISA 720 (G1) against both the Nef-Vif and Hsp27-Nef-Vif coated antigen ($p < 0.01$; Figures **3A** and **B**). Furthermore, the mean ratios of IgG2a/IgG1 were significantly increased in groups receiving Nef-Vif protein + Montanide ISA 720 (G1) and Hsp27-Nef-Vif protein (G2), indicating direction of the immune response toward Th1 response. However, this ratio was higher in the group receiving Hsp27-Nef-Vif protein (G2) than that in the group receiving Nef-Vif protein + Montanide ISA 720 (G1). On the other hand, the levels of total IgG, IgG1 and IgG2a were significantly enhanced in the group receiving Hsp27-Nef-Vif protein (G2) as compared to the group receiving Nef-Vif protein + Montanide ISA 720 (G1) against the Nef and Vif coated antigens ($p < 0.01$; Figures **3C** and **D**). Indeed, the Nef-Vif and Hsp27-Nef-Vif fusion proteins could approximately maintain the conformation of Nef and Vif proteins. The secretion of antibodies against the Nef coated antigen was more than those against the Vif coated antigen.

3.4. Cytokine Assay

The levels of IFN-γ and IL-5 cytokines in group receiving the recombinant Hsp27-Nef-Vif protein (G2) were

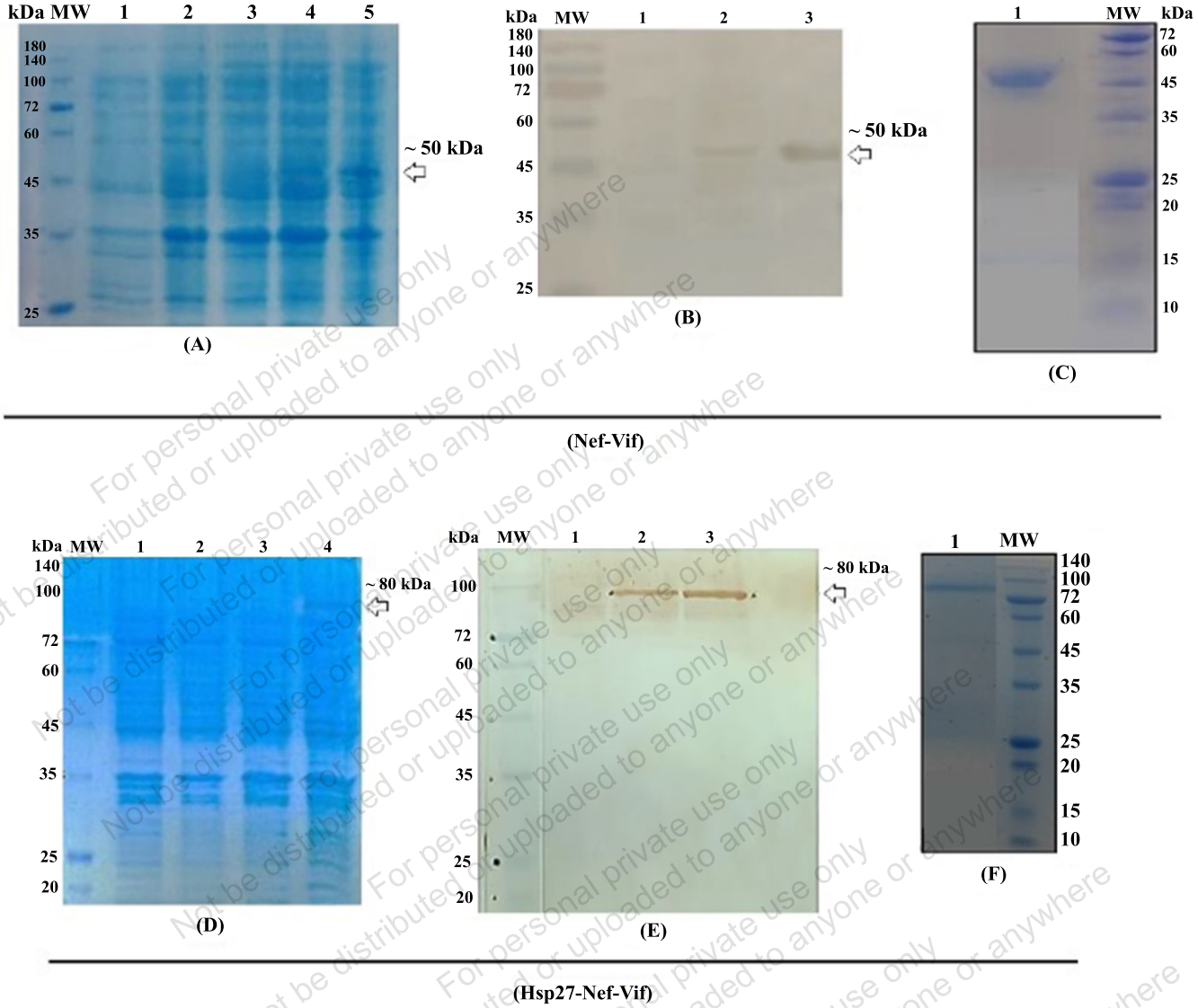

Figure 2. Confirmation and identification of the recombinant Nef-Vif (**A, B** & **C**) and Hsp27-Nef-Vif (**C, D** & **E**) proteins in *E. coli* using SDS-PAGE and western blotting, respectively: **A**) Confirmation of Nef-Vif protein expression in Rosetta strain on SDS-PAGE: Lane 1: Before induction; Lanes 2-5: 2, 3, 4 & 16 h after IPTG induction; **B**) Confirmation of Nef-Vif protein expression in Rosetta strain on western blotting: Lane 1: Before induction; Lane 2: 16 h after IPTG induction (for 15 mL bacterial culture); Lane 3: 16 h after IPTG induction (for 50 mL bacterial culture); **C**) The purified Nef-Vif protein; **D**) Confirmation of Hsp27-Nef-Vif protein expression in BL21 (DE3) strain on SDS-PAGE: Lane 1: Before induction; Lanes 2-4: 2, 4 & 16 h after IPTG induction; **E**) Confirmation of Hsp27-Nef-Vif protein expression in BL21 (DE3) strain on western blotting: Lane 1: Before induction; Lanes 2 & 3: 16 h after IPTG induction (for 50 mL bacterial culture); **F**) The purified Hsp27-Nef-Vif protein. MW: Molecular weight marker (Protein ladder, 10-180 kDa, Fermentas). The recombinant Nef-Vif and Hsp27-Nef-Vif proteins were generated in *E.coli* as clear bands of ~50 and ~80 kDa, respectively. *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

higher than those in group receiving the recombinant Nef-Vif protein + Montanide ISA 720 after re-stimulation of lymphocytes with the recombinant Nef-Vif or Hsp27-Nef-Vif proteins (G1, $p < 0.01$, Figures **4A** and **B**). Interestingly, after re-stimulation with the recombinant Nef or Vif proteins, the lymphocytes of both groups could secrete cytokines (Figures **4C** and **D**). However, the secretion of IFN-gamma in the group receiving the recombinant Hsp27-Nef-Vif protein (G2) was higher than that in the group receiving the recombinant Nef-Vif protein + Montanide ISA 720 after re-stimulation of lymphocytes with the recombinant Nef or Vif proteins (G1, $p < 0.01$, Figures **4C** and **D**); but there was no significant difference in IL-5 secretion between these groups ($p > 0.05$). The mean ratios of IFN-γ/IL-5 were significantly increased in groups receiving Nef-Vif protein + Montanide ISA 720 (G1) and Hsp27-Nef-Vif protein (G2) indicating the direction of the immune response toward Th1 response. However, this ratio

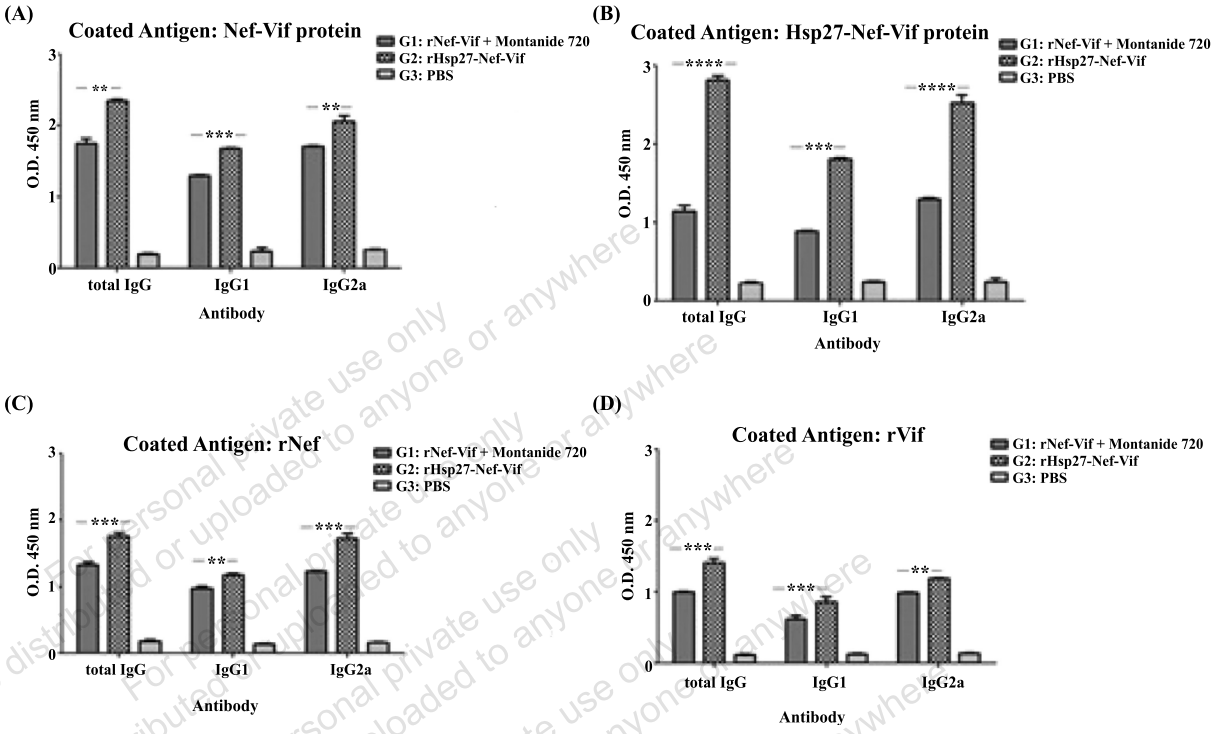

Figure 3. Evaluation of total IgG, IgG1 and IgG2a levels against the Nef-Vif (**A**), Hsp27-Nef-Vif (**B**), Nef (**C**), and Vif (**D**) coated antigens in different groups using indirect ELISA: All analyses were performed in duplicate for each sample shown as mean absorbance at 450 nm ±SD (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$). The background absorbance was subtracted from all data points. Moreover, the mean values were calculated from duplicates. Generally, the levels of total IgG, IgG1 and IgG2a in group receiving the recombinant Hsp27-Nef-Vif protein (G2) were significantly higher than those in group receiving the recombinant Nef-Vif protein + Montanide ISA 720 (G1) against both the Nef-Vif and Hsp27-Nef-Vif coated antigen ($p < 0.01$). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

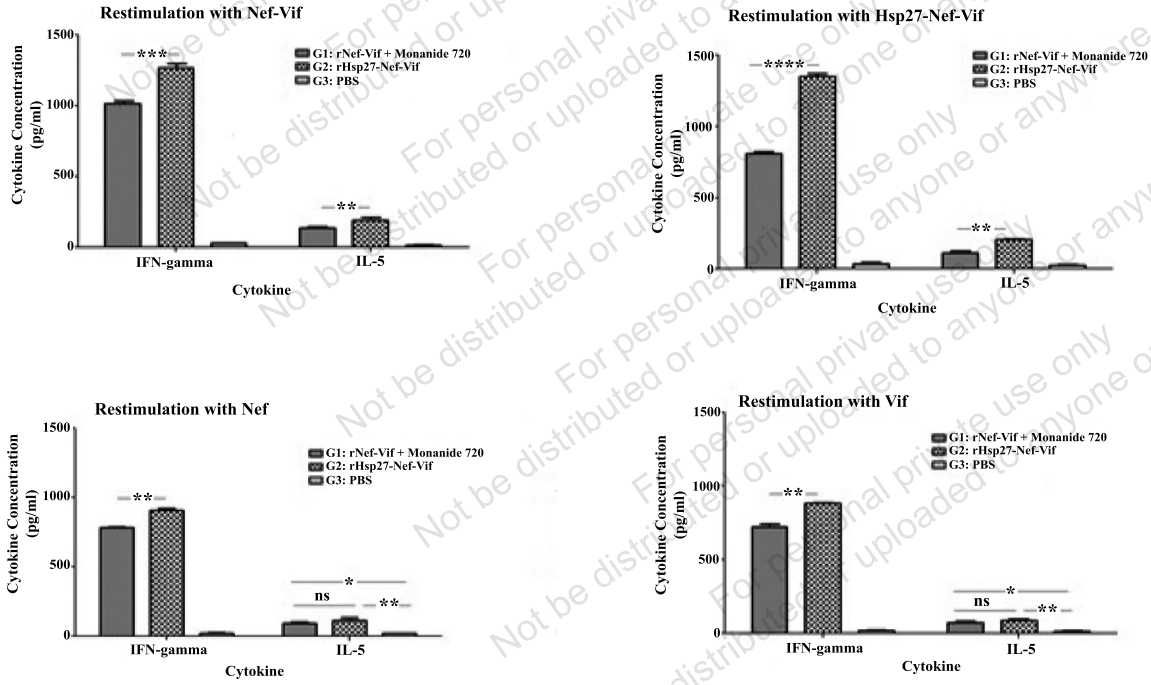

Figure 4. Evaluation of IFN-γ and IL-5 secretion in immunized groups with different modalities. All analyses were performed in triplicate for each sample shown as mean absorbance at 450 nm ±SD (ns: non-significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). Generally, the levels of IFN-γ and IL-5 cytokines in group receiving the recombinant Hsp27-Nef-Vif protein (G2) were higher than those in group receiving the recombinant Nef-Vif protein + Montanide ISA 720 after re-stimulation of lymphocytes with the recombinant Nef-Vif or Hsp27-Nef-Vif proteins (G1, $p < 0.01$). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

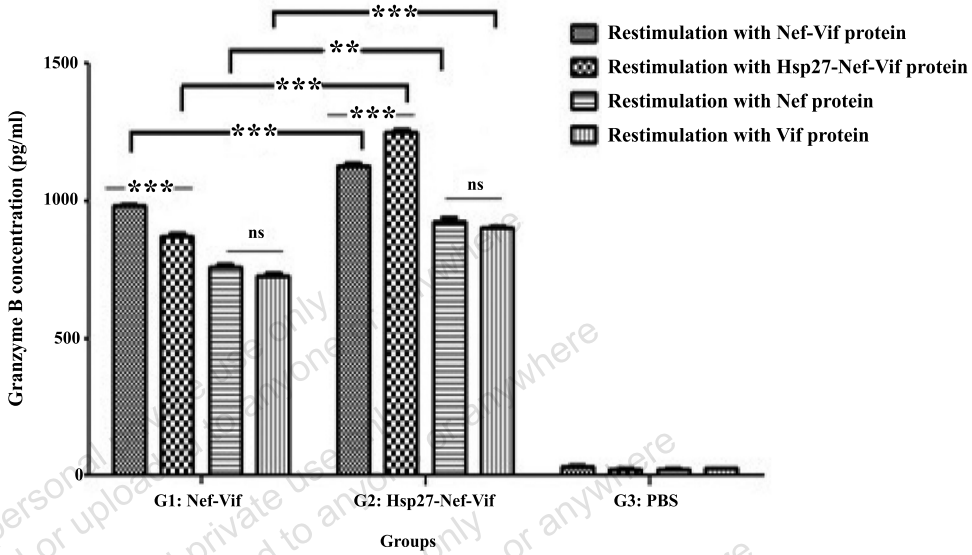

Figure 5. Evaluation of Granzyme B secretion in immunized groups with different modalities using ELISA: All analyses were performed in triplicate for each sample shown as mean absorbance at 450 nm ±SD (ns: non-significant; ** $p < 0.01$; *** $p < 0.001$). Generally, the higher secretion of Granzyme B was observed in group immunized with the recombinant Hsp27-Nef-Vif (G2) than in group immunized with the recombinant Nef-Vif + Montanide ISA 720 (G1) with respect to each recombinant protein such as Nef-Vif, Hsp27-Nef-Vif, Nef and Vif proteins ($p < 0.001$). *(A higher resolution / colour version of this figure is available in the electronic copy of the article).*

was higher in the group receiving Hsp27-Nef-Vif protein (G2) than that in the group receiving Nef-Vif protein + Montanide ISA 720 (G1). It should be mentioned that there was no significant difference in IFN-γ (G1: 44 pg/ml ± 4.1; G2: 51 pg/ml ± 2.8) secretion, and also in IL-5 (G1: 12 pg/ml ± 3.2; G2: 17 pg/ml ± 2.6) secretion between these groups after restimulation with the recombinant Hsp27 protein ($p > 0.05$). The levels of IFN-γ and IL-5 cytokines were 35 pg/ml ± 5.1 and 9 pg/ml ± 3.4, respectively in the PBS group (G3), as well. Thus, there was no significant difference in the IFN-γ and IL-5 levels in test groups (G1 & G2) as compared to the control group (G3; $p > 0.05$). Thus, Hsp27 did not show a considerable immune response alone.

3.5. Secretion of Granzyme B

The higher secretion of Granzyme B was observed in the group immunized with the recombinant Hsp27-Nef-Vif (G2) than in the group immunized with the recombinant Nef-Vif + Montanide ISA 720 (G1) with respect to each recombinant protein such as Nef-Vif, Hsp27-Nef-Vif, Nef and Vif proteins ($p < 0.001$; Figure **5**). It was interesting that the level of Granzyme B in both groups (G1 & G2) with respect to the recombinant Nef protein was similar to its level with respect to the recombinant Vif protein ($p > 0.05$; Figure **5**). The levels of Granzyme B were 19 pg/ml ± 1.2, 22 pg/ml ± 0.9, and 16 pg/ml ± 2.4 in groups 1, 2 and 3 after restimulation with the recombinant Hsp27 protein, respectively, indicating no significant difference between groups ($p > 0.05$). Thus, Hsp27 did not show a significant stimulation of Granzyme B alone.

4. DISCUSSION

Up to now, vaccination has played a key role in controlling infectious diseases. Cellular immune responses are thought to be important for controlling HIV-1 replication. Indeed, effective T-cell-mediated immunity has emerged as an essential component of the HIV vaccination. Thus, inducing an immune response against HIV proteins such as Nef and Vif accessory proteins with critical roles in HIV pathogenesis and immune evasion may lead to an effective approach [35-39]. Martins *et al.* showed that vaccinating rhesus macaques with minigenes encoding the fragments of Gag, Vif, and Nef led to broad cellular responses capable of controlling simian immunodeficiency virus (SIV) replication. However, broad T-cell responses induced by these gene fragments may not be sufficient for HIV-1 replication [40]. Our main objectives of this study include (a) Generation of the recombinant fusion protein harboring two main accessory proteins (Nef and Vif) in *E. coli*, (b) Generation of the recombinant fusion protein harboring Hsp27 fused to the N-terminal region of Nef-Vif fusion protein in *E. coli*; (c) Evaluation of the potency of Hsp27 as an endogenous adjuvant linked to antigen as compared to Montanide ISA-720 mixed with antigen in increasing antigen-specific immunity; and (d) Evaluation of immunostimulatory properties of the recombinant Nef-Vif protein + Montanide ISA-720 and Hsp27-Nef-Vif protein injected in mice against the Nef or Vif protein, alone.

In this study, two fusion constructs of pET24a-*nef-vif* and pET24a-*hsp27-nef-vif* were generated, and the presence of *nef-vif* and *hsp27-nef-vif* genes was confirmed as ~1200 bp and ~1900 bp bands on agarose gel using digestion and PCR. Then, the recombinant Nef-Vif and Hsp27-Nef-Vif proteins were produced in *E.coli* as ~50 and ~80 bp bands on SDS-PAGE. It was interesting that the recombinant Hsp27-Nef-Vif protein migrated as a band of ~90-95 kDa on SDS-PAGE and western blot likely due to the presence of hydrophobic amino acids and charge interaction.

Since the most important limitation of protein-based vaccines is their low immunogenicity [41], we tried to improve the vaccine construct's immunogenicity using adjuvants. The adjuvants activate a more effective cellular immune response by activating the IFN-γ producing $CD4^+$ T cells [42]. Montanide ISA-720 adjuvant was used in human vaccines against malaria, HIV, human papillomavirus (HPV), and coronavirus [17, 18]. The reports showed that not only Montanide ISA-720 has the ability to enhance cytotoxic T lymphocytes (CTL) activity but also improve antibody titers [17, 43, 44]. For example, Montanide ISA720 greatly enhanced humoral and cellular (Th1 type) immune responses against hepatitis C virus (HCV). The increased level of IgG2a was consistent with the induction of Th1 cells secreting IFN-γ [18].

On the other hand, among natural adjuvants, heat shock proteins were used as promising vaccine adjuvants by stimulating antigen-specific immunity to viral pathogens. Hsp-based vaccines target multiple innate and antigen-driven pathways [45, 46]. For example, vaccination with bacille Calmette-Guerin (BCG) peptides complexed to Hsps induced Th1 responses and conferred greater protection than BCG alone in tuberculosis [47]. Moreover, it was shown that binding of Hsp to viral complexes could enhance antiviral immunity including natural killer (NK) activity, antibody-dependent cellular cytotoxicity and CTL activities. Indeed, Hsp could stimulate antiviral host defense under stress conditions [48]. Since the Hsp27 interacts with toll-like receptors 2, 3 and 4 and induces the release of pro-inflammatory cytokines, it is one of the important natural adjuvants [29, 30]. In one study, it was shown that Hsp27 can be used as a suitable carrier in DNA vaccine design against HIV-1 infection [49]. Moreover, our previous study showed that Hsp27 in the mixed form with antigen could act as a more effective adjuvant in stimulation of antigen-specific immune responses and complete protection against tumor than other adjuvants such as Freund's adjuvant, Montanide and another small heat shock protein named as Hsp20 [34]. Hsp27 also possesses an effective ability to increase cell survival in response to a wide range of cellular stresses [50, 51]. Qazi *et al.* indicated that small Hsps are capable of delivering antigens to major histocompatibility complexes (MHC) for the stimulation of adaptive immunity [52]. In one report, the overexpression of Hsp27 in a breast cancer cell line increased its susceptibility to cytolysis by γδ T cells [53]. Our study demonstrated that the linkage of Hsp27 to antigen could elicit higher levels of IgG1, IgG2a, IFN-γ, IL-5 and Granzyme B than antigen mixed with Montanide ISA720. Furthermore, the mean ratios of IFN-γ/IL-5 and IgG2a/IgG1 were significantly increased in the group receiving Hsp27-Nef-Vif protein indicating the direction of the immune response pathway toward strong Th1 response. The secretion of Granzyme B was higher in the group receiving Hsp27-Nef-Vif protein than group receiving Nef-Vif protein + Montanide ISA720 suggesting CTL activity. These findings suggest that Hsp27 can be used as an effective adjuvant to enhance antigen-specific immune responses in HIV-1 infectious models for therapeutic vaccine development. However, the selection of adjuvant depends on some factors such as the antigen, the species to be vaccinated, the route of administration, and the likelihood of side-effects [54]. Hence, adjuvant compounds are important to potentiate an effective immunity against the antigen of interest.

CONCLUSION

In summary, these findings approved that Hsp27 not only has the ability to elicit antigen-specific T-cell immunity but also provides potentially higher IgG antibody levels than Montanide-ISA 720. However, further studies are required to confirm our results in large animals and infectious models in the future.

AUTHOR'S CONTRIBUTIONS

A. B. conceived and designed the experiments. N. K. and F. S. performed the experiments. N. K., E. A., and A. M. analyzed the data. N. K. and F. S. wrote the paper. A.B. edited the manuscript. All authors reviewed and approved the paper.

LIST OF ABBREVIATIONS

HIV-1 = Human Immunodeficiency Virus-1

DCs = Dendritic Cells

TLRs = Toll-like Receptors

SIV = Simian Immunodeficiency Virus

HPV = Human Papillomavirus

CTL = Cytotoxic T Lymphocytes

BCG = Bacille Calmette-guerin

CTL = Cellular Cytotoxicity

NK = Natural Killer

MHC = Major Histocompatibility Complexes

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Medical Ethics Committee of Pasteur Institute of Iran, (Approval No: IR.PII.REC.1400.037).

HUMAN AND ANIMAL RIGHTS

No humans were used in the study. All reported experiments on animals were performed based on approval protocols and care of laboratory animals in the Animal Experimentation Regulations of Pasteur Institute of Iran (national guideline) for scientific purposes.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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