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Research Article

Immunopotentiation by linking Hsp70 T-cell epitopes to Gag-Pol-Env-Nef-Rev multiepitope construct and increased IFN-gamma secretion in infected lymphocytes

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One sentence summary: General view of immunopotentiation by linking Hsp70 T-cell epitopes to Gag-Pol-Env-Nef-Rev multiepitope construct.
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Abstract

Therapeutic human immunodeficiency virus (HIV) vaccines can boost the anti-HIV host immunity to control viral replication and eliminate viral reservoirs in the absence of anti-retroviral therapy. In this study, two computationally designed multiepitope Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev constructs harboring immunogenic and highly conserved HIV T cell epitopes were generated in *E. coli* as polypeptide vaccine candidates. Furthermore, the multiepitope *gag-pol-env-nef-rev* and *hsp70-gag-pol-env-nef-rev* DNA vaccine constructs were prepared and complexed with MPG cell-penetrating peptide. The immunogenicity of the multiepitope constructs were evaluated using the homologous and heterologous prime/boost strategies in mice. Moreover, the secretion of IFN-γ was assessed in infected lymphocytes *in vitro*. Our data showed that the homologous polypeptide regimens could significantly induce a mixture of IgG1 and IgG2a antibody responses, activate T cells to secret IFN-γ, IL-5, IL-10, and generate Granzyme B. Moreover, *IFN-γ secretion was significantly enhanced in* single-cycle replicable (SCR) HIV-1 virions-*infected splenocytes in these groups compared to uninfected splenocytes. The linkage of* heat shock protein 70 *(Hsp70) epitopes to Gag-Pol-Env-Nef-Rev polypeptide in the homologous regimen increased significantly cytokines and Granzyme B levels, and IFN-γ secretion in virions-infected splenocytes.* Briefly, both designed constructs in the homologous regimens can be used as a promising vaccine candidate against HIV infection.

Keywords: Human immunodeficiency virus, multiepitope vaccine, cell penetrating peptide, MPG, heat shock protein 70, therapeutic vaccine

Introduction

In most human immunodeficiency virus (HIV)-infected people, the antiretroviral therapy (ART) can transform HIV into a manageable disease for a long time. However, ART can not eliminate the HIV reservoir from the body or cure the infection (Simon et al. 2006, Fidler et al. 2020). Therapeutic vaccination against HIV is an alternative strategy to eradicate HIV infection and enhance HIV-specific immune responses (Fomsgaard 2015, Chen and Julg 2020). Among different therapeutic vaccine platforms, subunit vaccines composed of the essential antigenic proteins are regarded safe due to their simplicity and low toxicity (Moyle 2017). Subunit peptide-based vaccine employs immunogenic epitopes of the target pathogens and can be generated by either genetic engineering or chemical synthesis easily (Forner et al. 2021). As reported, preclinical and clinical trials often focus on novel therapeutic vaccines that stimulate T-cell responses to the specific regions of HIV (Chen and Julg 2020). Indeed, optimization of vaccine immunogens design is an essential step for therapeutic vaccine development due to the high genetic diversity of HIV and its mutational escape from host immune responses (Gomes et al. 2020). Moreover, some parts of the HIV proteome are unnecessary for achieving full protection and lead to unwanted responses (Barouch 2008). In this sense, more attention should be given to peptide-based vaccines containing highly immunogenic epitopes of HIV and avoid of decoy epitopes (Barouch et al. 2010, Abdul-Jawad et al. 2016). The computationally designed vaccines are prepared in a way to cover multiepitope immunogens and are usually stable and highly immunogenic. These types of vaccines contain potential T-cell epitopes (PTEs) derived from various HIV antigens in form of multiple-antigenic polypeptide or mosaic immunogen. Moreover, these vaccines may carry long peptides harboring multiple overlapping epitopes or minimal-length epitopes (Korber and Fischer 2020). From fifteen HIV proteins, the Gag, Pol, and Nef are potential targets of the cytotoxic T lymphocytes (CTLs), and Env is the main object of humoral and cellular immunity (Kong et al. 2003, Chen and Julg 2020). There is a primary association between reduced infection risk and high levels of Env-specific $CD8^+$ T cell immunity (Prebensen et al. 2016). The Gag, Pol, Env, and Nef

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are the major proteins produced during viral infection. The conserved regions of these four proteins could induce high levels of specific $CD8^+$ and $CD4^+$ T cells, and interferon-gamma (IFN-γ) response in volunteers (Korber and Fischer 2020). Also, the Rev regulatory protein has functional regions that are relatively conserved among HIV-1 clades and can be applied in vaccine design (Yu et al. 2005). In a phase Ib clinical report, a peptide-based vaccine containing T-cell epitopes of the conserved Rev, Vif, Vpr, and Nef regions could elicit both cellular and humoral immunity in 45% of vaccinated individuals (Boffito et al. 2013). Furthermore, we previously designed two multiepitope HIV-1 immunogens composed of long peptides harboring various immunogenic T-cell epitopes of HIV-1 proteins (i.e. Gag, Pol, Env, Nef, & Rev) using bioinformatics prediction tools. These designed immunogens were restricted to frequent human leukocyte antigen (HLA) supertypes, and had high conservancy and population coverage in worldwide (Akbari et al. 2021). On the other hand, due to poor immunogenicity of multiepitope subunit vaccines (Li et al. 2014), we used the conserved and functional multiepitope long peptides of human heat shock protein 70 (Hsp70) linked to the designed construct (i.e. Gag-Pol-Env-Nef-Rev) as a potential immuno-stimulatory agent. Hsp70 has inherent adjuvant and immunogenic properties. It is capable of targeting associated viral peptides into antigen presenting cells (APCs) and promoting antigen processing and antiviral immunity (Moyle 2017). Furthermore, employing different delivery systems and prime/boost strategies were recommended to overcome the poor immunogenicity of subunit vaccines. Cell-penetrating peptides (CPPs, also known as protein transduction domains) were shown as one of the solutions to this problem (McClorey and Banerjee 2018). Among CPPs, a short amphipathic peptide, MPG, was known as an efficient DNA delivery system (Deshayes et al. 2008).

In the current study, two previously designed multiepitope Hsp70-Gag-Pol-Env-Nef-Rev and Gag-Pol-Env-Nef-Rev constructs containing several immunogenic major histocompatibility complex-I (MHC-I) and MHC-II binding epitopes (Akbari et al. 2021) were generated as polypeptide and DNA vaccine candidate constructs. Their immunogenicity (i.e. antibody secretion, lymphocyte proliferation, cytokine release, and Granzyme B secretion) was evaluated in the homologous (polypeptide prime/polypeptide boost) and heterologous (DNA prime/polypeptide boost) strategies. Moreover, the secretion of IFN-gamma was investigated in uninfected and single-cycle replicable (SCR) HIV-1 virions-infected lymphocytes re-stimulated by the recombinant Gag-Pol-Env-Nef-Rev polypeptide *in vitro*. The schematic model of study was shown in Graphical abstract.

Materials and methods

In silico design and synthesis of the multiepitope DNA construct

The non-toxic, non-allergen, immunogen, and highly conserved MHC-I and MHC-II binding epitopes from Gag, Pol, Env, Nef, Rev, and Hsp70 proteins had been predicted by *in silico* tools as described previously (Akbari et al. 2021). The final selected CTL and HTL (helper T lymphocyte) epitopes were joined by the AAY proteolytic linker (*i.e.* Hsp70-Gag-Pol-Env-Nef-Rev construct). The physicochemical and structural properties were predicted for the designed constructs (Hsp70-Gag-Pol-Env-Nef-Rev, and Gag-Pol-Env-Nef-Rev) (Akbari et al. 2021). The nucleotide sequence of the Hsp70-Gag-Pol-Env-Nef-Rev construct with appropriate restriction enzyme sites was synthesized commercially in pUC57 cloning vector (Biomatik Corporation, Canada) as described in our previous report (Akbari et al. 2021).

Construction of the recombinant expression vectors

The gag-*pol-env-nef-rev* and *hsp70-gag-pol-env-nef-rev* gene fragments were sub-cloned from pUC57 vector into the pET-24a (+) prokaryotic expression vector (Novagen) and also into the pcDNA 3.1 (−) eukaryotic expression vector (Invitrogen) using *Eco*RI/*Hind*III and *Bam*HI/*Hind*III (Thermo Fisher Scientific, Germany) sites, respectively. Then, ligation products were transformed into the DH5α *E. coli* strain, and the correct cloning procedures were validated by plasmid restriction mapping and sequencing. Finally, four recombinant plasmids were purified by an Endo-free plasmid Mega kit (Qiagen, Germany), and their concentration and purity were evaluated by NanoDrop spectrophotometer.

Expression of the recombinant multiepitope polypeptides

To express the recombinant multiepitope polypeptides in bacteria, the recombinant pET-24a(+)-*gag-pol-env-nef-rev* and pET-24a(+)-*hsp70-gag-pol-env-nef-rev* were transformed into the BL21 (DE3) and Rosetta *E. coli* strains. A single recombinant kanamycin-resistant colony from each strain was selected and grown in LB/kanamycin medium (Sigma, Germany) at 37°C overnight in a shaker incubator (150 rpm). Then, each culture was inoculated in the fresh 2xYT medium (Peptone 1.6%, Yeast 1%, NaCl 0.5%) to an optical density (OD_{600}) of 0.6–0.8. The expression was induced by adding 1 mM Isopropyl thiogalactopyranoside (IPTG, Sigma, Germany) at 37°C, and the optimized incubation time (2, 3, 4, and 16 h after IPTG induction) was determined. The bacterial pellet was harvested, and the expression of polypeptides was evaluated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purification of the recombinant multiepitope polypeptides

To evaluate the solubility of the recombinant products, we followed the Qiagen protocol. Accordingly, after the disruption of bacterial biomass using sonication in lysis buffer (Tris/NaCl, pH 8), the resulting pellet and supernatant were separated by centrifugation. Based on the manufacturer's instructions for denaturing conditions (*i.e.* 8M urea buffer and pH 4.5), both recombinant Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev polypeptides were purified by affinity chromatography using HisPur Ni-NTA resin (Thermo Fisher Scientific, Germany). An imidazole-SDS-Zn reverse staining protocol (Simpson 2007) was subsequently used for further purification. Western blot analysis using anti-His tag antibody (Abcam, UK; 1:10 000 v/v) was used for detection of the purified polypeptides. Afterward, the purified polypeptides 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 LAL assay, contamination with lipopolysaccharide (LPS) was less than 0.5 EU/mg (QCL-1000, Lonza). The purified multiepitope polypeptides were stored at −70∘C for long-term preservation.

Preparation of the MPG/DNA nanoparticles

The synthetic MPG (GALFLGFLGAAGSTMGAWSQPKKKRKV; BioMatik Company, Canada) was used as a DNA delivery system

to form the MPG/pcDNA-*gag-pol-env-nef-rev* and MPG/pcDNA-*hsp70-gag-pol-env-nef-rev* complexes. The recombinant plasmids (2 μg) were mixed with MPG at N/P ratio of 10:1 (Davoodi et al. 2021) in PBS 1X (pH 7.4) and incubated for 1 h at room temperature. The formation of MPG/DNA complexes was confirmed by agarose gel retardation assay and Zetasizer analysis (Malvern Instruments). The stability of MPG/DNA complexes was evaluated against DNA nucleases (DNase I) (Moret et al. 2001) and serum proteases (fetal bovine serum) (Sadeghian et al. 2012) *in vitro*. Briefly, to assess the stability of MPG/DNA complexes against DNA nucleases, DNase I (Fermentas, Germany) was added to the nanoparticles with a final concentration of 1.37 U enzyme per 1 μg DNA. The mixtures were incubated at 37°C for one hour followed by the addition of stop solution (200 mM sodium chloride, 20 mM EDTA and 1% SDS; Sigma, Germany). Also, to evaluate the serum stability, the serum was added to the nanoparticles, and the mixtures were incubated for 5 h at 37°C followed by adding 10% SDS solution for DNA plasmid release. Samples were analyzed by electrophoresis on 1% agarose gel, and the integrity of the DNA plasmid was visualized and compared with the naked DNA as a control (Moret et al. 2001, Sadeghian et al. 2012).

Mice immunization

Inbred BALB/c female mice, 6–8 weeks old, were purchased from the breeding stocks maintained at Pasteur Institute of Iran. Five mice in each group were considered and immunized subcutaneously at the footpad with different regimens as shown in Table 1. All experimental procedures were in accordance with the Animal Care and Use Protocol of Pasteur Institute of Iran (national guideline) for scientific purposes (Ethics code: IR.PII.REC.1399.035; Approval date: 2020–09–02). The Montanide 720 adjuvant (Seppic S.A., France) was used for injection of the recombinant polypeptides (5 μg) at the ratio of 30:70 v/v (antigen: adjuvant). Also, the MPG at N/P ratio of 10:1 was used for the delivery of DNA constructs (5 μg) into the cells. Immunization was repeated three times with a two-week interval (Days 0, 14, & 28).

Evaluation of antibody responses

To evaluate humoral immune responses in immunized mice, the pooled sera were prepared from each group three weeks after the last injection. The levels of antigen-specific total IgG (Sigma; 1:10 000 v/v) and its subclasses including IgG1 and IgG2a (Sigma, Germany; 1:10 000 v/v) were determined in the sera (1:100 v/v) using indirect ELISA (Davoodi et al. 2021). The coated antigens were the recombinant Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev polypeptides (~ 5 μg/mL) diluted in PBS1X. Each assay was repeated in duplicates, and all results were shown as mean ± SD for each sample.

Lymphocyte proliferation and cytokine secretion assay

Three weeks after the third immunization, five mice from each group were sacrificed, and their spleen tissues were dissected. For cellular assay, 2 × 10^6 cells/mL of the pooled splenocytes without red blood cells were re-suspended in complete RPMI-1640 medium supplemented by 5% fetal bovine serum (FBS, Gibco, Germany). All samples were cultured in 48-well plates in a humidified incubator under 5% CO_2 at 37°C for 72 h and in the presence of 5 μg/mL of each recombinant polypeptide. Concanavalin A (5 μg/mL; Sigma, USA) was included as a positive control. To evaluate cytokine secretion in immunized mice, the presence of IFN-γ, IL-5, and IL-10 in supernatants of the re-stimulated spleno-

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	Polypeptide/Polypeptide/Polypeptide	rGag-Pol-Env-Nef-Rev + Montanide	rGag-Pol-Env-Nef-Rev + Montanide	rGag-Pol-Env-Nef-Rev + Montanide
G2		rHsp70-Gag-Pol-Env-Nef-Rev	rHsp70-Gag-Pol-Env-Nef-Rev	rHsp70-Gag-Pol-Env-Nef-Rev
G3	Polypeptide/Polypeptide/Polypeptide	rHsp70-Gag-Pol-Env-Nef-Rev + Montanide	rHsp70-Gag-Pol-Env-Nef-Rev + Montanide	rHsp70-Gag-Pol-Env-Nef-Rev + Montanide
G4	DNA/Polypeptide/Polypeptide	pcDNA-*gag-pol-env-nef-rev* + MPG	rGag-Pol-Env-Nef-Rev + Montanide	rGag-Pol-Env-Nef-Rev + Montanide
G5	DNA/Polypeptide/Polypeptide	pcDNA-*hsp70-gag-pol-env-nef-rev* + MPG	rHsp70-Gag-Pol-Env-Nef-Rev	rHsp70-Gag-Pol-Env-Nef-Rev
G6	DNA/Polypeptide/Polypeptide	pcDNA-*hsp70-gag-pol-env-nef-rev* + MPG	rHsp70-Gag-Pol-Env-Nef-Rev + Montanide	rHsp70-Gag-Pol-Env-Nef-Rev + Montanide
G7	Control	PBS	PBS	PBS
G8	Control (Empty vector)	pcDNA3.1	pcDNA3.1	pcDNA3.1
G9	Control (Peptide)	MPG	MPG	MPG

r: recombinant

cytes was assessed by a sandwich-based ELISA kit (Mabtech, Swedish Biotech Company) according to the manufacturer's instructions. In addition, MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; Sigma, Germany) assay was performed to determine lymphocyte proliferation. The stimulation index (SI) was measured by the optical density (OD_{570}) ratio of the re-stimulated splenocytes to unstimulated splenocytes. Each assay was repeated in triplicates and all results were shown as mean ± SD for each sample.

Granzyme B secretion assay

To determine CTL activity *in vitro*, the release of Granzyme B (GrB) from effector splenocytes (E) was assessed by ELISA. Briefly, SP2/0 target cells (T; a standard murine myeloma cell line used as a target cell; provided by the National Cell Bank, Pasteur Institute of Iran) were seeded in triplicate into 96-well plates (2×10^4 cells/well) for 24 h in the presence of 5 μg/mL of each recombinant polypeptide. The pooled splenocytes of each group were added to the target cells at an E: T ratio of 100:1 and co-cultured in RPMI-1640 medium supplemented by 10% FBS (Gibco, Germany). After 6 h incubation, the supernatants were harvested, and Granzyme B concentrations were determined using an ELISA kit (eBioscience, USA) according to the manufacturer's instructions. All results were shown as mean ± SD for each sample.

Construction of SCR HIV-1 virion, and IFN-γ secretion assay in infected lymphocytes

The ability of the re-stimulated splenocytes to secrete IFN-γ after exposure to SCR HIV-1 virions, was assessed by a sandwich-based ELISA kit (Mabtech, Swedish Biotech Company). Construction of SCR HIV-1 virions was carried out according to our previous study (Soleymani et al. 2018). In brief, the human embryonic kidney (HEK 293T) cells (provided by the National Cell Bank, Pasteur Institute of Iran) were cultured and transfected with the lipofectamine (Sigma, Germany)/plasmid complex (6 μL of lipofectamine transfection reagent with 4 μg of psPAX2, pmzNL4-3, and pMD2.G plasmids (Soleymani et al. 2018)). The produced virions were quantified by p24 ELISA assay kit (Cell Biolabs, USA) (Cavrois et al. 2004; Soleymani et al. 2018), and virus stocks were prepared and stored at −70°C. The pooled splenocytes from each group (2×10^6 cells/mL) were re-stimulated with the recombinant Gag-Pol-Env-Nef-Rev polypeptide for 24 h, and then exposed to SCR virions (MOI: 20) for 72 h. The IFN-γ secretion was assessed in the cell supernatants using a sandwich-based ELISA kit (Mabtech, Swedish Biotech Company). Each assay was repeated in duplicates and all results were shown as mean ± SD for each sample.

Statistical analysis

The final results were statistically analyzed using one-way ANOVA to evaluate differences between groups (Prism 8, GraphPad Software, USA). Data were indicated as mean ± standard deviation (SD) for each group. A *P*-value < 0.05 was statistically considered significant.

Results

Construction of the recombinant expression vectors

The Hsp70-Gag-Pol-Env-Nef-Rev multiepitope peptide construct was designed, and validated by *in silico* tools in our previous study (Akbari et al. 2021). In general, ten CTL and eight HTL epitopes from HIV-1 Gag, Pol, Env, Nef and Rev proteins, and also two CTL and two HTL epitopes of Hsp70 were selected to design the Hsp70-Gag-Pol-Env-Nef-Rev construct as shown in Supplementary Figure 1. The designed multiepitope gene constructs (i.e. *gag-pol-env-nef-rev* and *hsp70-gag-pol-env-nef-rev genes*) were successfully subcloned in pET-24a (+) and pcDNA3.1 (−) expression vectors. The presence of *gag-pol-env-nef-rev* and *hsp70-gag-pol-env-nef-rev* fragments was confirmed by enzymatic digestion as clear bands of ∼894 bp and ∼1134 bp on agarose gel, respectively (Supplementary Figure 2). The concentration of endotoxin-free pcDNA-*gag-pol-env-nef-rev* and pcDNA-*hsp70-gag-pol-env-nef-rev* was about 1.8 and 3 mg/mL, respectively. The schematic representation of subcloning steps was shown in Supplementary Figure 3.

Formation of the stable MPG/DNA complexes

The MPG peptide could form stable non-covalent nanoparticles with pcDNA-*gag-pol-env-nef-rev* and pcDNA-*hsp70-gag-pol-env-nef-rev* at N/P ratio of 10:1. Indeed, the formation of the MPG/DNA complexes was confirmed by migration inhibition of the plasmids into the agarose gel. Moreover, agarose gel electrophoresis showed that unprotected plasmid DNAs were degraded in the presence of DNase and also FBS. In contrast, the recovered DNAs from nanoparticles remained intact after DNase and serum degradation assay (Supplementary Figure 4). The size and surface charge of both MPG/DNA complexes at N/P ratio of 10:1 was ∼ 200–300 nm and ∼18–21 mV, respectively.

Expression and purification of the multiepitope polypeptides

Our data showed that both multiepitope polypeptides were expressed in pET-24a/Rosetta or pET-24a/BL21 prokaryotic systems. The higher yield of *gag-pol-env-nef-rev* gene expression was observed in BL21 (DE3) strain at 16 h post-induction as compared to Rosetta strain and other incubation times. In contrast, a higher yield of *hsp70-gag-pol-env-nef-rev* gene expression was detected in the Rosetta strain at 4 h post-induction as compared to BL21 (DE3) strain and other incubation times. The recombinant *Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev* polypeptides were successfully purified using affinity chromatography under denaturing conditions followed by reverse staining. The clear bands of ∼35 and ∼43 kDa were detected for the purified *Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev* polypeptides in SDS-PAGE, respectively (Fig. 1A). Furthermore, the recombinant purified polypeptides were detected using anti-His antibody in western blotting (Fig. 1B). The concentrations *of Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev* polypeptides were between 0.6 and 0.8 mg/mL.

Antibody secretion

Our results showed a significant difference in the levels of antibody secretion between all test groups and control groups against both recombinant polypeptides ($P < 0.01$, Fig. 2). The highest levels of total IgG, IgG1, and IgG2a were observed in mice immunized with the homologous Gag-Pol-Env-Nef-Rev polypeptide construct (G1) against the Gag-Pol-Env-Nef-Rev coated antigen. Moreover, the highest levels of IgG1 and IgG2a were detected in groups immunized with the homologous Hsp70-Gag-Pol-Env-Nef-Rev (G2), and Hsp70-Gag-Pol-Env-Nef-Rev + Montanide (G3) regimens against the Hsp70-Gag-Pol-Env-Nef-Rev coated antigen ($p < 0.05$). Furthermore, there was no significant difference in the secretion level of total IgG between all groups immunized with different regimens of Hsp70-Gag-Pol-Env-Nef-Rev antigen ($P > 0.05$). It was interesting that the levels of total IgG and its isotypes were significantly lower in groups immunized with different regimens

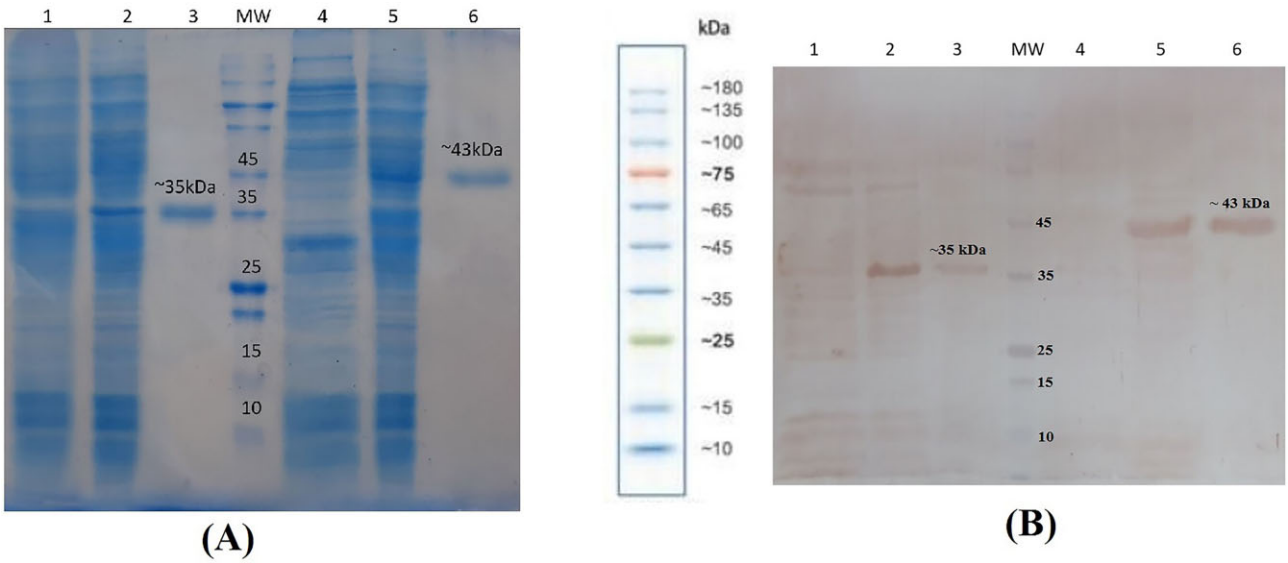

Figure 1. Confirmation and identification of the recombinant multiepitope polypeptides in *E. coli* using SDS-PAGE **(A)** and western blotting **(B)**, respectively: Lane 1: Before induction in BL21 (DE3) containing pET-24a(+)-*gag-pol-env-nef-rev*; Lane 2: 16 h after induction in BL21 (DE3) containing pET-24a(+)-*gag-pol-env-nef-rev*; Lane 3: The purified Gag-Pol-Env-Nef-Rev polypeptide; Lane 4: Before induction in Rosetta containing pET-24a(+)-*hsp70-gag-pol-env-nef-rev*; Lane 5: 4 h after induction in Rosetta containing pET-24a(+)-*hsp70-gag-pol-env-nef-rev*; Lane 6: The purified Hsp70-Gag-Pol-Env-Nef-Rev polypeptide. MW: Molecular weight marker (pre-stained protein ladder, 10–180 kDa, Fermentas).

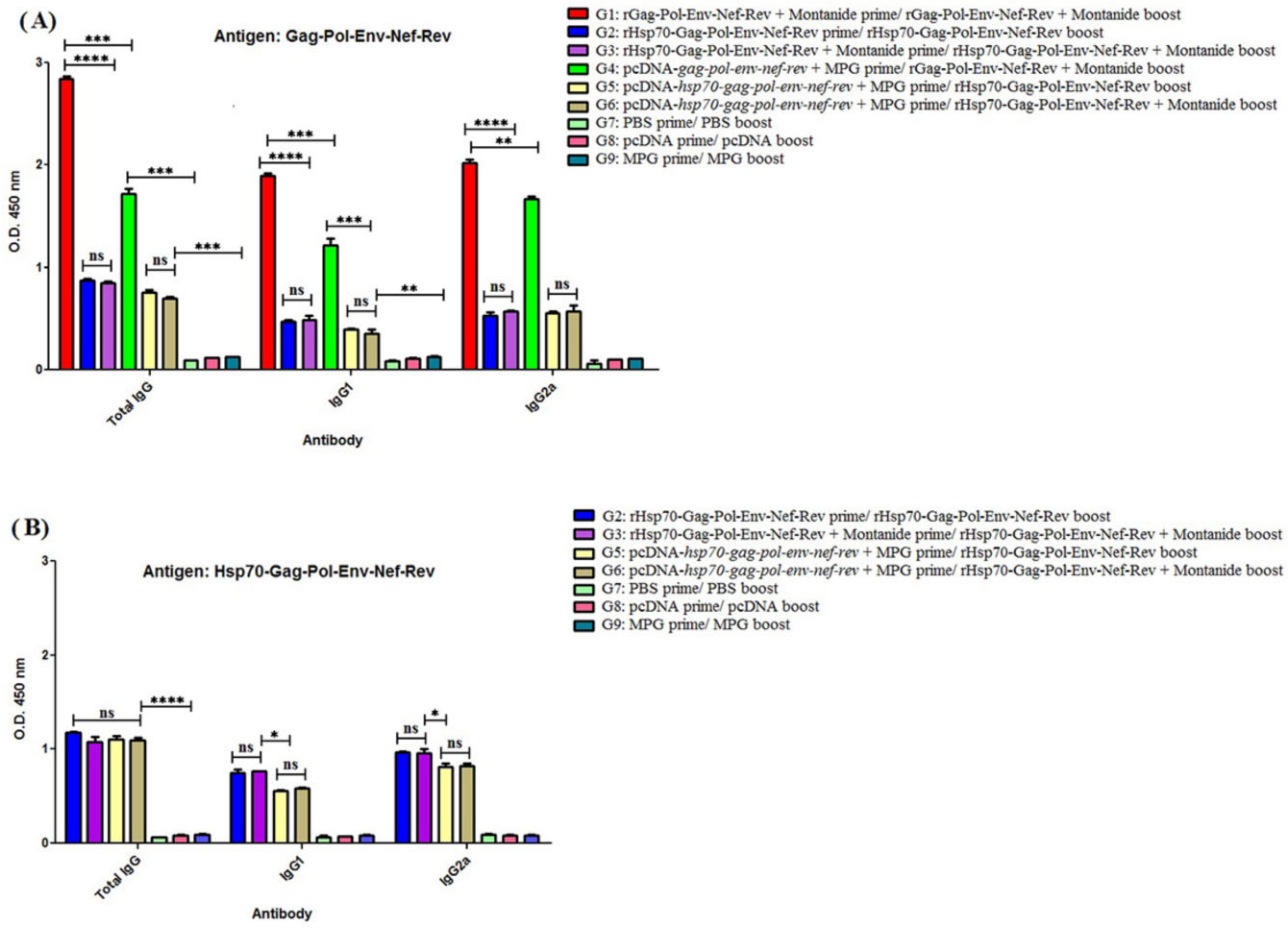

Figure 2. Evaluation of total IgG, IgG1 and IgG2a levels against the Gag-Pol-Env-Nef-Rev **(A)** and Hsp70-Gag-Pol-Env-Nef-Rev **(B)** antigens in different groups using indirect ELISA: All analyses were performed in duplicate for each sample shown as mean absorbance at 450 nm ± SD (ns: non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

of Hsp70-Gag-Pol-Env-Nef-Rev antigen than different regimens of Gag-Pol-Env-Nef-Rev antigen against the Gag-Pol-Env-Nef-Rev coated antigen ($P < 0.001$; Fig. 2). This result was likely due to the conformational change after adding Hsp70 epitopes and also the selection of Hsp70 T-cell epitopes. Moreover, the addition of Montanide adjuvant showed no significant difference in the levels of total IgG, IgG1, and IgG2a in groups immunized with the Hsp70-Gag-Pol-Env-Nef-Rev construct (G2, G3, G5, & G6; $P > 0.05$).

On the other hand, no significant antibody responses against Gag-Pol-Env-Nef-Rev or Hsp70-Gag-Pol-Env-Nef-Rev antigens were found in the control groups ($P > 0.05$), indicating that the seroreactivities were completely antigen-specific in mice (Fig. 2). Indeed, *MPG peptide (G9) had no effect on antibody secretion* against Gag-Pol-Env-Nef-Rev or Hsp70-Gag-Pol-Env-Nef-Rev antigens. *For this reason, this peptide was used to deliver low doses of DNA into the cells for increasing DNA uptake and thus its immunogenicity.* Generally, the mixture of IgG1 and IgG2a antibody responses were observed in all test groups indicating the direction of immune responses toward both Th1 and Th2 responses.

Lymphocyte proliferation and cytokine assay

The MTT results indicated that the recombinant Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev polypeptides could significantly stimulate the proliferation of lymphocytes in test groups as compared to the control groups after 72 h incubation ($P < 0.001$, Fig. 3A). In addition, our data showed a significant difference in the levels of IFN-γ and IL-10 secretion between test groups and control groups ($P < 0.05$, Fig. 3B and C). Among all the test groups, the highest levels of IFN-γ, IL-5, and IL-10 secretion were observed in mice immunized with the Gag-Pol-Env-Nef-Rev polypeptide + Montanide regimen (G1) after re-stimulation of splenocytes with the recombinant Gag-Pol-Env-Nef-Rev polypeptide (Figs 3B, C, and D). Indeed, the levels of all cytokines in the homologous Gag-Pol-Env-Nef-Rev polypeptide + Montanide regimen (G1) were significantly higher than those in the heterologous pcDNA-*gag-pol-env-nef-rev* + MPG/Gag-Pol-Env-Nef-Rev polypeptide + Montanide (G4) regimen after re-stimulation of splenocytes with the recombinant Gag-Pol-Env-Nef-Rev polypeptide ($P < 0.001$).

In addition, the highest levels of IFN-γ, IL-5, and IL-10 secretion were observed in mice immunized with the Hsp70-Gag-Pol-Env-Nef-Rev polypeptide + Montanide regimen (G3) after re-stimulation of splenocytes with the recombinant Hsp70-Gag-Pol-Env-Nef-Rev polypeptide (Fig. 3B, C, and D). Indeed, the levels of all cytokines were higher in groups receiving the homologous and heterologous Hsp70-Gag-Pol-Env-Nef-Rev regimens along with Montanide (G3 and G6) as compared to groups receiving the same regimens without Montanide (G2 and G5) after re-stimulation of splenocytes with the recombinant Hsp70-Gag-Pol-Env-Nef-Rev polypeptide (Fig. 3B, C, and D). The mean of IFN-γ/IL-10 and IFN-γ/IL-5 ratios were higher in all the test groups than control groups ($P < 0.05$) as shown in Table 2; indicating a stronger Th1 response in test groups.

Granzyme B assay

The results of Granzyme B assay indicated that all the test groups had considerably higher secretion of Granzyme B than control groups ($P < 0.0001$). Among all the test groups, the highest level of Granzyme B secretion was observed in mice immunized with homologous Gag-Pol-Env-Nef-Rev polypeptide + Montanide (G1) and Hsp70-Gag-Pol-Env-Nef-Rev polypeptide + Montanide (G3) regimens with respect to the recombinant Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev polypeptides, respectively (Fig. 4). The secretion of Granzyme B was higher in *Hsp70-Gag-Pol-Env-Nef-Rev polypeptide + Montanide-receiving group (G3) than that in Hsp70-Gag-Pol-Env-Nef-Rev polypeptide-receiving group (G2)* with respect to the recombinant Hsp70-Gag-Pol-Env-Nef-Rev polypeptide ($P < 0.0001$). *The same result was observed in the heterologous* Hsp70-Gag-Pol-Env-Nef-Rev regimens with and without Montanide (G5 and G6) with respect to the recombinant Hsp70-Gag-Pol-Env-Nef-Rev polypeptide ($P < 0.001$) indicating the effect of Montanide in Granzyme B secretion.

Table 2. The mean of IFN-γ/IL-5 and IFN-γ/IL-10 ratios in all groups.

Group	IFN-γ/IL-10 against Gag-Pol-Env-Nef-Rev polypeptide	IFN-γ/IL-10 against Hsp70-Gag-Pol-Env-Nef-Rev polypeptide	IFN-γ/IL-5 against Gag-Pol-Env-Nef-Rev polypeptide	IFN-γ/IL-5 against Hsp70-Gag-Pol-Env-Nef-Rev polypeptide
G1	5.11	-	13.54	-
G2	2.76	26.07	31.21	61.93
G3	2.00	3.98	26.69	15.47
G4	4.19	-	29.87	-
G5	7.58	9.12	30.33	18.53
G6	3.44	6.48	33.50	11.77
G7	0.54	0.63	1.80	1.00
G8	0.61	0.42	1.07	1.00
G9	0.60	0.38	0.85	0.62

In vitro IFN-γ secretion by single-cycle replicable (SCR) HIV-1 virions

Our data indicated a higher level of IFN-γ in the supernatant of the SCR virion-infected splenocytes which had been restimulated with the recombinant Gag-Pol-Env-Nef-Rev polypeptide compared to the control groups ($P < 0.001$; Fig. 5). The levels of IFN-γ were higher in homologous immunized groups (G1, G2, & G3) than heterologous immunized groups (G4, G5, & G6) ($P < 0.001$, Fig. 5). The highest levels of IFN-γ secretion were detected in mice immunized with the recombinant Hsp70-Gag-Pol-Env-Nef-Rev polypeptide emulsified with Montanide adjuvant (G3). Generally, infection of lymphocytes after their restimulation with the recombinant Gag-Pol-Env-Nef-Rev polypeptide led to a remarked increase in IFN-gamma secretion indicating the importance of different regimens in protection against the virus with the secretion of key cytokine.

Discussion

Therapeutic vaccination is one of the most curative strategies for inducing potent HIV-specific immune responses (Chen and Julg 2020). These vaccines can eliminate the viral reservoirs and facilitate viral control in the absence of ART (Korber and Fischer 2020). Mosaic polyvalent therapeutic vaccines engage potent cellular immunity. Moreover, targeting multiple epitopes may be the most efficient approach to overcome the high genetic diversity and variability of HIV (Hargrave et al. 2021). For example, HVTN705 mosaic Gag, Pol, and Env vaccine antigens in a Phase 2b clinical trial induced significantly more cross-reactive T-cell responses than the natural HIV proteins used in the HVTN505 trial (Chen and Julg 2020, Hargrave et al. 2021, Tatoud et al. 2021). As it is known, $CD8^+$ T-cell mediated immunity has a protective and suppressing effect on HIV-1 infection (Fomsgaard 2015). Also, the $CD4^+$ T-cell

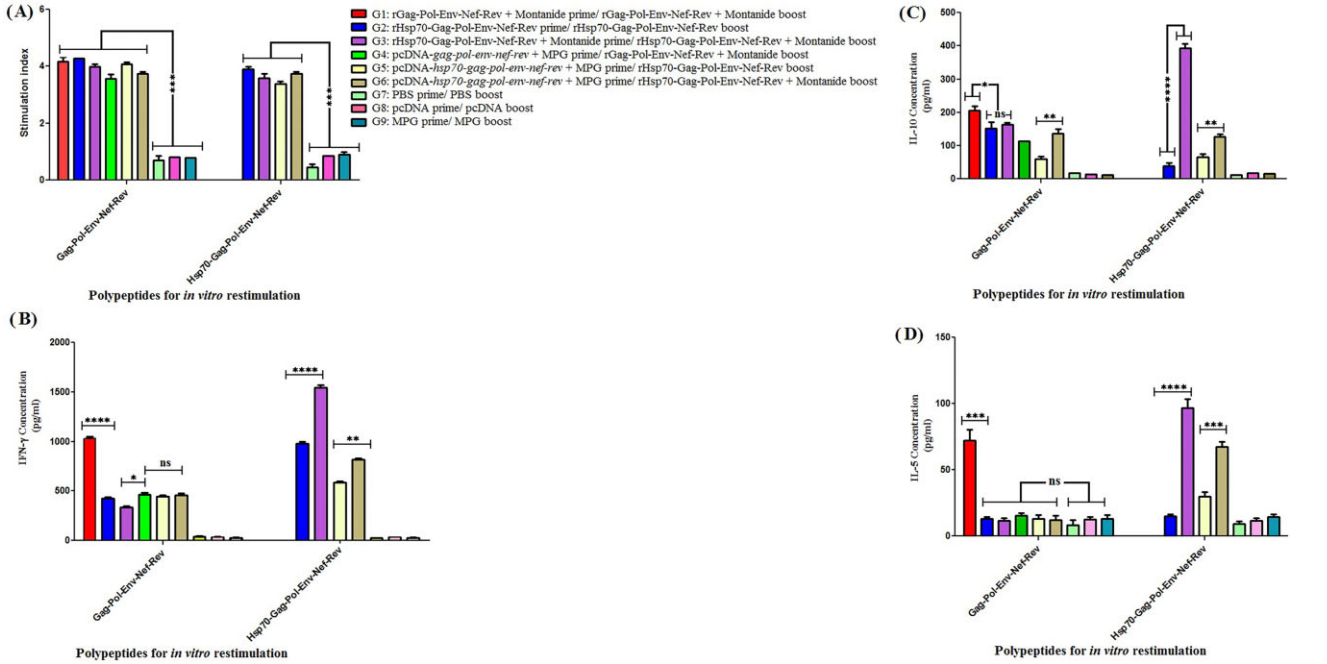

Figure 3. Evaluation of lymphocyte proliferation **(A)** and cytokines secretion **(B, C, & D)** in immunized groups with different modalities. All analyses were performed in triplicate for each sample shown as mean absorbance at 450 nm ± SD (ns: non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$).

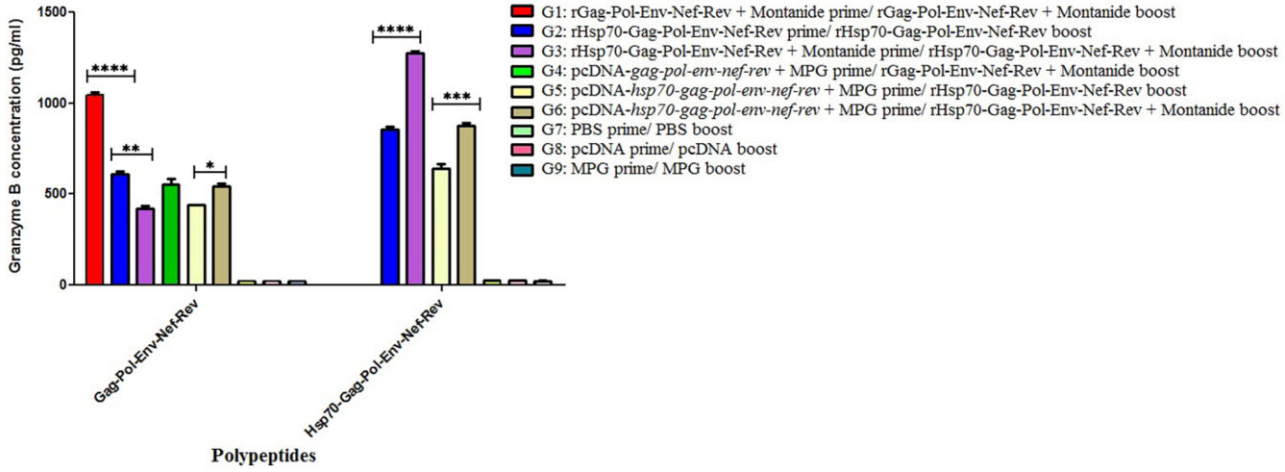

Figure 4. Evaluation of Granzyme B secretion in immunized groups with different modalities using ELISA: All analyses were performed in triplicate for each sample shown as mean absorbance at 450 nm ± SD (ns: non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$).

mediated immunity facilitates induction of $CD8^+$ T cell immunity and prolongs antibody immune responses leading to control of viremia (Chen and Julg 2020, Korber and Fischer 2020). Therefore, in the present study, we generated the multiepitope DNA and polypeptide constructs based on the immunogenic CTL and HTL epitopes of HIV Gag, Pol, Env, Nef and Rev proteins that were previously determined computationally by our group (Akbari et al. 2021). Furthermore, the functional epitopes of Hsp70 were linked to the construct (i.e. Gag-Pol-Env-Nef-Rev) as an immunostimulatory agent to potentiate an effective immune response. As a matter of fact, the mutational escape in the most conserved regions of the HIV genome may significantly lower viral fitness (Fomsgaard 2015). Thus, targeting a broad range of conserved HIV epitopes in our designed constructs can boost the magnitude and breadth of anti-HIV responses and overcome virus escape mutations (Chen and Julg 2020). Our designed polyvalent mosaic constructs have theoretically high population coverage that optimizes binding to diverse HLAs. These constructs are composed of long peptides containing T helper cell epitopes that overlap with the CTL epitopes linked by AAY linker to reduce the creation of junctional epitopes and optimize the proteasomal cleavage (Akbari et al. 2021). Several preclinical and clinical studies demonstrated that vaccination with long peptides induces higher immune responses compared to minimal epitopes since they elicit better effector $CD8^+$ T cell response due to sufficient stimulation of professional APC and memory T helper cell (Li et al. 2014, Ghaffari-Nazari et al. 2015). *Ondondo* et al. designed an HIV vaccine immunogen (tHIV-consvX) based on six highly conserved long fragments of Gag and

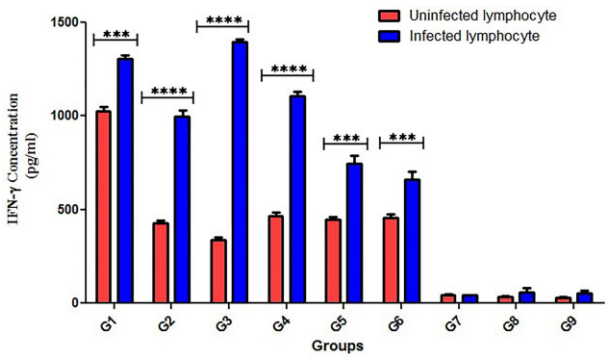

Figure 5. IFN-gamma secretion in SCR HIV-1-infected lymphocytes as compared to uninfected lymphocytes. The lymphocytes were restimulated with the Gag-Pol-Env-Nef-Rev polypeptides *in vitro* before viral infection. All analyses were performed in duplicate for each sample shown as mean absorbance at 450 nm ± SD (ns: non-significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ****$P < 0.0001$).

Pol using computational strategies. The tHIVconsvX was highly immunogenic in mice and was associated with lower viral loads and higher $CD4^+$ T-cell counts in untreated HIV-infected individuals (Ondondo et al. 2016, Korber and Fischer 2020). In our study, some of the selected epitopes were similar to those in tHIVconsvX (Ondondo et al. 2016, Akbari et al. 2021) or other vaccine candidate constructs (e.g. DALIA vaccine) that were potent immunogens in preclinical and clinical studies (Salmon-Céron et al. 2010, Mothe et al. 2015). The DALIA HIV-1 therapeutic vaccine (DCs loaded with five long peptides from Gag, Pol and Nef) in phase II trial were able to elicit HIV-specific sustained $CD8^+$ and $CD4^+$ T-cell responses in healthy volunteers. These data indicated that our designed multiepitope constructs may induce efficient immune responses against HIV-1 infection.

In our study, the recombinant Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev polypeptides were successfully generated in E. coli strains. Also, the endotoxin-free pcDNA-gag-pol-env-nef-rev and pcDNA-hsp70-gag-pol-env-nef-rev were prepared on a large scale and with high purity. The vaccine constructs were subcutaneously injected into BALB/c mice to assess immunological effects. Our data showed that all modalities could induce antibody secretion against the Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev antigens. Moreover, the mixture of IgG1 and IgG2a responses was observed for homologous and heterologous regimens. Among all groups, the homologous Gag-Pol-Env-Nef-Rev polypeptide + Montanide (G1) could significantly induce total IgG, IgG1, and IgG2a against the Gag-Pol-Env-Nef-Rev coated antigen as compared to other groups ($P < 0.05$). The reports showed that the full length of Hsp70 functions as an adjuvant for stimulating innate and cellular immunity, but it has a limited effect on inducing Th2 response (Lewis et al. 2014). In our previous study, the selection of Hsp70 epitopes was dependent on the specific MHC molecule binding and presenting the epitopes to T cells. Although, we predicted the probable B cell epitopes in the Gag-Pol-Env-Nef-Rev and Hsp70-Gag-Pol-Env-Nef-Rev constructs using bepipred linear epitope prediction tools, but Hsp70 B cell epitopes had lower prediction scores (Akbari et al. 2021). Moreover, most of the B cell epitope prediction tools typically identify linear stretches as epitopes, while most of the B cell epitopes on protein antigens are conformational and even discontinuous (El-Manzalawy and Honavar 2010, Sela-Culang et al. 2014). On the other hand, it was reported that Hsp70 and the Hsp70-derived peptides elicit CTL responses against antigenic peptides and poor Th2 cell activation (Shevtsov and Multhoff 2016). For example, immunization of macaques with a vaccine construct containing HIVgp120, SIVp27, and CCR5 peptides, all covalently linked to Hsp70 elicited T cell proliferation and CC chemokines but poor serum and vaginal fluid IgG and IgA antibody titers to the antigens (Bogers et al. 2004, Lewis et al. 2014). Another study indicated that Mage-a1 tumor antigen fused to Hsp70 could elevate the cellular immune response as compared to the humoral immune response in immunized mice (Jiang et al. 2013). Also, fusion of Hsp70 to Hantaan virus nucleocapsid protein (HTNV NP) induced the cellular immune response more than the humoral immune response to the HTNV NP in C57BL/6 mice (Li et al. 2008).

It should be noted that in our study, the full length of Hsp70 or its fragments were not used as an adjuvant similar to other studies. Herein, the T-cell epitopes of Hsp70 as an immunostimulator was determined to be located at the N-terminus of the Gag-Pol-Env-Nef-Rev construct. Linking the Hsp70 epitopes to the Gag-Pol-Env-Nef-Rev construct could likely cause conformational changes in the Gag-Pol-Env-Nef-Rev polypeptide. Thus, the levels of antibodies were lower in groups receiving Hsp70-Gag-Pol-Env-Nef-Rev construct than in groups receiving Gag-Pol-Env-Nef-Rev construct against the Gag-Pol-Env-Nef-Rev coated antigen. Moreover, the levels of antibodies generated in groups receiving Hsp70-Gag-Pol-Env-Nef-Rev construct against the Hsp70-Gag-Pol-Env-Nef-Rev coated antigen was lower than those in groups receiving Gag-Pol-Env-Nef-Rev construct against the Gag-Pol-Env-Nef-Rev coated antigen indicating induction of lower antibody responses due to the linkage of Hsp70 T cell epitopes. This result was similar to other studies as described above.

Several reports indicated that antigen-stimulated cells in HIV elite controllers are poly-functional, and release a mixture of cytokines and functional molecules (Rosa et al. 2011, Chen and Julg 2020). Also, analysis of IFN-γ secretion by T cells exposed to HIV antigens is a widely used assay for determining antigen-specific responses in vaccine trials (Collins et al. 2020). Thus, we evaluated lymphocyte proliferation and cytokine secretion in the immunized groups. MTT assay showed the proliferation of T cells, and thus secretion of the cytokines (i.e. IFN-γ, IL-5, and IL-10) in all test groups compared to control group. The highest levels of IFN-γ, IL-5 and IL-10 cytokines were detected in group immunized with the homologous Hsp70-Gag-Pol-Env-Nef-Rev polypeptide + Montanide regimen (G3) after re-stimulation of splenocytes with the recombinant Hsp70-Gag-Pol-Env-Nef-Rev polypeptide. Indeed, the use of Montanide 720 in the homologous or heterologous Hsp70-Gag-Pol-Env-Nef-Rev regimens (G3 and G6) could significantly enhance the levels of IFN-γ, IL-5, and IL-10 cytokines as compared to the same regimens without Montanide (G2 and G5) suggesting induction of both Th1 and Th2 responses in mice. However, the ratios of IFN-gamma/IL-10 and IFN-gamma/IL-5 were higher in the Hsp70-Gag-Pol-Env-Nef-Rev-receiving group (G2) than those in the Hsp70-Gag-Pol-Env-Nef-Rev + Montanide-receiving group (G3) with respect to the recombinant Hsp70-Gag-Pol-Env-Nef-Rev polypeptide (Table 2) indicating the importance of Hsp70 epitopes in direction of immune responses toward Th1 responses.

Previous studies showed the success of Montanide 720 in Phase I/II clinical trials of human therapeutic vaccines (Tehrani et al. 2016, Tifrea et al. 2020). This adjuvant was immunogenic and induced both cellular and humoral immune responses in vaccinated individuals (Tehrani et al. 2016). Herein, the secretion of Granzyme B was significantly increased in group immunized with the Gag-Pol-Env-Nef-Rev + Montanide regimen (G1) as compared to other groups in the presence of Gag-Pol-Env-Nef-Rev polypeptide. This level was further increased in group immu-

nized with the Hsp70-Gag-Pol-Env-Nef-Rev + Montanide regimen (G3) in the presence of Hsp70-Gag-Pol-Env-Nef-Rev polypeptide indicating the effects of Hsp70 epitopes in T-cell activation. Previous reports demonstrated the importance of high expression levels of perforin and granzyme B in HIV non-progressors individuals (Chen and Julg 2020). Our data showed that the homologous regimens induced significantly antibody, cytokine and granzyme B secretion as compared to the heterologous regimens (DNA prime/Polypeptide boost). Our previous study on in silico prediction of IFN-γ, IL-4 and IL-10 production by the used epitopes indicated that Hsp70 HTL epitopes are IFN-gamma and IL-10 inducer (Akbari et al. 2021). This result was similar to the experimental results. Similar to our results, other group reported that the levels of HIV antigen-specific IFN-γ and Granzyme B secretion elicited by immunization with HSPs/HIV protein complexes were significantly higher than groups immunized with HIV proteins without HSPs molecules (Krupka et al. 2015). Among Hsps, Hsp70 was known as an effective adjuvant in vaccine development studies (Moyle 2017).

It should be explained that the direction and stimulation of immune responses by the Montanide adjuvant depends on the nature of antigen. It was used as an adjuvant combined with HIV and malaria antigens in clinical trials, and showed promising results in promoting T cell responses (Qiu et al. 2008). The Hsp70-Gag-Pol-Env-Nef-Rev construct was designed based on Th cell and CTL activity, and induction of the cellular immune response in immunized mice. Thus, regarding the nature of vaccine construct, the Montanide adjuvant could not increase antibody response in time of interest. As know, commercial hepatitis B vaccine is based on HBsAg that elicits a strong antibody production. In one study, immunization of mice with HBsAg formulated in Montanide adjuvant increased antibody production and humoral immune responses compared to the commercially available HBsAg vaccine (Savoji et al. 2019). In contrast, mice immunization with the recombinant hepatitis C virus (HCV) core and coreE1E2 proteins formulated in Montanide resulted in a significant Th1 cellular immune response (Mehrlatifan et al. 2016). These findings suggested that the direction of immune response toward cellular or humoral immunity by Montanide adjuvant depends on the nature of antigen and the type of immune response elicited by it.

A major point in this study was the use of MPG CPP for DNA delivery. The data showed that the heterologous DNA + MPG prime/polypeptide boost could significantly increase humoral and cellular immune responses as compared to control groups. Although these immune responses were lower than those in the homologous polypeptide responses; it should be noted that the MPG/DNA nanoparticles with a low dose of plasmid DNA (~5 μg) could induce immune responses in immunized mice. *Generally, CPPs can deliver the DNA directly into the cytoplasm of APCs, and also protect the DNA from degradation before the nucleus entry for transcription. For instance, mice immunization with DNA constructs encoding HCV core and coreE1E2 genes complexed with MPG peptide stimulated strong HCV-specific cellular and humoral immune responses in lower concentrations than the naked DNA constructs* (Mehrlatifan et al. 2016). *Moreover, mice immunization with the human papillomavirus (HPV) E7 DNA complexed with MPG peptide induced a stronger Th1 cellular immune response than that induced by E7 DNA alone* (Saleh *et al.* 2015). *Thus, MPG peptide was proposed as a gene carrier in the development of therapeutic vaccines.*

One of the most important goals of HIV therapeutic vaccination is the elimination of the virus reservoirs by killing the infected cells (Chen and Julg 2020). In this study, the SCR virions were used to infect mouse lymphocytes. We assessed the ability of infected lymphocytes in IFN-γ secretion as an indicator of virus-specific T cell responses. The SCR virions (Soleymani et al. 2018) are capable to induce strong immune responses in vaccine development as an important source of HIV antigens. Herein, we studied the effects of virus exposure in the re-stimulated splenocytes with the Gag-Pol-Env-Nef-Rev polypeptide for each group. These results demonstrated a major increase in IFN-γ secretion after virus exposure as compared to control groups. It can suggest expansion of already existing T cells, priming new T cell responses, or re-establishment of responses lost early after vaccination following the in vivo viral challenge. As detected, IFN-γ secretion was significantly enhanced in infected splenocytes for all test groups as compared to uninfected splenocytes ($p < 0.05$). The highest IFN-γ secretion was observed in groups immunized with Hsp70-Gag-Pol-Env-Nef-Rev polypeptide + Montanide (G3) and Gag-Pol-Env-Nef-Rev polypeptide + Montanide (G1), respectively. As known, the CD4 molecule plays a direct role in $CD8^+$ T cell function by modulating IFN-γ secretion. Some previous studies showed that using the dendritic cells (DCs) pulsed with non-replicating virus (i.e. AT-2 HIV-1) could activate T cells, reduce viral loads, and enhance HIV-specific immunity (Buseyne et al. 2001, Larsson et al. 2002, Lubong Sabado et al. 2009). Previous data also showed that the cross-presentation of HIV proteins by DCs led to higher CTL responses specific for immunodominant epitopes (Dinter *et al.* 2015).

In conclusion, our data indicated that the Hsp70-Gag-Pol-Env-Nef-Rev and Gag-Pol-Env-Nef-Rev constructs can be considered as potent immunogens for developing multiepitope-based vaccine candidates against HIV-1 infection. Regarding the antibody and cytokine secretion, the vaccine constructs elicited the mixed Th1/Th2 responses more towards Th1 activation in immunized mice. The linkage of Hsp70 epitopes to the Gag-Pol-Env-Nef-Rev construct could significantly increase cytokines and Granzyme B secretion with respect to the recombinant Hsp70-Gag-Pol-Env-Nef-Rev polypeptide. In addition, IFN-gamma secretion from restimulated splenocytes was considerably enhanced in all test groups especially in the homologous Hsp70-Gag-Pol-Env-Nef-Rev + Montanide regimen (G3) after virion exposure as compared to restimulated splenocytes in the lack of virion infection. However, further studies are required to assess durability and longevity of the immune responses and determine their effects in reduction of HIV-1 replication in animal models (e.g. macaque) in future.

Supplementary Figure 1 The designed Hsp70-Gag-Pol-Env-Nef-Rev multiepitope peptide construct containing CTL and HTL epitopes of each protein

Supplementary Figure 2 Confirmation of the *gag-pol-env-nef-rev* and *hsp70-gag-pol-env-nef-rev* gene constructs in the pET-24a (A) and pcDNA 3.1 (B) using enzymatic digestion on agarose gel electrophoresis: Lane 1: double digestion of pET-24a(+)-*gag-pol-env-nef-rev* (A) and pcDNA 3.1(-)-*gag-pol-env-nef-rev* (B) by *Eco*RI/*Hind*III restriction enzymes; Lane 2: double digestion of pET-24a(+)-*hsp70-gag-pol-env-nef-rev* (A) and pcDNA 3.1(-)-*hsp70-gag-pol-env-nef-rev* (B) by *Bam*HI/*Hind*III restriction enzymes; DNA ladder (1 kb, Fermentas)

Supplementary Figure 3 The schematic model of subcloning the multiepitope constructs in expression vectors: (A) Subcloning the *gag-pol-env-nef-rev* gene in pET-24a (+) and pcDNA3.1 (-) vectors; (B) Subcloning the *hsp70-gag-pol-env-nef-rev* gene in pET-24a (+) and pcDNA3.1(-) vectors

Supplementary Figure 4 Formation of the MPG/DNA nanoparticles, and their stability against DNase and FBS: (A) The naked pcDNA-*gag-pol-env-nef-rev* and MPG/pcDNA-*gag-pol-env-nef-rev* nanoparticles; (B) The naked pcDNA-*hsp70-gag-pol-env-nef-rev* and MPG/pcDNA-*hsp70-gag-pol-env-nef-rev* nanoparticles; As

shown, the formation of the MPG/DNA complexes was confirmed by migration inhibition of the plasmids into the agarose gel. Moreover, the naked plasmid DNAs were degraded in the presence of DNase and also FBS. In contrast, the recovered DNAs from nanoparticles remained intact after DNase and serum degradation assay.

Supplementary data

Supplementary data are available at *FEMSPD* online.

Ethical Approval

All experimental procedures for animal studies were in accordance with the Animal Care and Use Protocol of Pasteur Institute of Iran (national guideline) for scientific purposes (Ethics code: IR.PII.REC.1399.035; Approval date: 2020–09–02).

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Conflict of Interest statement. The authors have no conflict of interest to declare.

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