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ORIGINAL ARTICLE

Immunostimulatory effects of Hsp70 fragments and Hsp27 in design of novel HIV-1 vaccine formulations

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Abstract

Background: Heat shock proteins (HSPs) as an adjuvant induce antigen-specific immunity through facilitating antigen presentation and stimulating T cells. In this study, the immunostimulatory properties of two major fragments of Hsp70 (N-Hsp70$_{(aa\ 1–387)}$ with ATPase property and C-Hsp70 $_{(aa\ 508–641)}$ with peptide-binding capacity) and the full length of Hsp27 as vaccine adjuvants were evaluated to boost HIV-1 Nef antigen-specific immunity in both in vitro and in vivo experiments.

Methods: At first, the nanoparticles harbouring DNA fusion constructs (i.e. N-Hsp70-Nef, C-Hsp70-Nef and Hsp27-Nef) complexed with HIV Rev $_{(34–50)}$ cell-penetrating peptide were generated to deliver DNA into the cells. Then, the recombinant Nef, Hsp27-Nef, N-Hsp70-Nef and C-Hsp70-Nef proteins were generated in *E.coli* expression system. Next, the immunostimulatory properties of these fusion constructs were evaluated in both in vitro and in vivo studies. Finally, the secretion of main cytokines from single-cycle replicable (SCR) HIV-1 virion-exposed splenocytes was investigated.

Results: Our data showed that the stable and non-toxic DNA/Rev nanoparticles could successfully deliver the genes of interest into the cells. Moreover, higher secretion of antibodies and cytokines was detected in mice receiving the Hsp-Nef constructs than in mice receiving Nef antigen. The C-Hsp70 was also superior for inducing Nef-specific Th1 and CTL immunity compared with N-Hsp70 and Hsp27. The T-cell activity was maintained in the SCR-exposed splenocytes, especially the splenocytes of mice receiving the C-Hsp70-Nef regimen.

Conclusion: Altogether, these findings demonstrate the significance of Hsps as enhancers of antigen-specific immunity. Notably, the C-Hsp70 region showed better adjuvant properties for inducing cellular immunity in the improvement of HIV-1 therapeutic vaccines.

KEYWORDS

cell-penetrating peptide, heat shock protein, HIV-1 Nef, Hsp27, Hsp70, SCR

INTRODUCTION

Vaccine candidates are currently under development using various platforms, including vaccines based on recombinant protein, viral vector (e.g. adenoviral vector), DNA and mRNA. However, these platforms may lack the immunological profiles needed to mediate the enhanced adaptive immunity. As a result, efforts have been made to increase vaccine immunogenicity through the integration of appropriate adjuvants, specific antigen targeting and effective carriers. Innovative approaches are imperative in the quest to create non-toxic adjuvants and less immunogenic delivery carriers for treatment of various infectious diseases [1, 2]. In recent years, there has been a significant focus on continuous pre-clinical and clinical trials aimed at creating an efficacious HIV vaccine. Among various HIV-1 proteins under consideration, Nef has been proposed as a main antigen candidate for vaccine improvement [3]. Additionally, cell-penetrating peptides (CPPs) have been considered as a viable approach for transporting antigens into cellular compartments, specifically antigen-presenting cells (APCs), in the field of vaccine development [4]. Different protein types, such as protamines, secretory proteins and G-protein-coupled receptors, were recognized as DNA/RNA binders. Databases also revealed viral coat proteins with arginine-rich segments, potentially acting as CPPs. These viral protein segments might be related to their infection-related roles [5]. HIV-1 Rev is a crucial viral protein that assists in the nuclear export of viral RNAs containing introns. These RNAs encode vital viral structural and enzymatic proteins, while also supplying complete genomes for packaging [6]. The reports have explored the RNA-binding segment of HIV-1 Rev as a potential carrier for DNA delivery. The transfection results demonstrated that HIV-1 Rev [amino acid (aa): 34–50] exhibits a translocation activity similar to HIV-1 Tat (aa 48–60) [7].

Furthermore, heat shock proteins (HSPs) have been suggested as adjuvants for new vaccine designs needing cell-mediated immunity against diseases. HSPs, a protein family expressed during the cell cycle, are widely conserved in all organisms, and induced by various stressors (e.g. environment, metabolism and pathophysiology conditions). This universality positions HSPs as crucial components of cellular stress responses [8, 9]. Besides regulating cellular homeostasis during both normal and stressful situations, certain members of the HSP family, such as Hsp27 and cytoplasmic Hsp70, possess strong immunostimulatory properties. These proteins have been utilized as vaccine adjuvants, specifically tailored for combating cancer and infections [10, 11]. The studies showed various functions of the HSP70 family within the immune system. These functions encompass intracellular responsibilities, such as involvement in antigen presentation and the expression of innate receptors, as well as extracellular duties, such as participation in tumour immunosurveillance and autoimmunity [9]. HSP70 comprises two separate functional regions: the peptide-binding domain at the C-terminus and the ATPase domain at the N-terminus [12]. Some studies have demonstrated that immune functions are associated with either the N-terminal or C-terminal segments of HSP70. For example, when human papillomavirus (HPV) E7 antigen is fused to the N-terminal segment of mouse Hsp70, it not only induces a specific cytotoxic T-cell (CTL) response against E7 but also protects mice against E7-expressing tumours. Furthermore, this investigation emphasized that the region responsible for binding peptides in Hsp70 is crucial for the effectiveness of the E7-Hsp70 DNA vaccine [13]. Wang et al. showed the C-terminal peptide-binding region of Hsp70 stimulates human monocytes to generate interleukin 12 (IL-12), tumour necrosis factor-alpha (TNF-α), and C-C chemokines [14]. Furthermore, this specific region of Hsp70 played a crucial role in eliciting an anti-tumour response and activating natural killer cells (NK cells) when exposed to murine tumour B16 expressing the Mela tumour antigen [15].

The small HSP family (sHSPs), including Hsp27 and α-crystallins known as ATP-independent chaperones, showed anti-apoptotic, proangiogenic, anticancer and anti-inflammatory effects through client protein interactions [16]. Hsp27 and other members of sHSPs contain a C-terminal alpha-crystallin domain (about 40% of the protein) and a less conserved N-terminal domain harbouring a hydrophobic WDPF motif [17]. It has been demonstrated that within the extracellular environment, Hsp27 can function as a signalling molecule, interacting with membrane receptors such as toll-like receptors (TLRs). This interaction leads to the induction of nuclear factor-kappa-light-chain-enhancer of activated B cells (NF-κB) through phosphorylation [18]. Furthermore, when human macrophages were exposed to the recombinant Hsp27, it resulted in heightened NF-κB transcriptional activity. This, in turn, caused the elevation of several genes, including the proinflammatory markers interleukin 1 β (IL-1β) and TNFα, alongside anti-inflammatory factors like interleukin 10 (IL-10) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [19]. These data indicate that the function of Hsp27 may vary depending on its localization. Furthermore, Small Hsp27 has been suggested as a promising adjuvant for boosting HIV-1 antigen-specific immunity [20].

In this study, we assessed and compared two fragments of cytoplasmic Hsp70 and the full length of Hsp27 as an adjuvant for enhancement of antigen-specific immunity in vitro and in vivo. In this regard, HIV-1 Nef antigen candidate was linked to two major Hsp70

fragments: an N-terminal ATPase region (N-Hsp70: aa 1–387) and a C-terminal peptide-binding region (C-Hsp70: aa 508–641). The N-Hsp70-Nef, C-Hsp70-Nef and Hsp27-Nef fusion proteins were employed to stimulate the secretion of TNF-α, interferon gamma (IFN-γ) and IL-10 cytokines from mouse dendritic cells (DCs), mouse splenocytes and splenocytes co-cultured with DCs. Additionally, HIV-1 Rev peptide (aa 34–50) was used to facilitate DNA delivery in vitro and in vivo for the first time. Then, the immunostimulatory effects of N-terminal and C-terminal regions of Hsp70 and Hsp27 were studied for enhancing HIV-1 Nef antigen-specific immunity using heterologous DNA prime/protein boost vaccine formulations in mice. Finally, in vitro secretion of the main cytokines (TNF-α, IFN-γ and IL-10) from splenocytes exposed to single-cycle replicable (SCR) HIV-1 virions was evaluated to detect the efficiency of immunization modalities.

METHODS

DNA construction

DNA encoding the N-terminal ATPase domain (aa 1–387) and the C-terminal peptide-binding domain (aa 508–641) of Hsp70 (human heat shock 70 kDa protein 1A: UniProtKB-P0DMV8) was amplified by polymerase chain reaction (PCR) and then cloned into the pET-23a (Novagen, Merck, Germany) vector harbouring the HIV-1 Nef gene (pET-23a-Nef; previously provided by our group [20]) using *Nde*I/*Eco*RI restriction enzymes (Fermentas, Germany). Then, for generation of DNA constructs in a eukaryotic expression vector, the N-Hsp70-Nef and C-Hsp70-Nef fusion constructs were subcloned from pET-23a vector into the pcDNA3.1 (−) vector (Invitrogen, Germany) using *Bam*HI/*Hind*III (Fermentas) restriction enzymes. The full length of HIV-1 Nef (from HIV-1 pNL4.3 vector, accession no: AF324493.2) and the Hsp27-Nef fusion gene (*Mus musculus* HSP27, accession no: NM_013560) were previously cloned by our research team into the pET-23a vector using *Eco*RI/*Sal*I and *Nhe*I/*Sal*I, respectively, and the pcDNA3.1 (−) vector using *Bam*HI/*Hind*III and *Bam*HI/*Afl*II, respectively [20]. The DNA constructs were purified using an Endo-free plasmid Giga kit (Qiagen, Valencia, CA, USA), and their concentration and purity were determined using NanoDrop spectrophotometry (Thermo Scientific, Germany).

Preparation of CPP/DNA nanoparticles

The RNA-binding segment of HIV Rev peptide (aa 34–50; TRQARRNRRRRWRERQR) was considered as a CPP [7], and synthesized by Biomatik Co. (Canada). To obtain the CPP/DNA complexes such as pcDNA3.1-N-Hsp70-Nef/Rev, pcDNA3.1-C-Hsp70-Nef/Rev, pcDNA3.1-Hsp27-Nef/Rev and pcDNA3.1-Nef/Rev, 1 μg of each DNA was mixed with Rev peptide at the nitrogen-to-phosphate ratios (N/P ratios) of 5, 10, 15 and 20, followed by an incubation at 37°C for 60 min. The nanoparticle formation was confirmed by gel retardation electrophoresis assay, and a certain N/P ratio was selected for zeta potential (HORIBA SZ-100) and scanning electron microscope (SEM EM-8000) analysis. Moreover, the stability of nanoparticles against DNA nucleases (DNase I; 1 U/μL, Fermentas) and proteases of fetal bovine serum (FBS) was investigated in a certain N/P ratio according to our previous study [21].

DNA transfection and MTT assay

To monitor the efficiency of Rev peptide to deliver DNA constructs, different N/P ratios of peptide/DNA nanoparticles with a 10 μg of DNA were used. For this purpose, human embryonic kidney (HEK)-293 T cell line (ATCC, Cat# CRL-3216) was seeded at a density of 6.0×10^4 cells/well in 24-well plates in complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FBS (Gibco, Germany). The DNA/Rev nanoparticles at the N/P ratio of 15 for pcDNA3.1-Nef/Rev, and the N/P ratio of 20 for pcDNA3.1-N-Hsp70-Nef/Rev, pcDNA3.1-C-Hsp70-Nef/Rev and pcDNA3.1-Hsp27-Nef/Rev were formed, and incubated at 37°C for 60 min (the N/P ratios were selected based on Section 2.2). Then, the nanoparticles were added to the cells in the presence of serum-free medium for 4 h. After that, the culture medium was replaced with complete DMEM 10% heat-inactivated FBS (Gibco). The detection of protein expression was performed by Western blotting at 48 h post-transfection using anti-Nef monoclonal antibody (Abcam, USA; diluted to 1:5000 v/v) conjugated to horseradish peroxidase (HRP) and 3, 3′-diaminobenzidine (DAB) substrate (Sigma, Germany). The cytotoxicity of DNA/Rev complexes was assessed in the HEK-293 T-cell line using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay kit (Sigma).

Generation of the recombinant proteins in *E. coli*

For expression of the recombinant N-Hsp70-Nef and C-Hsp70-Nef proteins, the pET-23a-N-Hsp70-Nef and pET-23a-C-Hsp70-Nef fusion constructs were transformed into the Rosetta and BL21 *E. coli* strains. The expression of proteins was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Subsequently, we optimized the incubation time at 2, 4 and 16 h

post-induction. Purification was carried out using affinity chromatography on a Ni-NTA agarose column under denaturing conditions (8 M urea, pH = 4.5). Their expression was detected by Western blot analysis using an anti-Nef monoclonal antibody (Abcam; diluted to 1:10 000 v/v) conjugated to HRP. On the other hand, the recombinant HIV-1 Nef (~ 30 kDa) and Hsp27-Nef (~ 57 kDa) proteins were expressed in the *E. coli* Rosetta strain (1 mM IPTG, 16 h post-induction) and purified by affinity chromatography under denaturing conditions. It should be mentioned that the Nef and Hsp27-Nef proteins were previously generated by our group [20]. Herein, these proteins were prepared at a large scale for in vitro and in vivo studies. Finally, after dialysis of all recombinant proteins against phosphate-buffered saline (PBS) 1X, their amounts were assessed by Bradford protein assay kit (Sigma). The Limulus Amebocyte Lysate assay (LAL; QCL-1000 kit, Lonza, USA) showed the level of endotoxin contamination < 0.4 EU/mg of proteins.

In vitro cytokine assay

We investigated the effects of exogenous recombinant N-Hsp70-Nef, C-Hsp70-Nef, Hsp27-Nef and Nef proteins on the secretion of TNF-α, IFN-γ and IL-10 cytokines from mouse DCs, splenocytes, and DCs co-cultured with splenocytes. For this purpose, DCs were isolated from the bone marrow cells of 5- to 7-week-old male BALB/c mice (Naïve mice; Pasteur Institute of Iran), and cultured in the presence of 20 ng GM-CSF (Peprotech, England) and 10 ng IL-4 (Peprotech) cytokines according to the previous studies [20, 22]. Briefly, femurs and tibias were isolated from mice. Then, the cleaned bones were immersed in 70% ethanol for 2 min. Both ends of each bone were trimmed, and bone marrow cells were obtained by flushing each bone shaft with culture medium (RPMI 1640, ~ 1 mL). Next, erythrocytes were lysed using ACK lysis buffer. The remaining cells were cultured at a density of 5.0×10^6 cells/cm^2 in RPMI 1640, supplemented with 20% FBS and 3% penicillin/streptomycin (Gibco). Every 2 days, the cells were replenished with RPMI 1640 supplemented with 10% FBS, 20 ng GM-CSF and 10 ng IL-4. Finally, non-adherent and loosely adherent cells, which were in the form of immature DCs, were harvested on the fifth day. Moreover, splenocytes were isolated from mice spleens in the presence of ACK lysis buffer for depletion of red blood cells according to the previous studies [20]. The entire procedure was carried out in accordance with the approval protocols and ethical standards for laboratory animal care established by the research ethics committees at the Pasteur Institute of Iran, in adherence to national guidelines, for scientific research purposes (ethics code: IR.PII.REC.1400.059; approval date: 1 November 2021). After isolation of DCs and splenocytes, these cells were cultured at a density of 5.0×10^5 cells/well (DCs), and a density of 2.0×10^6 cells/well (splenocytes), individually. Then, these cells were exposed to the recombinant N-Hsp70-Nef, C-Hsp70-Nef, Hsp27-Nef and Nef proteins at a concentration of 5 μg/mL for 72 h. Furthermore, DCs (T, target cells) were pulsed with each recombinant protein for 24 h, and then co-cultured with the splenocytes (E, effector cells) at an E: T ratio of 10:1 for 48 h. Finally, the secretion of TNF-α, IFN-γ and IL-10 cytokines was evaluated using a Sandwich-based enzyme-linked immunosorbent assay (ELISA) (Mabtech, Sweden).

Mice immunization

Female BALB/c mice, aged between 5 and 7 weeks, were administered with different vaccine formulations. These mice were procured from the breeding facilities at the Pasteur Institute of Iran, and housed in a controlled environment following the institute's established protocols for maintaining specific pathogen-free conditions (ethics code: IR.PII.REC.1400.059; approval date: 1 November 2021). Six groups of five mice were divided randomly and immunized subcutaneously three times with 2-week intervals (days 0, 14 and 28) using heterologous prime/boost modalities as indicated in Table 1. The DNA (10 μg)/Rev nanoparticles were used at the N/P ratios of 15 for the pcDNA3.1-Nef/Rev complex and 20 for the pcDNA3.1-Hsp27-Nef/Rev, pcDNA3.1-N-Hsp70-Nef/Rev and pcDNA3.1-C-Hsp70-Nef/Rev complexes. The amount of recombinant protein was 10 μg in each mouse. The Rev peptide and PBS1X were used as control groups.

Evaluation of humoral immune responses

To investigate humoral immune responses induced by heterologous prime/boost regimens, 3 weeks after the third immunization, serum samples of each group were obtained and pooled. The levels of total immunoglobulin G (IgG) and its isotypes (IgG1 and IgG2a) in the pooled sera of each group (diluted to 1:100 v/v) were evaluated by goat anti-mouse antibodies conjugated to HRP (Sigma; diluted to 1:10 000 v/v) using indirect ELISA. The recombinant Nef protein (5 μg/mL) and tetramethylbenzidine (TMB) were used as the coated antigen and the substrate, respectively. The optical density (OD) was measured at a wavelength of 450 nm. All data are represented as means ± SD for each set of samples.

TABLE 1 Immunization schedule using heterologous DNA prime/protein boost regimens.

Group	No.	First injection (day 0)	Second injection (day 14)	Third injection (day 28)
G1	5	pcDNA-Nef/CPP	rNef protein	rNef protein
G2	5	pcDNA-Hsp27-Nef/CPP	rHsp27-Nef protein	rHsp27-Nef protein
G3	5	pcDNA-N-Hsp70-Nef/CPP	rN-Hsp70-Nef protein	rN-Hsp70-Nef protein
G4	5	pcDNA-C-Hsp70-Nef/CPP	rC-Hsp70-Nef protein	rC-Hsp70-Nef protein
G5	5	Rev CPP	Rev CPP	Rev CPP
G6	5	PBS	PBS	PBS

Note: HIV-1 Rev's RNA-binding segment (34–50) considered as cell-penetrating peptide (CPP).
Abbreviations: C-HSP70, C terminal of heat shock protein 70; HSP27, heat shock protein 27; N-HSP70, N terminal of heat shock protein 70; PBS, phosphate-buffered saline; r, recombinant.

Evaluation of cellular immune responses

Three weeks after the last injection, a total of five mice from each experimental group were euthanized. Then, the splenocytes were isolated from spleens according to Section 2.4 [20]. The pooled splenocytes from each group (2.0×10^6 cells/mL) were cultivated in 24-well plates in the presence of 5 μg/mL of Nef protein for 72 h. Concanavalin A (ConA, 5 μg/mL) and RPMI 10% were considered as positive and negative controls, respectively. The detection of TNF-α, IFN-γ and IL-10 cytokines was carried out using a Sandwich-based ELISA kit (Mabtech). All data were represented as means ± SD for each set of samples.

Granzyme B secretion assay

For evaluation of the release of Granzyme B (GrB) from effector splenocytes (E), SP2/0 target cells (T) (ATCC, catalogue no. CRL-1581; a standard murine myeloma cell line) were seeded at a density of 2.0×10^4 cells/well in triplicate in 96-well plates. The cells were incubated for 24 h in the presence of 5 μg/mL of the recombinant Nef protein. Subsequently, the pooled splenocytes from each group were added to the target cells at a ratio of 100:1 (E:T ratio), and co-cultured in RPMI-1640 medium supplemented with 10% FBS (Gibco). After a 6 h incubation period, the supernatants were collected, and the concentrations of Granzyme B were quantified using an ELISA kit (eBioscience, USA). All data were represented as means ± SD for each set of samples.

In vitro virion-specific cytokine assay

At first, the generation of single-cycle replicable human immunodeficiency virus-1 (SCR HIV-1) was carried out in accordance with the previous reports [20]. Briefly, the HEK-293 T-cell line was co-transfected with 4 μg of pmzNL4.3, psPAX2 and pMD2G vectors using TurboFect reagent (Fermentas). Subsequently, culture supernatants containing virions were gathered, and then concentrated by centrifuging at 45 000 ***g*** for 120 min. The quantity of virions was determined using a p24 ELISA kit (rETRO-TEK, USA). Then, the pooled splenocytes from each group (2.0×10^6 cells/well) were exposed to SCR virions (50 μg of p24 antigen) for 72 h. Finally, the cell culture supernatants were harvested and used to assess the levels of TNF-α, IFN-γ and IL-10 cytokines using a sandwich-based ELISA kit (Mabtech).

Statistical analysis

Prism 8 software (GraphPad) was utilized to evaluate statistical differences between the control and test groups using one-way ANOVA. The data were indicated as means ± SD for each group. A p-value <0.05 was considered statistically significant ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$).

RESULTS

Confirmation of DNA constructs

Amplification of the N-terminal $_{(1161\ bp)}$ and C-terminal $_{(399\ bp)}$ fragments of Hsp70 by PCR was confirmed on 1% agarose gel electrophoresis. Moreover, clear bands of ∼1800 bp for the N-Hsp70-Nef and ∼ 1050 bp for the C-Hsp70-Nef fusion genes were observed on the agarose gel after pET23a-N-Hsp70-Nef and pET23a-C-Hsp70-Nef double-digestion using *Nde*I/*Hind*III restriction enzymes. On the other hand, the recombinant endotoxin-free pcDNA3.1-N-Hsp70-Nef and pcDNA3.1-C-Hsp70-Nef vectors were confirmed by the presence of clear bands of ∼1800 bp and ∼ 1050 bp for fusion genes in agarose gel after *Bam*HI/*Hind*III double-digestion (Figure 1). Finally, the pET23a and pcDNA3.1 (−) vectors harbouring HIV-1 Nef (∼ 648 bp), Hsp27-Nef (∼ 1368 bp),

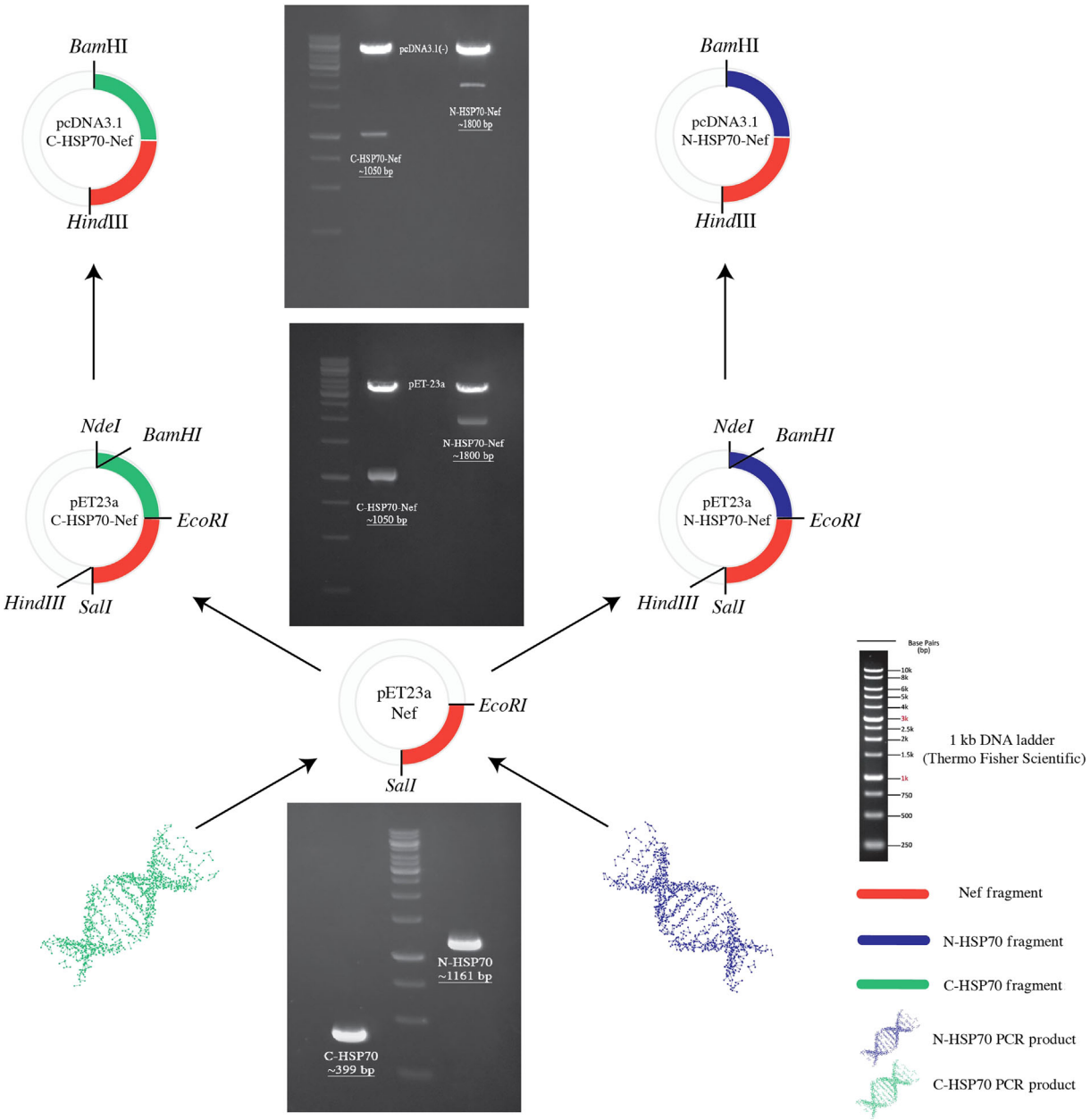

FIGURE 1 Schematic representation of the C-Hsp70-Nef and N-Hsp70-Nef gene constructs in prokaryotic and eukaryotic expression vectors. The C-terminal and N-terminal fragments of Hsp70 were amplified by polymerase chain reaction (PCR) and cloned into the pET23a-Nef using *Nde*I/*Eco*RI restriction enzymes. Then, these constructs were subcloned into the pcDNA3.1 (−) using *Bam*HI/*Hind*III restriction enzymes.

N-Hsp70-Nef and C-Hsp70-Nef genes were purified at a large scale as endotoxin-free plasmids. The accuracy of all DNA constructs was confirmed by PCR, digestion and Sanger sequencing.

Confirmation of the CPP/DNA complexes

The interaction between positive charge of Rev peptide and negative charge of DNA constructs was analysed by gel retardation assay. Our data showed that DNA did not migrate into the agarose gel at the N/P ratio of 15 for pcDNA3.1-Nef and the N/P ratio of 20 for pcDNA3.1-N-Hsp70-Nef, pcDNA3.1-C-Hsp70-Nef and pcDNA3.1-Hsp27-Nef, suggesting the formation of CPP/DNA complexes. Moreover, the scanning electron microscopy (SEM) analysis of nanoparticles showed that the diameter of Rev/DNA complexes was in the range 150–250 nm with a uniform and cubic shape. Figure 2 shows the results of gel retardation assay and

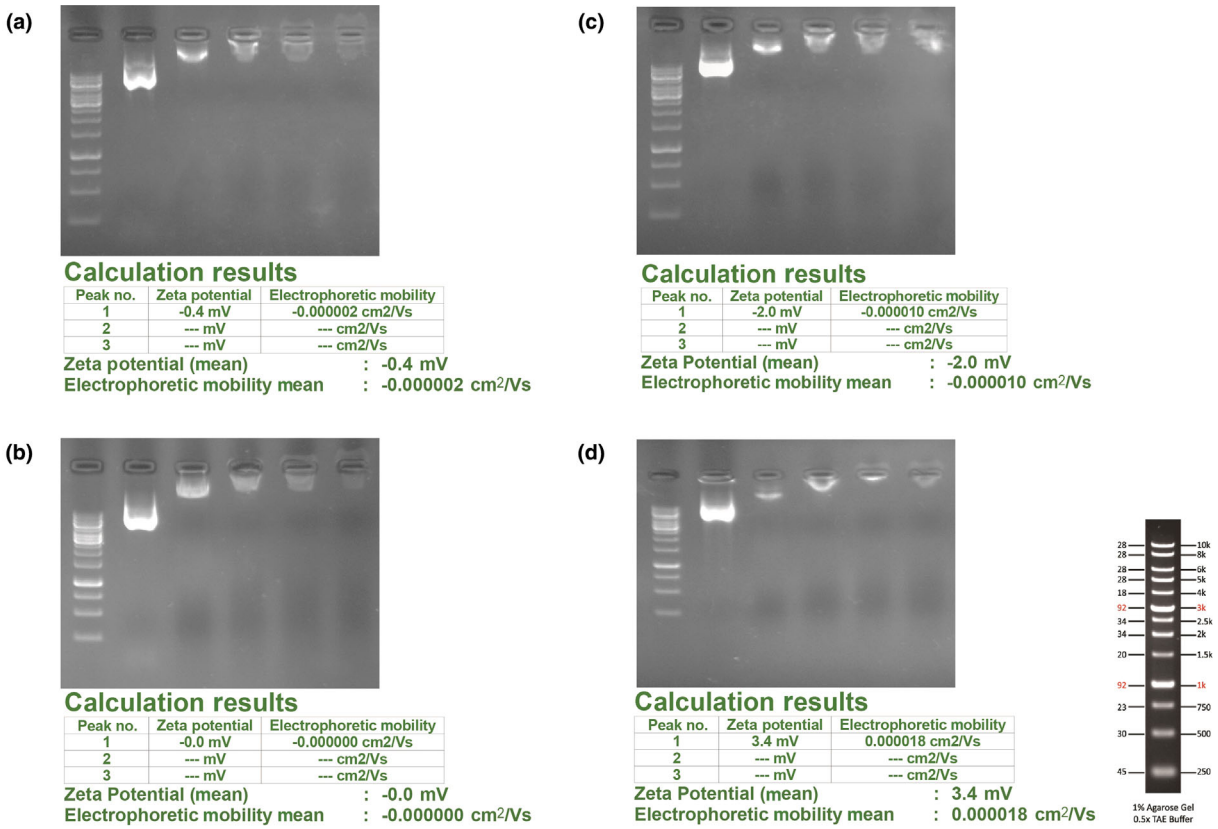

FIGURE 2 The formation of DNA/Rev complexes at different nitrogen-to-phosphate (N/P) ratios using gel retardation electrophoresis, and their zeta potential at a certain N/P ratio using dynamic light scattering analysis: (a) pcDNA3.1-C-HSP70-Nef/Rev (N/P: 20); (b) pcDNA3.1-N-HSP70-Nef/Rev (N/P: 20); (c) pcDNA3.1-Nef/Rev (N/P: 15); and (d) pcDNA3.1-HSP27-Nef/Rev (N/P: 20) nanoparticles.

zeta potential of Rev/DNA complexes at the desired N/P ratios. The peptide/DNA complex exhibited protection against DNase I degradation and serum proteases at certain N/P ratios. Indeed, agarose gel electrophoresis revealed that the DNA recovered from nanoparticles remained structurally intact.

Efficiency of gene transfer mediated by rev-based nanoparticles

Based on the MTT results, no cytotoxic effects were observed in the cells transfected with Rev-based nanoparticles over a 48 h period. Moreover, Western blotting revealed the presence of dominant bands of approximately 42 kDa for C-Hsp70-Nef, 70 kDa for N-Hsp70-Nef, 27 kDa for Nef, and 54 kDa for Hsp27-Nef at 48 h post-transfection using anti-Nef monoclonal antibody. These corresponding bands were not present in the untransfected cells, confirming the targeted generation of the desired proteins solely in the transfected cells **(**Figure 3**).**

Generation of the recombinant proteins

The expression of recombinant C-Hsp70-Nef and N-Hsp70-Nef proteins was evaluated in *E. coli* Rosetta and BL21 strains. Our data showed that both recombinant proteins were expressed in *E. coli* Rosetta strain using 1 mM IPTG at 37°C. The recombinant C-Hsp70-Nef was expressed at 4 h post-induction, but the recombinant N-Hsp70-Nef was expressed at 16 h post-induction. The purification of recombinant proteins was performed through the denaturing method (8 M urea buffer and pH = 4.5) and migrated as the clear bands of ∼42 for C-Hsp70-Nef and ∼ 70 kDa for N-Hsp70-Nef in sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis **(**Figure 4a,b**).** Moreover, the purified proteins were detectable using anti-Nef monoclonal antibody in Western blotting, as indicated in Figure 4c. On the other hand, the purified Nef and Hsp27-Nef proteins migrated as clear bands of ∼27 kDa and ∼54 kDa in SDS-PAGE, respectively, as previously reported [20]. The concentration of the recombinant proteins was in the range 0.7–1 mg/mL.

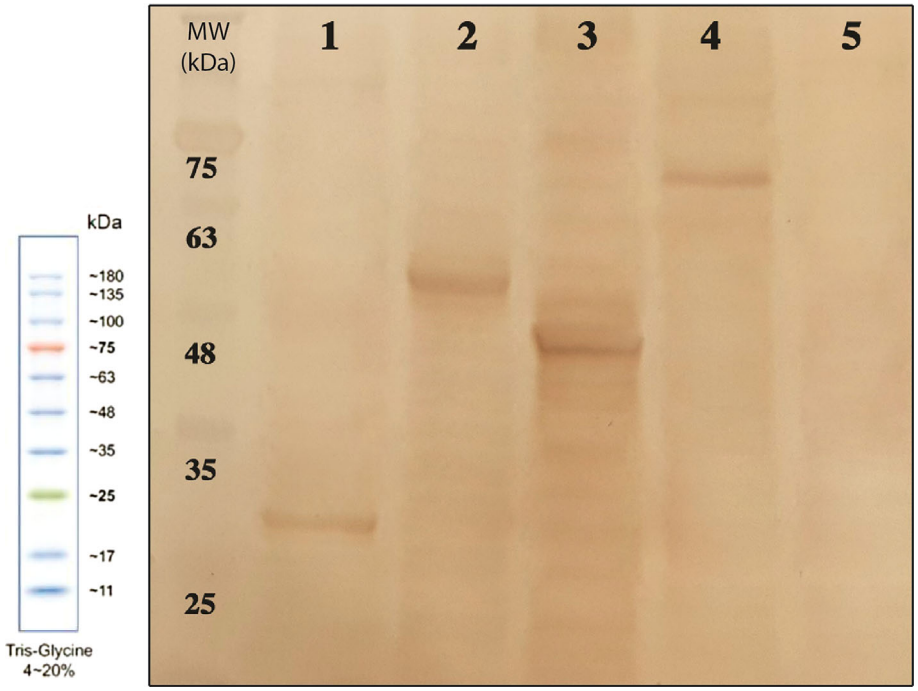

FIGURE 3 Analysis of gene transfer into the HEK-293 T cells by Rev-based nanoparticles using Western blotting. The expression of Nef (lane 1: ∼27 kDa), HSP27-Nef (lane 2: ∼54 kDa), C-HSP70-Nef (lane 3, ∼42 kDa) and N-HSP70-Nef (lane 4, ∼70 kDa) proteins was monitored by Western analysis using an anti-Nef monoclonal antibody as compared with the untransfected cells (lane 5). MW, molecular weight marker (prestained protein ladder, 10–180 kDa; Thermo Scientific).

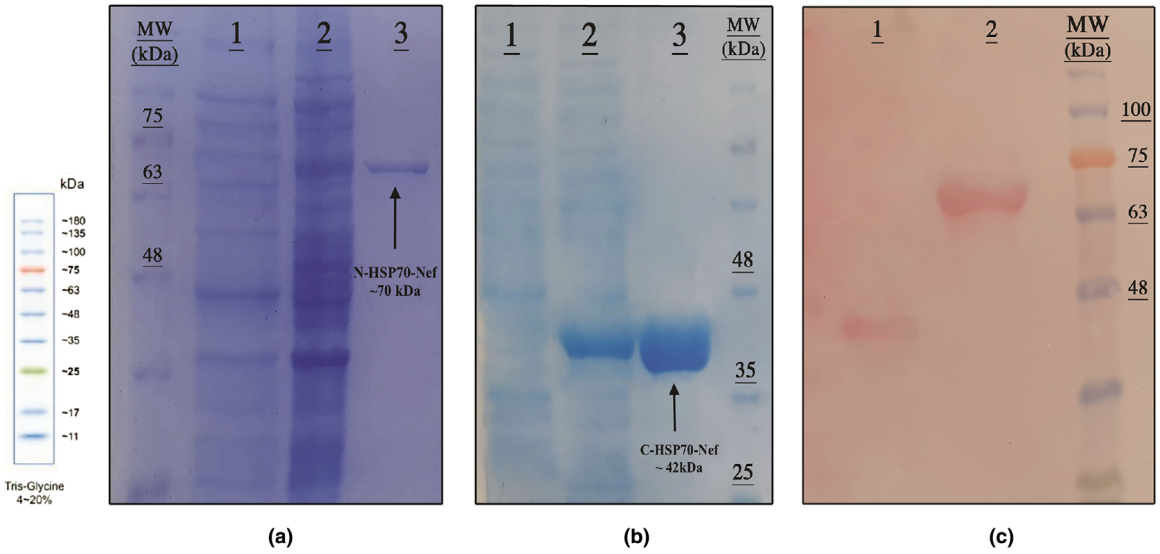

FIGURE 4 Generation of the recombinant proteins in Rosetta strain using sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. (a) Recombinant N-Hsp70-Nef protein; (b) recombinant C-Hsp70-Nef protein (lane 1, before induction; lane 2, after induction; lane 3, purified protein); (c) identification of the recombinant proteins by Western blotting using anti-Nef monoclonal antibody [lane 1, purified C-Hsp70-Nef protein (∼42 kDa); lane 2, purified N-HSP70-Nef protein (∼70 kDa)]. MW, molecular weight marker (prestained protein ladder, 10–180 kDa, Thermo Scientific).

Evaluation of in vitro cytokine assay

To assess the immunostimulatory effects of the recombinant proteins prior to performing in vivo experiments, an in vitro analysis of cytokine secretion was conducted using mouse DCs, splenocytes and co-culturing of DCs with splenocytes. As depicted in Figure 5(a), our findings indicated that DCs exposed to the recombinant proteins

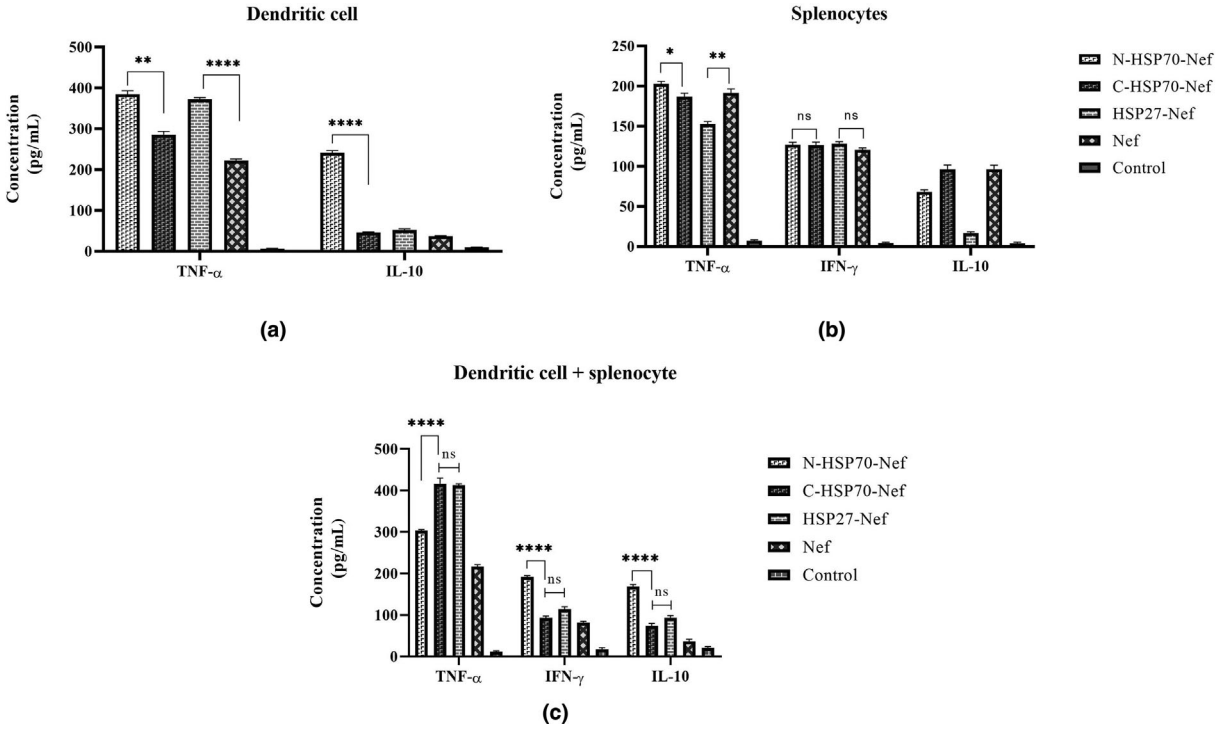

FIGURE 5 In vitro secretion of cytokines from dendritic cells (a) and splenocytes (b) exposed to the recombinant proteins, and splenocytes co-cultured with the recombinant protein-exposed dendritic cells (c) was evaluated using a Sandwich-based enzyme-linked immunosorbent assay (ELISA). The unexposed cells to the recombinant proteins were considered as controls. All analyses were performed in duplicate for each sample (ns, non-significant; *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

could significantly induce the secretion of TNF-α and IL-10 cytokines as compared to the control group (unexposed DC cells; $p < 0.05$). Furthermore, the splenocytes exposed to the recombinant proteins could secrete TNF-α, IFN-γ and IL-10 cytokines to a greater extent than the control group (unexposed DC cells; $p < 0.05$; Figure 5b). However, the levels of TNF-α and IFN-γ cytokines were higher than the level of IL-10 cytokine in the cells exposed to the recombinant proteins ($p < 0.05$). These results demonstrated the immunostimulatory effects of the recombinant proteins on immune cells such as DCs and splenocytes. Evaluation of the immunostimulatory effects of the recombinant proteins on interaction between DCs and splenocytes revealed that the C-Hsp70-Nef protein could induce higher secretion of TNF-α cytokine than the N-Hsp70-Nef protein ($p < 0.0001$), but no significant difference in TNF-α secretion was observed between the C-Hsp70-Nef and Hsp27-Nef proteins ($p > 0.05$). Moreover, the N-Hsp70-Nef protein could elicit more IFN-γ secretion than the C-Hsp70-Nef and Hsp27-Nef proteins ($p < 0.0001$). The N-Hsp70-Nef protein elicited a higher level of IL-10 than the Hsp27-Nef and C-Hsp70-Nef proteins as well ($p < 0.0001$). However, all these recombinant proteins induced lower IL-10 level than TNF-α and IFN-γ levels ($p < 0.05$). Additionally, there was no notable difference detected in the secretion of IL-10 between C-Hsp70-Nef and Hsp27-Nef proteins ($p > 0.05$; Figure 5c**)**.

Evaluation of humoral immune responses

The levels of total IgG, IgG2a and IgG1 antibodies were evaluated at 3 weeks after the last immunization using ELISA. Our data indicated that in response to the Nef antigen, the C-Hsp70-Nef regimen (G4) elicited higher levels of total IgG, IgG2a and IgG1 than the N-Hsp70-Nef and Hsp27-Nef regimens (G2 & G3; $p < 0.0001$). Additionally, the N-Hsp70-Nef regimen (G3) showed higher secretion of IgG1 than the Hsp27-Nef regimen (G2) in immunized mice ($p < 0.0001$), but no significant difference was observed between the N-Hsp70-Nef and Hsp27-Nef regimens in total IgG and IgG2a secretion (G2 & G3; $p > 0.05$, Figure 6**)**. The ratios of IgG2a/IgG1 for groups receiving Hsp27-Nef (G2), N-Hsp70-Nef (G3) and C-Hsp70-Nef (G4) regimens were ∼ 0.7, ∼ 0.4 and ∼ 0.6, respectively. These data indicated that the N-Hsp70-Nef regimen (G2) had a greater impact on the secretion of IgG1 compared with IgG2a in the immunized groups, suggesting its influence in the induction of a humoral immune response. Generally, the C-Hsp70, N-Hsp70 and Hsp27 enhanced Nef-specific antibodies to a

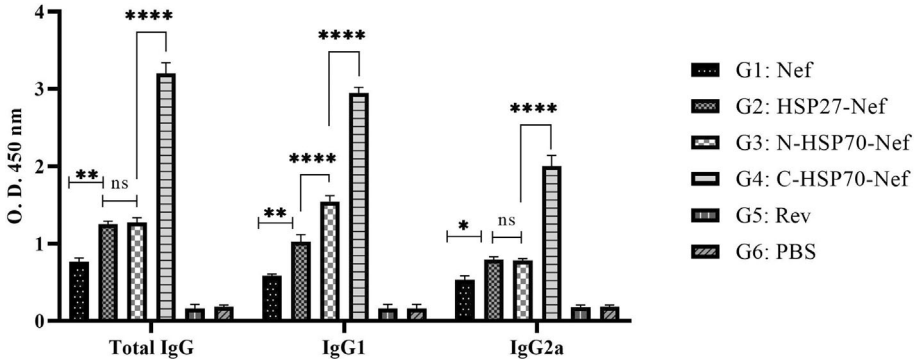

FIGURE 6 Antibody responses (total IgG, IgG1, and IgG2a) in various regimens were assessed by an indirect enzyme-linked immunosorbent assay (ELISA) against Nef-coated antigen. Each sample was analysed in duplicate, and the results were presented as the mean absorbance at 450 nm ± SD (ns, non-significant; *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

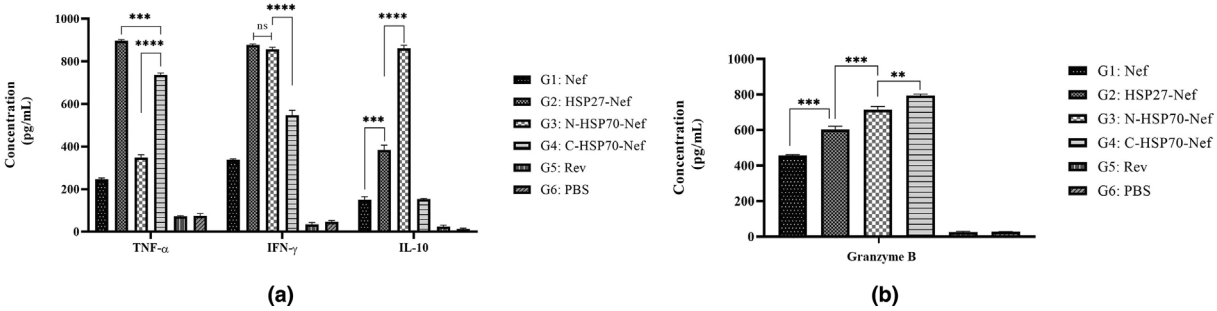

FIGURE 7 The secretion of interferon gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α) and interleukin 10 (IL-10) cytokines (a) and Granzyme B (b) by the restimulated splenocytes with Nef antigen using a sandwich-based enzyme-linked immunosorbent assay (ELISA). All analyses were performed in duplicate for each sample (ns, non-significant; **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

significantly greater degree than the Nef regimen alone (G1, $p < 0.05$; Figure 6).

Evaluation of cellular immune responses

Cellular immune responses in immunized mice were evaluated by measuring TNF-α, IFN-γ and IL-10 secretion. In general, the data suggested that all Hsp candidates (G2-G4) had the potential to enhance the secretion of cytokines **(**Figure 7a**)**. In this regard, our results showed that there was no significant difference between the N-Hsp70-Nef and Hsp27-Nef regimens (G2 and G3) in secretion of IFN-γ ($p > 0.05$), but these levels were higher than the C-Hsp70-Nef regimen (G4, $p < 0.0001$). By contrast, both the C-Hsp70-Nef and Hsp27-Nef regimens (G4 and G2) could elicit more TNF-α secretion than the N-Hsp70-Nef regimen (G3; $p < 0.0001$). Moreover, the N-Hsp70-Nef regimen (G3) was superior in IL-10 secretion in comparison with other groups ($p < 0.0001$). The group immunized with Hsp27-Nef regimen (G2) showed higher IL-10 secretion than the group immunized with C-Hsp70-Nef regimen **(**G4; Figure 7a**)**. The ratios of IFN-γ/IL-10 for the Hsp27-Nef (G2), N-Hsp70-Nef (G3) and C-Hsp70-Nef (G4) regimens were ∼ 2.3, ∼ 1 and ∼ 3.7, respectively. Additionally, the ratios of TNF-α/IL-10 for the Hsp27-Nef (G2), N-Hsp70-Nef (G3) and C-Hsp70-Nef (G4) regimens were ∼ 2.4, ∼ 0.4 and ∼ 4.9, respectively. These results indicated that the C-Hsp70-Nef regimen (G4) has more potential in inducing cellular immune responses than the Hsp27-Nef and N-Hsp70-Nef regimens (G2 and G3). All Hsp formulations could significantly induce the secretion of IFN-γ and TNF-α cytokines as compared with the Nef antigen alone (G1; $p < 0.001$).

Evaluation of Granzyme B secretion

Three weeks after the last immunization, the findings exhibited that the group immunized with the C-Hsp70-Nef regimen (G4) generated a notably greater concentration of Granzyme B (CTL activity) compared with other groups ($p < 0.01$; Figure 7b). Additionally, the secretion of Granzyme B was significantly elevated in the group immunized with the N-Hsp70-Nef regimen (G3) in comparison with

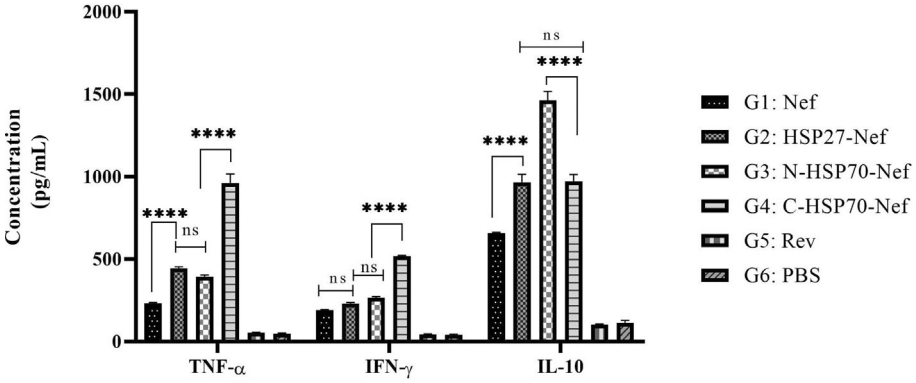

FIGURE 8 The secretion of cytokines by single-cycle replicable (SCR) HIV-1-infected splenocytes compared with control groups. All analyses were performed in duplicate for each sample (ns, non-significant; ****$p < 0.0001$).

the Hsp27-Nef regimen (G2; $p < 0.001$). All Hsp formulations induced the secretion of Granzyme B to a significantly greater degree than the Nef antigen alone (G1; $p < 0.001$).

Evaluation of in vitro virion-specific cytokine

The secretion of cytokines from the pooled splenocytes exposed to SCR virions showed that all immunized groups could significantly secrete IFN-γ, TNF-α and IL-10 cytokines in comparison with control groups (Figure 8; $p < 0.05$). The results demonstrated that the secretion of IL-10 cytokine was higher than other cytokines in all test groups. Furthermore, the secretion of TNF-α was higher than IFN-γ in all groups. Our data indicated that the secretion of IL-10 cytokine from splenocytes was higher in the N-Hsp70-Nef group (G3) than in the C-Hsp70-Nef and Hsp27-Nef groups (G4 and G2) when exposed to SCR ($p < 0.0001$). Furthermore, mice immunized with the C-Hsp70-Nef regimen (G4) exhibited a higher level of TNF-α secretion than mice immunized with the N-Hsp70-Nef regimen ($p < 0.0001$). The secretion of IFN-γ cytokine was higher in the C-Hsp70-Nef group (G4) than in the N-Hsp70-Nef group (G3; $p < 0.0001$; Figure 8**).**

DISCUSSION

Recombinant vaccines including proteins and DNA are two prominent approaches in the field of vaccinology that have shown great promise in the prevention and control of infectious diseases. The primary aim of this research was to evaluate the immunostimulatory efficacy of two cytoplasmic Hsp70 fragments and the full length of Hsp27 protein in a heterologous DNA/protein regimen targeted towards HIV-1 Nef antigen. Moreover, the in vitro potency of mice splenocytes exposed to SCR HIV-1 virions was evaluated by measuring TNF-α, IFN-γ and IL-10 secretion.

The heterologous DNA prime/protein boost strategy is an innovative approach in vaccinology to enhance immune response against infectious diseases. DNA vaccines induce lasting immunological memory, whereas protein-based vaccines provide rapid and robust responses [23, 24]. The combination of the DNA and protein regimens can potentially lead to durable protection against the target pathogen. This approach is adaptable and applicable to a wide range of infectious diseases, including viral infections (e.g. HIV, influenza, SARS-CoV-2 and bacterial infections) [25, 26].

In vaccine development, adjuvants could play a crucial role in enhancing the immune response. In this regard, heat shock proteins (HSPs) could offer a promising avenue for improving vaccine efficacy and immunogenicity as a potential adjuvant. Early studies showed that HSP families like HSP70 or HSP90 were able to produce specific anti-tumour immunity [27]. Subsequently, the potency of HSPs was shown for binding to antigenic peptides, thereby aiding in the presentation of antigens by the major histocompatibility complex class I (MHC I) [28], and finally activation of CD8 T cells [29]. Several studies have highlighted that the immune functions of heat shock proteins are within specific regions (i.e. the N-terminal and C-terminal fragments) named as mini-chaperones. These influential regions of HSPs play a pivotal role in the design and formulation of diverse vaccines, encompassing DNA-based vaccines as well as protein or peptide-based vaccines. Remarkably, heat shock protein 70 has emerged as a highly promising candidate due to its ability to facilitate the cross-presentation of tumour-associated antigens (TAAs) by APCs, thereby

eliciting a potent and robust T-cell-mediated immune response [30]. The HSP70 family has an N-terminal ATPase domain (nucleotide-binding domain) and a C-terminal substrate-binding domain [31]. *Mycobacterium tuberculosis* Hsp70, abbreviated as m. Hsp70, holds a significant role in immunity due to its ability to trigger the innate immune system and induce targeted immune responses against both tumours and viral infections. According to the results of an in silico analysis, the multi-epitope vaccine containing T-cell epitopes from E5 and E7 proteins of HPV16/18 linked to the C-terminal domain of Hsp70 was structurally stable, non-allergic and non-toxic [32]. In this regard, in an hepatitis B virus (HBV) transgenic mouse model, the CT-Hsp70 (aa 359–610) fusion vaccine could enhance HBsAg-specific CTL responses, facilitate clearance of circulating HBsAg and down-regulate HBV replication [33]. Another study indicated that the fusion DNA vaccine carrying CT-Hsp70 linked to MPT51 antigen led to a greater production of MPT51-specific IFN-γ by CD4 T cells compared with MPT51 or MPT51-NT-Hsp70 DNA vaccines. These findings indicated that the C-terminal domain could stimulate a more robust antigen-specific Th1 immune response than DNA antigen alone [34]. It is interesting that mice do not exhibit substantial baseline expression of Hsp70, but they show induction of this protein in response to the same stimuli observed in humans. Furthermore, the genes responsible for encoding Hsp70 (HSPA1A) in humans and mice share a high degree of similarity [35]. Moreover, the anti-tumour efficacy of a DNA vaccination based on mouse Hsp70 in targeting a tumour-associated antigen was assessed, focusing on the HPV type 16 E7 protein. By attaching E7 to the N-terminus of mouse Hsp70, this approach not only induced a specific CTL response against E7 but also conferred protection to mice against tumours expressing E7 [13].

Based on these data, the origin of Hsp70 and the target antigen may have different effects on the adjuvanticity of Hsp70 domains. In this study, we evaluated the effects of both N-terminal and C-terminal fragmants of Hsp70 (cytoplasmic HspA1A) in enhancing HIV-1 Nef-specific immune responses. Our in vitro and in vivo findings demonstrated that C-Hsp70 triggered a higher secretion of TNF-α compared with N-Hsp70 in response to the Nef antigen ($p < 0.0001$). Additionally, the levels of total IgG and IgG2a secretion were higher in the group immunized with C-Hsp70-Nef (G4) than in the group immunized with N-Hsp70-Nef (G3; $p < 0.0001$). These data suggest that C-Hsp70 significantly promotes immune response towards Th1 cellular immunity. Moreover, the C-Hsp70-Nef regimen (G4) induced higher secretion of Granzyme B than the N-Hsp70-Nef regimen (G3) in response to the Nef antigen ($p < 0.01$), indicating more CTL activity.

Heat shock protein 27 (Hsp27) has been extensively studied among the small HSP family. Interestingly, elevated levels of Hsp27 were observed in various diseases, including renal injury, renal fibrosis, cancer, cardiovascular disease, neurodegenerative disease and neuronal injury [36]. Hsp27 has also been implicated in viral infections. In the cells infected with human adenovirus, Hsp27, p38 MAPK and NFκB-p65 formed a signalling complex that influenced downstream pro-inflammatory mediators [37]. Hsp27, along with other HSPs such as Hsp70, has been recognized as a potent adjuvant for stimulating the immune system against cancer and infectious diseases [9, 38]. Furthermore, the engagement between Hsp27 and TLRs triggers subsequent signalling pathways that lead to the activation of NF-κB, and ultimately cell maturation and the release of Th1-type cytokines [18, 39, 40]. In this regard, another goal of this study was the comparison of immunostimulatory properties of Hsp27 with the N-terminal and C- terminal of Hsp70. Our in vitro results (i.e. co-culturing the pulsed DCs with splenocytes) showed that the Hsp27-Nef protein could induce higher secretion of TNF-α cytokine than the N-Hsp70-Nef protein ($p < 0.0001$), but there was no significant difference between the Hsp27-Nef and C-Hsp70-Nef proteins ($p > 0.05$). By contrast, the N-Hsp70-Nef protein could elicit more IFN-γ and IL-10 levels than the C-Hsp70-Nef and Hsp27-Nef proteins in vitro ($p < 0.0001$). As is known, IL-10 is an anti-inflammatory cytokine that suppresses IFN-γ production through its effects on macrophages and dendritic cells, thus controlling immunopathology [41]. However, IL-10 cytokine has both inhibitory and stimulatory effects on IFN-γ production, depending on the specific context and immune environment. In some cases, IL-10 can also promote the production of IFN-γ. This occurs when the immune system needs to mount a robust response against a particular pathogen or when IFN-γ is necessary for other immune functions [42, 43]. In relation to this matter, the elevated levels of IFN-γ and IL-10 in the peripheral blood mononuclear cells of individuals suffering from systemic lupus erythematosus (SLE) provided evidence that IFN-γ and IL-10 may play a role in the increased B-cell activity, the presence of polyclonal hypergammaglobulinemia, and the production of autoantibodies observed in SLE [44]. Moreover, emergency myelopoiesis is essential to control infection, characterized by inflammation-induced haematopoiesis to replenish immune cells in the periphery. Cardoso and colleagues demonstrated an unexpected finding that IL-10 has the capacity to alter the activation state of CD4 and CD8 T cells in vivo, leading to the production of IFN-γ by these specific T-cell subsets. This discovery expands our understanding of IL-10 beyond its well-known anti-inflammatory properties, suggesting that it can also

stimulate IFN-γ production. This novel insight opens new possibilities for the development of immunotherapies based on IL-10 [45]. In this context, our current study has revealed intriguing findings regarding the impact of the N-Hsp70 fragment on high secretion of IFN-γ and IL-10, both in vitro and in vivo. Although the precise underlying mechanism remains elusive, our data suggest a synergistic effect. To elucidate this phenomenon, further investigations are warranted, and additional research is needed to unravel the underlying reasons behind this intriguing observation. In vivo studies showed that the N-Hsp70-Nef regimen could significantly induce Granzyme B secretion as compared with the Hsp27-Nef regimen with respect to Nef antigen ($p < 0.001$), suggesting higher CTL activity.

Besides the main roles of HSPs as an adjuvant in vaccine improvement, the involvement of CPPs in vaccine delivery systems showed the potential to enhance the uptake of antigens by APCs, making it a viable and safe option to complement traditional adjuvant formulations [4, 46, 47]. The abundance of arginine residues in various DNA/RNA-binding segments has been suggested. In this regard, the internalization efficiency of the RNA-binding segment derived from HIV-1 Rev protein (Rev peptide: aa 34–50) was shown by transfection of COS-7 cells with the plasmid as efficiently as polyarginine and polylysine [48]. Moreover, Futaki et al. found that various arginine-rich peptides like HIV-1 Rev (aa 34–50) have a translocation activity very similar to Tat peptide (aa 48–60) in mouse macrophage RAW264.7 cells. Based on these promising findings, we utilized HIV-1 Rev peptide (34–50) as a carrier for delivery of antigens in our DNA immunization regimen for the first time. The analysis of the interaction between the positive charge of the Rev peptide and the negative charge of DNA constructs confirmed the DNA/CPP complex formation at different N/P ratios, preventing DNA migration into agarose gel. Additionally, SEM revealed that the diameter of Rev/DNA complexes was in the range 150–250 nm. No significant nanoparticle-associated toxicity was observed in the transfected HEK-293 T cells. In vitro successful gene delivery of Rev-based nanoparticles and their stability and safety confirmed their use for in vivo studies. Moreover, these nanoparticles help to use lower amounts of DNA (10 μg) compared with the naked DNA (50 or 100 μg).

Single-cycle replicable virions offer a controlled and safe approach to study viral replication and antigen expression in host cells without the risks associated with wild-type viruses [49]. They are valuable tools in virology research and vaccine development, enabling investigations into viral pathogenesis, immune responses and antiviral drugs. In situations where an HIV animal model is unavailable or impractical, SCR viruses serve as useful alternatives, providing valuable insights into viral biology and facilitating the development of targeted therapeutics and vaccines [49]. In this investigation, 3 weeks after the last immunization, the pooled splenocytes of each group were exposed to SCR virions. The results of cytokine secretion showed that the secretion of IL-10 was greater than that of other cytokines in all test groups. Elevated IL-10 levels have been detected in individuals with HIV infection, and the reports have indicated that blocking IL-10 can improve T-cell function in certain HIV-infected individuals [50]. Two points should be considered in our findings. First, we did not restimulate the splenocytes with antigens. Indeed, restimulation with antigens was replaced with the SCR exposure for detecting the active lymphocytes and memory maintenance. Second, it was interesting that the C-Hsp70-Nef regimen (G4) maintained the levels of TNF-α and IFN-γ cytokines. Indeed, the secretion of TNF-α was higher in the splenocytes exposed to SCR than in those restimulated with antigen in this group (G4). However, the IL-10 secretion was higher in the N-Hsp70-Nef regimen than in the C-Hsp70-Nef and Hsp27-Nef regimens after SCR exposure, similar to the results of restimulated splenocytes with each antigen (unexposed groups with SCR virions; $p < 0.0001$). Thus, increasing IL-10 secretion after SCR exposure in these regimens and their potential as a therapeutic agent should be further studied in animal models. Overall, among the vaccine constructs, the C-Hsp70-Nef regimen could further activate T-cells and maintain their activity against viral infection in vitro.

In summary, our findings reveal that both N- and C-terminal fragments of Hsp70 can serve as adjuvants, enhancing immune responses against HIV-1 Nef antigen. However, Hsp27 and the C-terminal fragment of Hsp70 have a more pronounced influence on cellular immune responses than the N-terminal fragment of Hsp70 in response to Nef antigen. Moreover, the HIV-1 Rev peptide was successfully used for DNA delivery and its protection in both in vitro and in vivo settings. On the other hand, the studies of SCR exposure to splenocytes showed higher efficiency of the C-Hsp70-Nef regimen in cytokine secretion (higher TNF-α and IFN-γ levels and lower IL-10 level) than of the N-Hsp70-Nef and Hsp27-Nef regimens, indicating its use as a promising vaccine candidate in the next experiments in vivo.

AUTHOR CONTRIBUTIONS

AM: project administration, methodology, data curation, writing – original draft; EA: methodology, data curation; PMP: project administration; FJ: investigation; SG: validation, visualization; SAS: validation, visualization; AB: supervision, validation, writing – review and editing. All authors approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

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 STATEMENT

All data are available in the manuscript.

ETHICAL STATEMENT

The entire procedure was carried out in accordance with the approval protocols and ethical standards for laboratory animal care established by the Research Ethics Committees at the Pasteur Institute of Iran, in adherence to national guidelines, for scientific research purposes (ethics code: IR.PII.REC.1400.059; approval date: 1 November 2021).

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