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Investigation of Immunostimulatory Effects of IFN-γ Cytokine and CD40 Ligand Costimulatory Molecule for Development of HIV-1 Therapeutic Vaccine Candidate

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The most crucial disadvantage of DNA-based vaccines is their low immunogenicity; therefore, finding an effectual adjuvant is essential for their development. Herein, immunostimulatory effects of IFNγ cytokine and a CD40 ligand (CD40L) costimulatory molecule are evaluated as combined with an antigen, and also linked to an antigen in mice. For this purpose, after preparation of the HIV-1 Nef, IFNγ, and CD40L DNA constructs, and also their recombinant protein in an *Escherichia coli* expression system, nine groups of female BALB/c mice are immunized with different regimens of DNA constructs. About 3 weeks and also 3 months after the last injection, humoral and cellular immune responses are assessed in mice sera and splenocytes. Additionally, mice splenocytes are exposed to single-cycle replicable (SCR) HIV-1 virions for evaluating their potency in the secretion of cytokines in vitro. The data indicate that the linkage of IFNγ and CD40L to Nef antigen can significantly induce the Th-1 pathway and activate cytotoxic T lymphocytes compared to other regimens. Moreover, groups receiving the IFNγ-Nef and CD40L-Nef fusion DNA constructs show higher secretion of IFNγ and TNF-α from virion-infected lymphocytes than other groups. Therefore, the IFNγ-Nef and CD40L-Nef fusion DNA constructs are suggested to be a potential option for development of an efficient HIV-1 vaccine.

1. Introduction

Combating human immunodeficiency virus (HIV) infection is a challenging issue of human health.[1] HIV invades the immune system and the ultimate result is dysregulation and dysfunction of the immune cells, which impede induction of protective cellular and humoral immune response.[2] Hence, harnessing vaccine-induced immunity would aid in re-shaping immune responses, and thus prevention or treatment of HIV infection.[3] Considering the necessity of HIV-1 Nef protein in induction of HIV infection and AIDS progression, it has been incorporated in various vaccine platforms.[4,5] Despite the established importance of DNA-based and protein-based vaccines in reconstructing the immune responses, their inadequate immunogenicity is the main obstacle.[6] Over the last decade, discovery of adjuvants has made a lot of progress in the field of vaccine research.[7] Up to now, numerous adjuvants have been presented, ranging from aluminum hydroxide (alum) and molecular adjuvants to ligand- and cytokine-based adjuvants.[7] CD40 ligand (CD40L), an immunostimulatory molecule, is attractive vaccine adjuvant that has been utilized in various DNA vaccines. Incorporating CD40L as an adjuvant in the designed DNA vaccines against modified *vaccinia virus Ankara* (MVA),[8] Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and *bovine herpesvirus-1*[9] showed promising results for enhancement of vaccine functional quality. Among cytokine-based adjuvants, IFNγ has proven to be safe and effective in cancer,[9] allergy[10], and viral[11] vaccines. Importantly, the published reports showed that codelivery of the full-length CD40L[12,13] and IFNγ[14,15] with HIV-1 proteins augment vaccine-induced immune responses. Accordingly, we assessed and compared the efficacy of CD40L and IFNγ as a vaccine adjuvant to enhance HIV-1 Nef antigen-specific immunity in DNA-based vaccine strategies. In general, our specific objectives were included: a) Preparation of the endotoxin-free DNA constructs alone or fused forms (i.e., pcDNA-Nef, pcDNA-IFNγ, pcDNA-CD40L, pcDNA-CD40L-Nef, pcDNA-IFNγ-Nef, pcDNA-CD40L + pcDNA-Nef, and pcDNA-IFNγ + pcDNA-Nef); b) Generation of the recombinant Nef, IFNγ and CD40L proteins in *Escherichia coli*expression system; c) Preparation of single-cycle replicable virions (SCR HIV-1 virions); d) Determination of the best vaccine strategy through antibody, cytokine, and Granzyme

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B secretion assay; and e) Analysis of in vitro antiviral effects using the exposure of mice splenocytes to SCR virions through cytokine assay. Generally, adjuvant properties of CD40L and IFNγ were compared in increasing HIV-1 Nef antigen-specific immunity. Moreover, the most effective form of CD40L and IFNγ (i.e., the combined or linked forms) was determined to enhance antigen-specific immunity (Graphical Abstract).

2. Experimental Section

2.1. Design of the Fusion DNA Constructs

The orientation of linking CD40L and IFNγ to the N-terminal or C-terminal of Nef antigen using different immunoinformatics studies was previously determined. Briefly, interaction of vaccine constructs (i.e., CD40L-Nef, Nef-CD40L, IFNγ-Nef & Nef-IFNγ) with toll-like receptors (TLRs), IFN-γ receptor and CD40 was performed by ClusPro server (https://cluspro.bu.edu/). Moreover, their immunogenicity, toxicity and allergenicity were investigated using IEDB, ToxinPred and AllerTOPv2.0 servers, respectively.

2.2. Construction of the Recombinant Expression Vectors

At first, the full length of *Mus musculus* IFNγ gene (Accession No: NP_032363.1) and *Mus musculus* CD40L (Accession No: NP_035746.2) with appropriate restriction enzyme sites were synthesized in pUC57 cloning vector (GenScript Biotech company, China). Then, for generation of pcDNA3.1(−)-IFNγ, pcDNA3.1(−)-CD40L, pET-24a(+)-IFNγ, and pET-24a(+)-CD40L constructs, the IFNγ and CD40L fragments were subcloned from pUC57 vector into the pcDNA 3.1(−) eukaryotic expression vector (Invitrogen) and the pET-24a(+) prokaryotic expression vector (Novagen) using *Bam*HI/*Hind*III (Thermo Fisher Scientific, Germany) restriction enzymes. Moreover, to prepare the eukaryotic expression vectors containing IFNγ-Nef and CD40L-Nef fusion DNA constructs, at first, the Nef gene was digested from pET-23a-Nef (designed in our previous study[16]) with NheI/ SalI restriction enzymes, and ligated to the linearized pUC57-IFNγ and pUC57-CD40L (i.e., pUC57-IFNγ-Nef and pUC57-CD40L-Nef). Then, the IFNγ-Nef and CD40L-Nef fusion genes were subcloned from pUC57-IFNγ-Nef and pUC57-CD40L-Nef into the XbaI/HindIII cloning sites of pcDNA3.1 (−) expression vector. Finally, all recombinant plasmids were purified in large-scale using an endotoxin-free plasmid Giga kit (Qiagen, Germany), and their concentration and purity were evaluated by NanoDrop spectrophotometer. It should be mentioned that the group previously prepared the pcDNA-Nef construct.[16] Herein, this construct was generated as endotoxin-free plasmid in a large scale for immunization study.

2.3. Transfection of DNA Constructs into the Mammalian Cells

Transfection of DNA constructs (i.e., pcDNA-Nef, pcDNA-IFNγ, pcDNA-CD40L, pcDNA-CD40L-Nef or pcDNA-IFNγ-Nef; 2 µg) into human embryonic kidney 293T cells (HEK-293T, CRL-3216; provided from the cell bank of Pasteur Institute of Iran) was performed using Lipofectamine 2000 transfection reagent (Sigma, Germany). Moreover, HEK-293T cells transfected with pcDNA3.1 (−) and pEGFP-N1 using Lipofectamine 2000 reagent were used as the negative and positive controls, respectively. The expression of the Nef, IFNγ, CD40L, CD40L-Nef, and IFNγ-Nef proteins was confirmed by western blot analysis using an anti-His tag antibody or anti-Nef monoclonal antibody (Abcam, USA; 1:10000 v/v) conjugated to horseradish peroxidase (HRP), and diaminobenzidine (DAB; Sigma) substrate.

2.4. Production of the Recombinant Proteins in Bacterial Expression System

The recombinant Nef, IFNγ and CD40L proteins were generated in bacteria for using in immunological analyses. The pET-23a (+) prokaryotic expression vector (Novagen) harboring HIV-1 Nef gene was previously designed by our group. Moreover, the recombinant Nef protein was previously generated by our group in *E. coli* Rosetta strain under the optimized conditions (i.e., 16 h incubation after induction with 1×10^{-3} M isopropyl thiogalactopyranoside (IPTG, Sigma, Germany) at 37 °C.[16] Herein, we generated the recombinant Nef protein in large scale based on our previous study.[16] Moreover, for expression of the recombinant IFNγ and CD40L proteins, the *E. coli* BL21 and Rosetta strains were transformed with the recombinant pET-24a-CD40L and pET-24a-IFNγ constructs. A single colony from each strain was selected, and cultured in liquid Luria-Bertani (LB) medium. Then, the grown bacteria were inoculated in the fresh TY2X medium (Peptone 1.6%, Yeast 1%, NaCl 0.5%; Sigma, Germany) to an optical density of 0.7–0.8 at 600 nm. Next, the protein expression was induced by adding 1×10^{-3} M IPTG at different incubation times (i.e., 2, 3, 4, and 16 h), and temperature scales (i.e., 25 °C and 37 °C) after induction. Finally, the cell pellets were harvested and analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and identified by western blotting using anti-His tag antibody conjugated to HRP (Abcam, USA; 1:10000 v/v).

2.5. Purification and Assessment of the Recombinant Nef, IFNγ, and CD40L Proteins

The recombinant IFNγ and CD40L proteins were purified by affinity chromatography using a Ni-NTA agarose column under denaturing conditions (8 M urea buffer and pH = 4.5) according to the manufacturer's instructions (Qiagen). Moreover, the recombinant Nef protein was purified by affinity chromatography under denaturing conditions as reported previously.[16] Then, the purified proteins were dialyzed against phosphate buffer saline (PBS) 1X using a dialysis membrane (10 kDa, Thermo Fisher Scientific, Germany). Finally, their concentrations were measured by Bradford protein assay kit and NanoDrop spectrophotometry, and stored at −70 °C for long-term preservation. The endotoxin contamination was less than 0.5 EU per mg protein as monitored by LAL assay (QCL-1000, Lonza).

2.6. Mice Immunization

Inbred BALB/c female mice, 6–8 weeks old, 20–22 g, were purchased from the breeding stocks maintained at Pasteur

Table 1. Different immunization modalities in mice.

Group	First injection (prime: Day 0)	Second injection (booster 1: Day 14)	Third injection (booster 2: Day 28)
G1	pcDNA-Nef	pcDNA-Nef	pcDNA-Nef
G2	pcDNA-Nef + pcDNA-CD40L	pcDNA-Nef + pcDNA-CD40L	pcDNA-Nef + pcDNA-CD40L
G3	pcDNA-Nef + pcDNA-IFNγ	pcDNA-Nef + pcDNA-IFNγ	pcDNA-Nef + pcDNA-IFNγ
G4	pcDNA-CD40L-Nef	pcDNA-CD40L-Nef	pcDNA-CD40L-Nef
G5	pcDNA-IFNγ-Nef	pcDNA-IFNγ-Nef	pcDNA-IFNγ-Nef
G6	pcDNA-CD40L	pcDNA-CD40L	pcDNA-CD40L
G7	pcDNA-IFNγ	pcDNA-IFNγ	pcDNA-IFNγ
G8 (control)	PBS 1X	PBS 1X	PBS 1X
G9 (control)	Empty vector (pcDNA3.1)	Empty vector (pcDNA3.1)	Empty vector (pcDNA3.1)

Institute of Iran. Seven mice in each group were considered and immunized subcutaneously at the footpad with different DNA regimens (50 μg) three times with a two-weeks interval (Days 0, 14 & 28) as shown in **Table 1**. The whole process was done based on approval protocols and care of laboratory animals in the Animal Experimentation Regulations of Pasteur Institute of Iran (national guideline) for scientific purposes (Ethics code: IR.PII.REC.1400.032; Approval date: 2021-06-22). Groups receiving empty vector (pcDNA3.1) and PBS1X were used as control groups. The immunization program was indicated in **Figure 1**. It should be mentioned that before bleeding and sacrificing, mice were anaesthetized by Ketamine and Xylazine according approval protocols. Animal health and behavior were monitored daily for well-being with evaluation of appetite level, general activity, hair coat condition, and grooming behavior.

2.7. Antibody Secretion Assay

To evaluate humoral immune responses in immunized mice, the pooled sera were prepared from each group (n = 4 per group) three weeks after the last injection. The levels of goat anti-mouse antibodies conjugated to HRP (total IgG, IgG1 and IgG2a; Sigma, Germany; 1:10000 v/v) were determined in the pooled sera (1:100 v/v in 1% bovine serum albumin (BSA)/PBS-Tween) using sandwich enzyme-linked immunosorbent assay (ELISA). The coated antigens were the recombinant IFNγ, CD40L, and Nef proteins (≈5 μg mL^{-1}) diluted in PBS1X. Tetramethylbenzidine (TMB) was used as a substrate for HRP-conjugated antibodies. The optical density (OD) was assessed at the wavelength of 450 nm. Each assay was repeated in duplicate, and all results were shown as mean ± SD for each sample.

2.8. Cytokine Secretion Assay

Three weeks after the last immunization, four mice from each group (n = 4 per group) were sacrificed, spleens were removed and homogenized to prepare mice splenocytes. Then, 2 × 10^6 cells mL^{-1} of the pooled splenocytes without red blood cells was adjusted in complete culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 UI mL^{-1} of

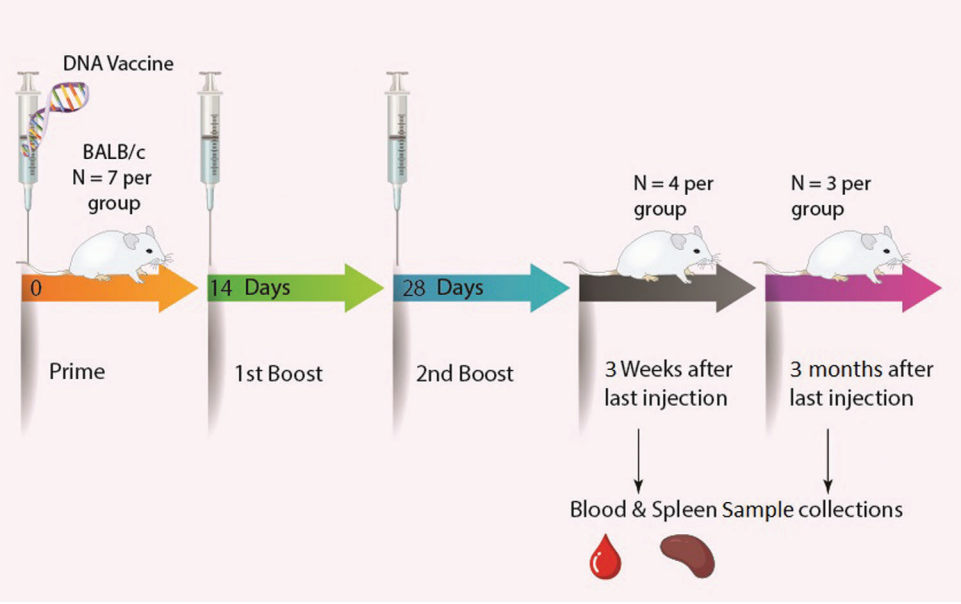

Figure 1. Graphic description of immunized mice with 50 μg of the DNA constructs per mouse three times with two-week intervals.

Table 2. Protein-protein docking results (lowest energy in the best model) of the Nef protein linked to IFNγ or CD40L protein with TLRs, IFN-γ receptor and CD40.

Construct	TLR2	TLR3	TLR4	TLR5	IFNγ receptor	CD40
CD40L$^{(\text{whole sequence})}$-Nef $^{(\text{whole sequence})}$	−1247.5	−1157.3	−1328.3	−1653.1	–	−1142.6
Nef $^{(\text{whole sequence})}$- CD40L$^{(\text{whole sequence})}$	−1223.2	−1121.5	−1280.7	−1567.4	–	−1107.8
IFNγ $^{(\text{whole sequence})}$-Nef $^{(\text{whole sequence})}$	−1402.9	−1277.9	−1496.9	−1743.3	−1127.1	–
Nef $^{(\text{whole sequence})}$- IFN$\gamma$ $^{(\text{whole sequence})}$	−1266.8	−1153.1	−1138.8	−1474.1	−1083.7	–

penicillin and 20 μg mL^{-1} of streptomycin). Next, splenocytes were re-stimulated with 5 μg mL^{-1} of the recombinant proteins (IFNγ, CD40L and Nef), and 5 μg mL^{-1} of concanavalin A (Con A mitogen, positive control, Sigma, USA), and incubated at 37 °C and 5% CO_2 for 72 h. Finally, the cell-free supernatants were used to assess IFN-γ, TNF-α, and IL-10 using sandwich-based ELISA kit (Mabtech, Swedish Biotech Company). The detection limit of cytokines was 4 pg mL^{-1}. All results were shown as mean ± SD for each sample.

2.9. 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; CRL-1581) were seeded in triplicate into 96-well plates (2×10^4 cells per well) for 24 h in the presence of 5 μg mL^{-1} of each recombinant protein (IFNγ, CD40L and Nef). 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 to assess Granzyme B concentration using an ELISA kit (eBioscience, USA) according to the manufacturer's instructions. All results were shown as mean ± SD for each sample.

2.10. Construction of SCR HIV-1 Virions, and Cytokine Secretion Assay

In the first step, construction of SCR HIV-1 virions was performed according to the previous studies of the group.[17] Briefly, HEK-293T cells were cotransfected with three pmzNL4.3, psPAX2 and pMD2G plasmids using TurboFect reagent (Fermentas, Germany) to generate the SCR HIV-1 virions. The supernatants of the transfected cells were harvested and ultracentrifuged in 45 000 *g* for 120 min. Then, the pellet of virions was resuspended in a culture medium, and the amount of virions was quantified by p24 ELISA assay kit.

In the next step, the secretion of cytokines (IFN-γ, IL-10 and TNF-α) from the pooled splenocytes (2×10^6 cells mL^{-1}) was evaluated after exposure to the generated SCR virions (equivalent to 50 μg of p24 antigen) for 72 h using sandwich ELISA kit. Each test was repeated in duplicate, and all results were indicated as mean ± SD for each sample.

2.11. Evaluation of Long-Term Immunity

To assess long-lasting immunity against HIV-1 Nef antigen, three months after the last immunization, the remaining mice from each group ($n = 3$ per group) were applied to assess the secretion of antibodies, cytokines and Granzyme B according to above sections.

2.12. Statistical Analysis

To assess the differences between the test and control groups, one-way ANOVA was performed by Prism 6.0 software (GraphPad, San Diego, California, USA). Data were indicated as mean ± standard deviation (SD) for each group (7 mice per group). A p-value < 0.05 was statistically considered significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). The studies were performed in two independent experiments.

2.13. 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.1400.032; Approval date: 2021-06-22).

3. Results

3.1. Verification of the Recombinant DNA Constructs

Our previous immunoinformatics results showed stronger interaction of the IFNγ-Nef and CD40L-Nef fusion proteins with TLRs, IFN-γ receptor and CD40 than the Nef-IFNγ and Nef-CD40L fusion proteins for inducing immune responses (**Table 2**). Moreover, these studies showed high immunogenicity, low toxicity, and no allergenicity for the IFNγ-Nef and CD40L-Nef fusion proteins as compared to the Nef-IFNγ and Nef-CD40L fusion proteins. Thus, we selected the CD40L-Nef and IFNγ-Nef fusion constructs for design of the DNA vaccine constructs. At first, the IFNγ, CD40L, IFNγ-Nef, and CD40L-Nef genes were successfully subcloned in pET-24a (+) and pcDNA3.1 (-) expression vectors. The presence of IFNγ, CD40L, IFNγ-Nef, and CD40L-Nef fragments was confirmed by digestion as clear bands of ≈539 bp, ≈866 bp, ≈1187 bp and ≈1514 bp migrated in agarose gel, respectively (**Figure 2**). Moreover, the endotoxin-free pcDNA-Nef was confirmed after digestion as a clear band of ≈30 bp on agarose

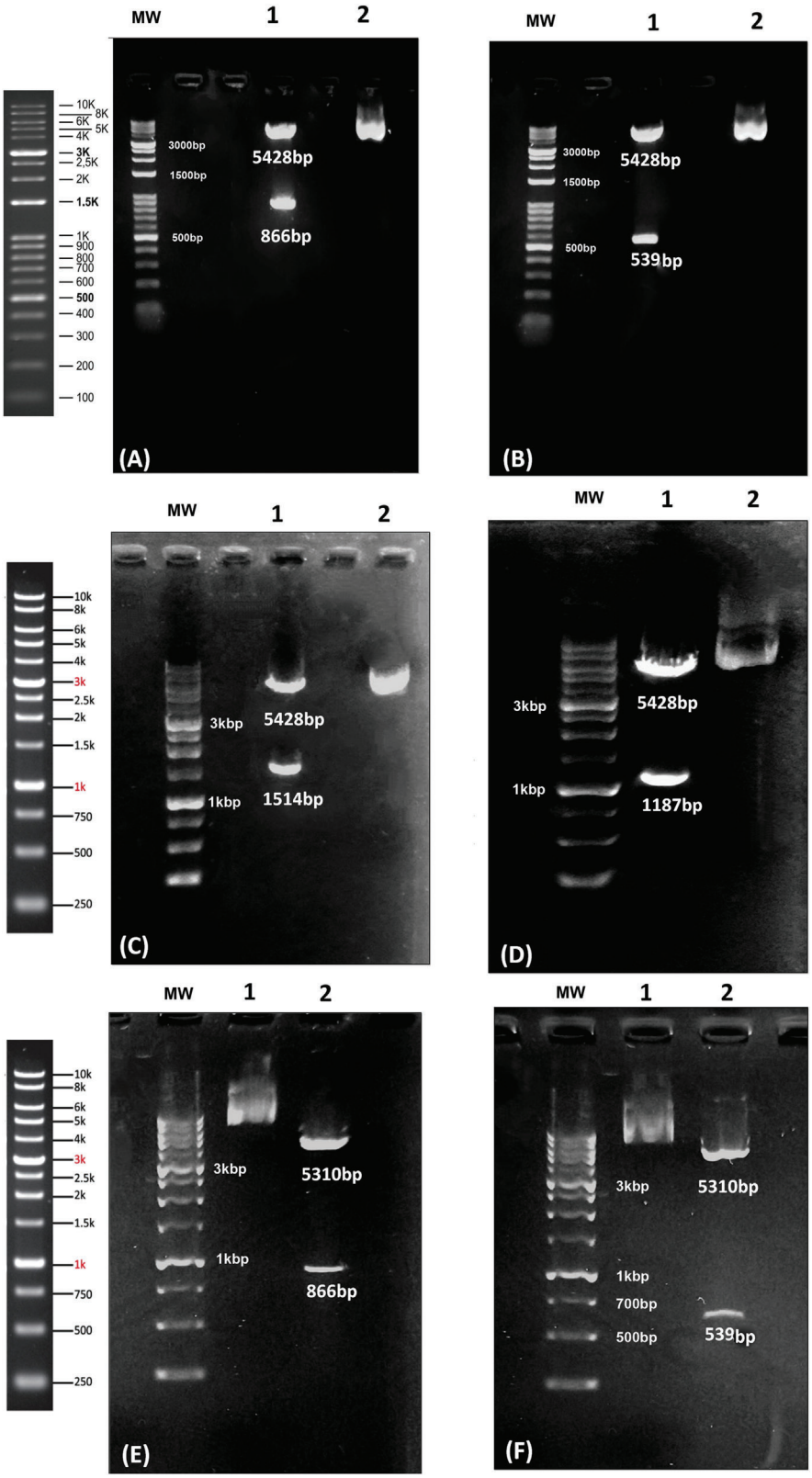

Figure 2. Confirmation of CD40L (A), IFN-γ (B), CD40L-Nef (C) and IFN-γ-Nef (D) genes subcloned in pcDNA3.1 (-) vector; Confirmation of CD40L (E) and IFN-γ (F) genes subcloned in pET-24a (+) vector; Lane 1: the recombinant plasmid digested with the restriction enzymes; Lane 2: the extracted recombinant plasmid. MW is DNA ladder (10 kb; Fermentas).

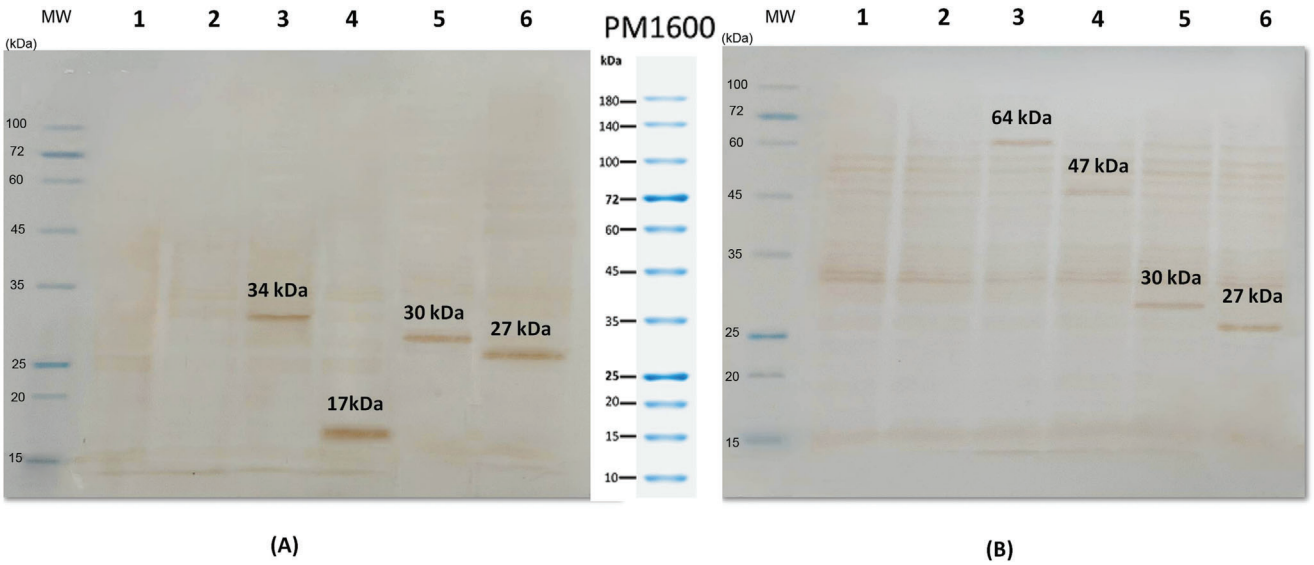

Figure 3. (A) Confirmation of CD40L (Lane 3), IFN-γ (Lane 4), and Nef (Lane 5) proteins expression in the HEK-293T cells by western blot analysis using anti-His tag antibody (1:10000 v/v); (B) Confirmation of CD40L-Nef (Lane 3), IFN-γ-Nef (Lane 4), and Nef (Lane 5) proteins expression in the HEK-293T cells by western blot analysis using anti-Nef monoclonal antibody (1:10000 v/v). No band was detected in untransfected cells (Lane 1) and the transfected cells with pcDNA3.1 vector (Lane 2) using anti-His tag antibody (A) and anti-Nef monoclonal antibody (B) as negative controls. Moreover, the positive control (the transfected cells with pEGFP-N1, Lane 6) showed a clear band of ≈27 kDa for expression of green fluorescent protein (GFP) by western blot analysis using anti-GFP polyclonal antibody (1:5000 *v/v*, Abcam, USA) for accuracy of transfection process. MW is molecular weight marker. (Protein ladder, 10–180 kDa, Fermentas).

gel as reported previously.[16] The concentration of endotoxin-free pcDNA-IFNγ, pcDNA-CD40L, pcDNA-IFNγ-Nef, pcDNA-CD40L-Nef, pcDNA-Nef and pcDNA3.1 was 1.82, 2.30, 1.93, 2.11, 1.76, and 2.01 mg mL^{-1}, respectively.

3.2. Protein Expression in Mammalian Cell Line

For in vitro delivery of the DNA constructs (pcDNA-IFNγ, pcDNA-CD40L, pcDNA-IFNγ-Nef, pcDNA-CD40L-Nef, and pcDNA-Nef) into HEK293T cells, Lipofectamine 2000 was used as a commercial transfection reagent. The expression of proteins was evaluated by western blotting at 48 h after transfecting HEK-293T cells. The results indicated the presence of Nef, IFNγ, CD40L, IFNγ-Nef and CD40L-Nef proteins with the expected size of ≈30, ≈17, ≈34, ≈47, and ≈64 kDa, respectively in the transfected cells (**Figure 3**). The untransfected cells and also the cells transfected with pcDNA3.1 vector showed no band in these regions upon incubation with the same antibodies.

3.3. Generation of the Recombinant Proteins in *E. Coli* Expression System

The expression of IFNγ and CD40L proteins was evaluated in pET-24a/Rosetta and pET-24a/BL21 systems. Our data showed that IFNγ protein was generated at 37°C in Rosetta strain for 16 h. In contrast, CD40L expression was observed in BL21 strain at 4 h after induction and 37 °C. Moreover, the recombinant HIV-1 Nef protein was expressed in *E. coli* Rosetta strain at 16 h after induction and 37 °C as previously reported.[16] The results indicated that all recombinant proteins could be successfully purified under denaturing conditions. The purified Nef, IFNγ, and CD40L proteins migrated as clear bands of ≈30 kDa, ≈17 kDa, and ≈34 kDa in SDS-PAGE, respectively (**Figure 4**A). Furthermore, the recombinant proteins were detectable using anti-His tag antibody in western blotting (Figure 4B). The recombinant proteins had a concentration range between 0.7 and 0.9 mg mL^{-1}.

3.4. Evaluation of Short-Term and Long-Term Antibody Responses

Evaluation of total IgG and the related subclasses against the recombinant Nef, IFN-γ and CD40L proteins in different groups was performed using indirect ELISA method. Our data indicated that the levels of total IgG, IgG1 and IgG2a in the sera of mice immunized with pcDNA-Nef accompanied by CD40L and IFNγ (G2, G3, G4, & G5) against the Nef coated antigen were significantly higher than those in group immunized with pcDNA-Nef (G1) and control groups ($p < 0.0001$, **Figure 5**A–C). Moreover, the levels of total IgG, IgG1 and IgG2a in the sera of mice immunized with the fusion DNA constructs (pcDNA-CD40L-Nef and pcDNA-IFNγ-Nef: G4 & G5) were significantly higher than groups immunized with the combined DNA constructs (pcDNA-Nef + pcDNA-CD40L and pcDNA-Nef + pcDNA-IFNγ: G2 & G3; $p < 0.0001$, Figure 5A–C). Furthermore, the highest levels of IgG1 and IgG2a were detected in group immunized with pcDNA-IFNγ-Nef (G5) against the Nef coated antigen ($p < 0.0001$). However, no significant antibody responses (total IgG, IgG1 & IgG2a) against CD40L or IFN-γ coated antigens were detected in the immunized groups (OD_{450}: 0.1-0.2) as compared to control groups (OD_{450}: 0.1–0.2; $p > 0.05$; Figure S1, Supporting Information). In addition, the results of long-lasting study showed that antibody responses were stable after three months. Moreover, the use of

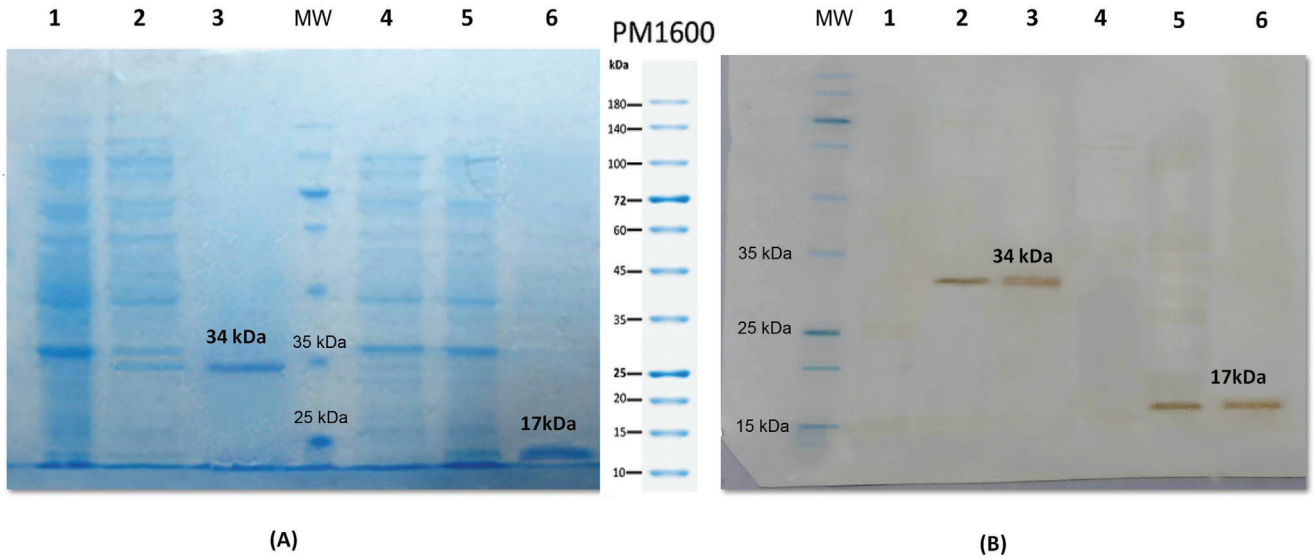

Figure 4. A) SDS-PAGE analysis of the purified proteins in *E. coli* expression system; B) Western blot analysis of the purified proteins using anti-His tag antibody. Lanes 1 and 4: before induction; Lane 2: The expressed CD40L protein after induction; Lane 3: The purified CD40L protein; Lane 5: The expressed IFN-γ protein after induction; Lane 6: The purified IFN-γ protein; MW is molecular weight marker (Protein ladder, 10–180 kDa, Fermentas).

CD40L or IFNγ adjuvants as combined or linked forms in vaccination regimens could significantly increase antibody responses against Nef coated antigen in long term assay (i.e., three months after the last injection) as compared to those in short term assay (i.e., three weeks after the last injection; $p < 0.05$; Figure 5). On the other hand, the mean ratios of IgG2a to IgG1 were increased in groups immunized with Nef DNA along with CD40L DNA or IFNγ DNA as combined or linked forms (G2, G3, G4, & G5) in both short and long-term antibody responses. Generally, groups immunized with Nef DNA along with CD40L DNA or IFNγ DNA as combined or linked forms (G2, G3, G4, & G5) induced the mixture of IgG1 and IgG2a antibodies directed toward more IgG2a response especially in group immunized with pcDNA-IFNγ-Nef (G5). It should be mentioned that the levels of antibody responses in each mouse individually for each group were similar to the pooled sera of each group as shown in Figure S2 (Supporting Information). Moreover, we obtained the serum ratio of 1:100 (v/v) through serial dilution with the sera samples as shown in Figure S3 (Supporting Information).

3.5. Evaluation of Short-Term and Long-Term Cytokine Secretion

The short-term and long-term cytokine results for the pooled splenocytes of mice in each group showed a significant difference in the levels of IFN-γ, IL-10 and TNF-α secretion between test and control groups ($p < 0.0001$, **Figure 6**). The levels of IFN-γ and TNF-α were significantly higher in mice immunized with Nef DNA construct as combined with or linked to IFNγ and CD40L adjuvants (G2, G3, G4, & G5) compared to Nef DNA construct, alone (G1, $p < 0.0001$, Figure 6C). Among all the test groups, the highest levels of IFN-γ and TNF-α secretion were observed in mice immunized with the pcDNA-CD40L-Nef (G4) after restimulation of splenocytes with the recombinant Nef protein ($p < 0.0001$, Figure 6A,B). In contrast, no significant IL-10 secretion was observed in immunized groups as compared to control groups in three weeks and three months after the last injection (IL-10 concentration range: 2–5 pg mL^{-1}). It was interesting that the levels of IFN-γ and TNF-α were stable in three months after the last injection. Moreover, a significant increase in the secretion of IFN-γ and TNF-α ($p < 0.0001$) was detected in all immunized groups especially groups receiving pcDNA-CD40L-Nef (G4) and pcDNA-IFNγ-Nef (G5) in three months after the last injection. Generally, group immunized with pcDNA-CD40L-Nef (G4) showed the highest levels of IFN-γ and TNF-α, and the lowest levels of IL-10 in three weeks and three months after the last injection (Figure 6). Regarding to high secretion of IFN-γ and TNF-α, and low secretion of IL-10, all immunized groups especially groups receiving pcDNA-CD40L-Nef (G4) and pcDNA-IFNγ-Nef (G5) could induce T-helper 1 (Th1) response.

3.6. Evaluation of Short-Term and Long-Term Granzyme B Secretion

The results of Granzyme B assay for the pooled splenocytes in each group indicated that secretion of Granzyme B after restimulation with the Nef protein in groups receiving Nef DNA construct along with IFNγ and CD40L adjuvants as combined or linked forms (G2, G3, G4 & G5) was considerably higher than group immunized with pcDNA-Nef (G1) and control groups ($p < 0.0001$; **Figure 7**). Also, groups immunized with pcDNA-CD40L-Nef (G4) and pcDNA-IFNγ-Nef (G5) showed higher levels of Granzyme B than groups immunized with pcDNA-Nef + pcDNA-CD40L (G2) and pcDNA-Nef + pcDNA-IFNγ (G3) ($p < 0.0001$). Moreover, the highest level of Granzyme B secretion was observed in mice immunized with pcDNA-IFNγ-Nef (G5) regimen ($p < 0.0001$). On the other hand, the Granzyme B secretion was stable in three months after the last injection. Indeed, the Granzyme B secretion was significantly increased in all

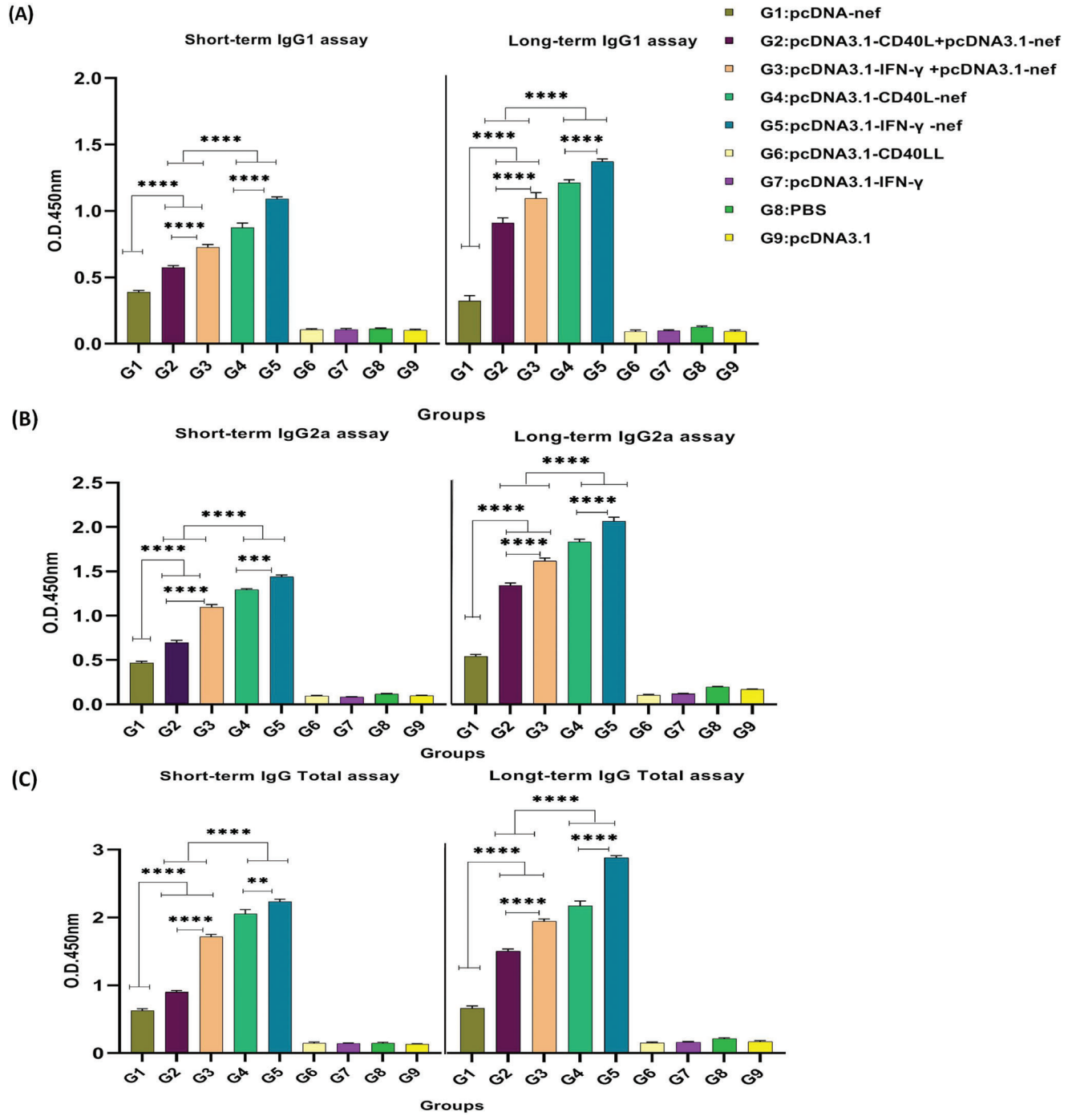

Figure 5. Evaluation of short-term ($n = 4$) and long-term ($n = 3$) antibody responses against the Nef coated antigen in different groups using indirect ELISA: (A) IgG1, (B) IgG2a, and (C) total IgG. The levels of total IgG, IgG1 and IgG2a in the sera of mice immunized with pcDNA-Nef accompanied by CD40L and IFNγ (G2, G3, G4 & G5) against the Nef coated antigen were significantly higher than those in the group immunized with pcDNA-Nef (G1) without adjuvant. The levels of total IgG and IgG2a in the sera of mice immunized with pcDNA-Nef linked to IFNγ and CD40L adjuvants (G4, G5) were significantly higher than groups immunized with pcDNA-Nef mixed with IFNγ and CD40L adjuvants (G3, G4). Each experiment was performed in duplicate for each sample which was shown as the mean absorbance at 450 nm ± SD (** $p < 0.01$; *** $p < 0.001$; ****$p < 0.0001$).

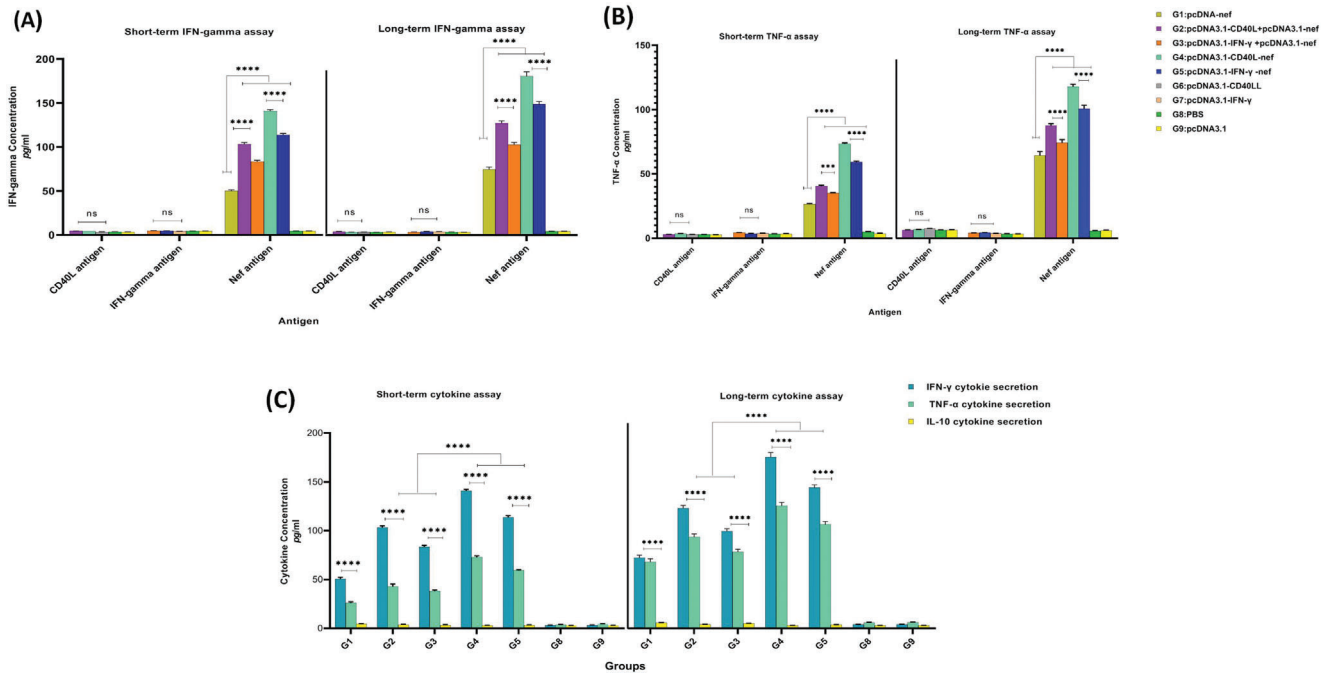

Figure 6. Evaluation of short-term ($n = 4$) and long-term ($n = 3$) cytokine secretion after restimulation of splenocytes with the recombinant Nef, CD40L, and IFN-γ proteins: A) IFN-γ and B) TNF-α ; C) Comparison of cytokines secretion after restimulation of splenocytes with the recombinant Nef protein in different groups using ELISA. The levels of IFN-γ and TNF-α were significantly higher in mice immunized with pcDNA-Nef as combined with or linked to IFNγ and CD40L adjuvants (G2, G3, G4, & G5) compared to pcDNA-Nef without adjuvant (G1). The highest levels of IFN-γ and TNF-α were observed in mice immunized with the pcDNA-CD40L-Nef (G4). Each experiment was performed in duplicate for each sample which was shown as the mean absorbance at 450 nm ± SD (ns: non-significant; *** $p < 0.001$; ****$p < 0.0001$).

immunized groups in three months after the last immunization as compared to that in three weeks after the last immunization ($p < 0.0001$; Figure 7).

3.7. Secretion of Cytokines by Lymphocytes Exposed to Single-Cycle Replicable (SCR) HIV-1 Virions In Vitro

Our data demonstrated that the secretion of IFN-γ, TNF-α and IL-10 were high in the supernatant of the SCR virion-infected splenocytes as compared to the control groups ($p < 0.0001$; **Figure 8**D). The secretion of IFN-γ and TNF-α was significantly higher in groups receiving pcDNA-Nef + pcDNA-CD40L (G2), pcDNA-Nef + pcDNA-IFNγ (G3), pcDNA-CD40L-Nef (G4), and pcDNA-IFNγ-Nef (G5) than group immunized with pcDNA-Nef (G1) ($p < 0.0001$, Figure 8). Among all groups, the levels of IFN-γ and TNF-α secretion were uppermost in mice immunized with the pcDNA-CD40L-Nef (G4). In general, infected lymphocytes could significantly secret IFN-γ, TNF-α and IL-10 in comparison with non-infected lymphocytes ($p < 0.0001$, Figure 8A–C) indicating active T cells induced after immunization and maintaining memory against virions.

4. Discussion

Therapeutic vaccination such as DNA vaccination is one of the most curative strategies for inducing potent HIV-specific immune responses. DNA vaccines possess the potential to elicit long-lasting antibody responses and T cell-mediated immunity especially CD8$^+$ T-cell responses.[18–20] To improve the immunogenicity of DNA vaccines, one key strategy mentioned in many current clinical HIV-1 DNA vaccine formulations is the use of novel molecular adjuvants.[21–24] Numerous studies highlighted the potential of IFNγ and CD40 ligand as immune adjuvants.[25–27] Indeed, binding IFN-γ to IFN-γ receptor on antigen-presenting cells (APCs) enhanced the expression of costimulatory molecules and cytokines essential for T cell activation.[28] Also, CD40L plays an important role in stimulating the production of cytokines and inducing the expression of costimulatory molecules by interacting with CD40 on antigen-presenting cells. Overall, this interaction enhanced the ability of APCs to effectively activate and differentiate T-cells.[29] The use of these adjuvants in vaccine design could boost CD4$^+$ and CD8$^+$ immune responses against viral infections such as influenza, herpes simplex virus (HSV), HIV, and in the 4T1 mouse breast cancer model.[30–34] For instance, the efficacy of Env and Gag-encoding DNA vaccines was increased by co-immunization with IFNγ and IFNγ-inducing cytokine (IL-12) as an adjuvant.[35,36] Furthermore, CD40L-adjuvanted Env DNA/modified *vaccinia virus Ankara simian immunodeficiency virus* (SIV) vaccine led to an enhanced functional quality of anti-Env antibody response, breadth of anti-SIV CD8$^+$ CD4$^+$ T cell responses, and improved viral control. In fact, co-injection of CD40L with Env-encoding DNA showed a potent effect on inducing immune responses.[8] Kwa et al. also indicated that CD40L adjuvant improved the anti-Env antibodies, anti-SIV CD8$^+$ and CD4$^+$ T cell responses,

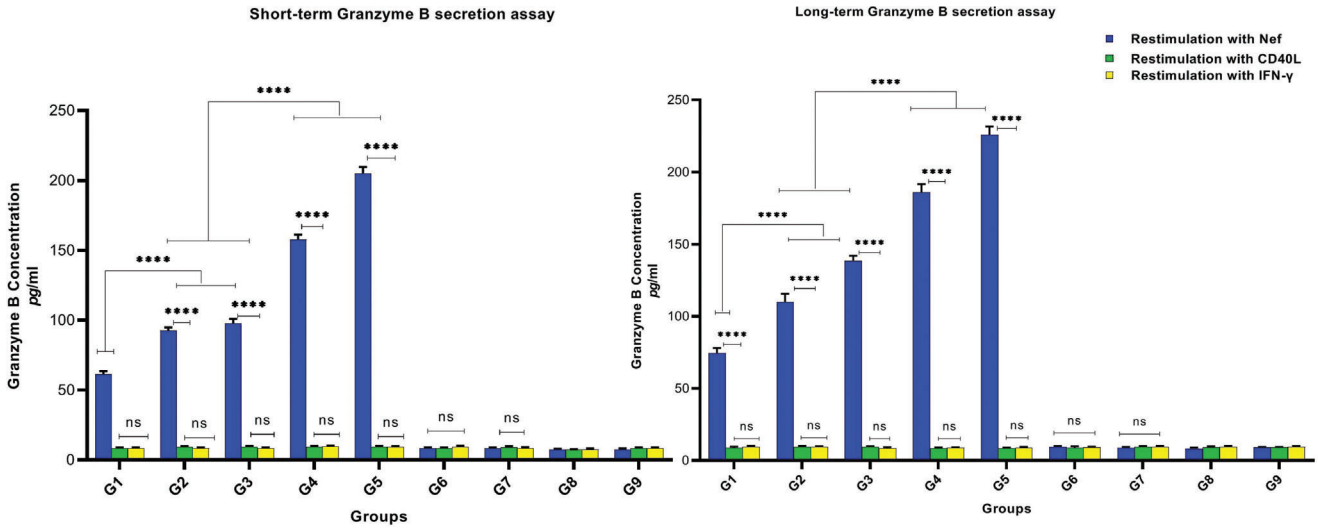

Figure 7. Evaluation of short-term ($n = 4$) and long-term ($n = 3$) granzyme B secretion in different groups using ELISA: Concentration of Granzyme B (pg mL^{-1}) in groups receiving pcDNA-Nef along with IFNγ and CD40L adjuvants as combined or linked forms (G2, G3, G4 & G5) was considerably higher than group immunized with pcDNA-Nef without adjuvant (G1) and control groups (G6-G9). Each experiment was performed in duplicate for each sample which was shown as the mean absorbance at 450 nm ± SD. (ns: non-significant; ****$p < 0.0001$).

and viral control in an Env-based DNA vaccine.[31] Moreover, co-expressing CD40L with SIVmac251 encoding gag, pol and env genes (ALVAC-SIV/CD40L) could increase SIV-specific humoral and cytotoxic responses in Macaques.[37]

We studied the effects of IFNγ and CD40L cytokines as an adjuvant to improve B-cell and T-cell immune responses elicited by HIV-1 Nef DNA-based vaccine construct. These responses were evaluated in short-term and long-term times for showing the stability of immune responses. Moreover, the potency of lymphocytes isolated from the immunized and control mice was investigated after in vitro exposure to SCR HIV-1 virions through cytokines' secretion assay.

Our data showed that immunization with pcDNA-Nef along with pcDNA-CD40L or pcDNA-IFN-γ adjuvants increased the secretion of Nef-specific antibodies (especially IgG2a isotype), IFN-γ and TNF-α, and Granzyme B as compared to immunization with pcDNA-Nef without adjuvant. Furthermore, our findings confirmed that the linkage of IFNγ or CD40L gene to Nef gene in the DNA constructs (pcDNA-IFNγ-Nef or pcDNA-CD40L-Nef) led to significantly higher Nef-specific immune responses than their combination with the Nef DNA construct (pcDNA-Nef + pcDNA-CD40L; pcDNA-Nef + pcDNA-IFN-γ). As known, the immune system regulates the production of IgG1 and IgG2a through different cytokines. Cytokine IL-10 induces IgG1 production, while IFN-γ primarily stimulates IgG2a production. Additionally, the IgG2a to IgG1 ratio can provide valuable insights into the type of immune response against HIV-1 infection. A higher IgG2a/IgG1 ratio indicates a Th1-type response, which is associated with a more robust cellular immune response. Conversely, a higher IgG1/IgG2a ratio suggests a Th2-type response, which is associated with a stronger humoral immune response.[38,39] Balancing the Th1/Th2 immune response is crucial for enhancing the protective effect of the vaccine and preventing pathological enhancement post-immunity.[40] In the present study, the mixture of IgG1 and IgG2a antibody responses was observed in test groups indicating the direction of immune responses toward both Th1 and Th2 responses. However, a significant increase in IgG2a level compared to IgG1 level was illustrative of stronger cellular immune response than humoral immune response against HIV-1 Nef antigen. The highest level of IgG2a was detected in group immunized with pcDNA-IFNγ-Nef against the Nef coated antigen. In this line, Nimal et al. reported that the IFNγ-gp120 fusion DNA vaccine could induce antigen-specific IgG2a response promoting Th1 responses.[41] Also, Wang et al. showed the immunoadjuvant effects of IFN-γ as a DNA construct (pcDNA3.1-IFN-γ) in combination with outer membrane protein A (OmpA) that led to an increased level of serum antibodies against *Edwardsiella tarda*.[42]

On the other hand, our study indicated that the levels of IFN-γ and TNF-α were significantly higher in groups immunized with pcDNA-CD40L-Nef and pcDNA-IFNγ-Nef fusion constructs compared to groups receiving pcDNA-Nef + pcDNA-CD40L and pcDNA-Nef + pcDNA-IFNγ constructs. Among all the test groups, the highest level of IFN-γ and TNF-α secretion was observed in mice immunized with the pcDNA-CD40L-Nef after restimulation of splenocytes with the recombinant Nef protein. High levels of IFN-γ and TNF-α, and low level of IL-10 showed induction of Th1 immune response against Nef antigen. The ex vivo studies demonstrated that IFNγ can boost the activities of cytotoxic T lymphocytes (CTLs) and NK cells against the cells infected with HIV-1. Upregulation of MHC-I by IFNγ enhanced CTL activity to recognize intracellular pathogens. Moreover, IFNγ could upregulate MHC-II for induction of antigen-specific $CD4^+$ T cells.[28] IFN-γ secretion had a significant correlation with CD4 count in HIV^+ patients and antigen-specific $CD8^+$ T cell response. Therefore, scientific advancements have concentrated on utilizing IFN-γ as a biological indicator to analyze the type of immunity caused by potential HIV vaccines.[43] Moreover, depending upon the clinical phase of HIV-1 infection, the increase of multifunctional cytokines such as TNF-α led

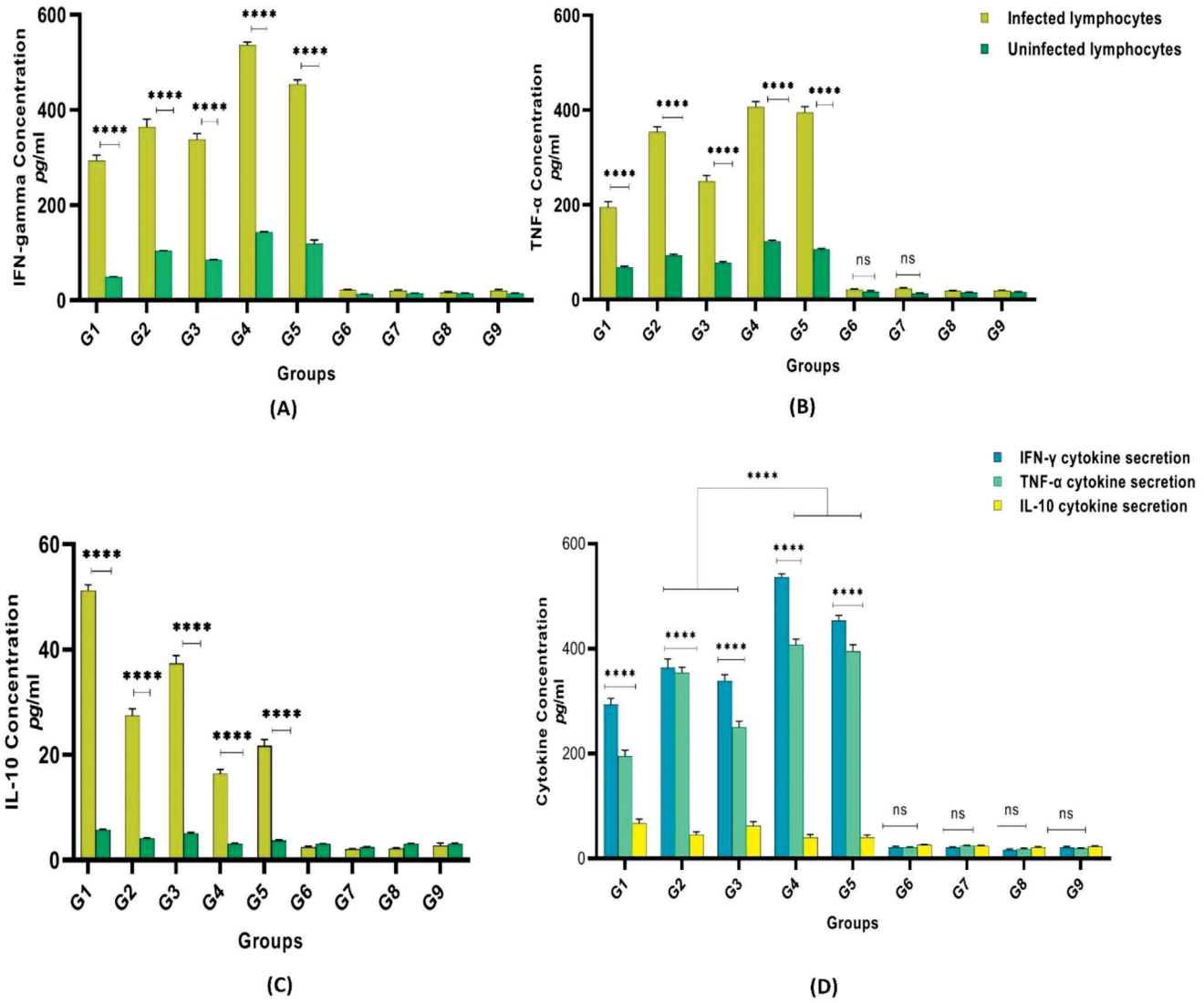

Figure 8. Evaluation of cytokines secretion in SCR HIV-1-infected lymphocytes as compared to uninfected lymphocytes: IFN-γ (A), TNF-α (B) and IL-10 (C); Comparison of cytokines levels (concentration: pg mL^{-1}) in SCR HIV-1-infected lymphocytes (D). Secretion of IFN-γ, TNF-α and IL-10 were higher in the supernatant of the SCR virion-infected splenocytes than the control groups and uninfected lymphocytes. Each experiment was performed in duplicate for each sample which was shown as the mean absorbance at 450 nm ± SD (ns: non-significant; ****$p < 0.0001$).

to the control of HIV-1 infection. HIV-1 non-progression was linked to the presence of polyfunctional T cells expressing IFN-γ along with the inflammatory cytokine TNF-α and/or cytotoxins (perforin or granzyme B). On the other hand, TNF-α inhibited HIV-1 replication in a variety of cell types including recently infected peripheral blood monocytes, alveolar macrophages, and thymic cortical dendritic macrophages (TCDM).[44] Additionally, TNF-α induced a number of HIV suppressive factors (e.g., RANTES) in lymphoid cells.[45] Interestingly, the data showed that inclusion of IFNγ in vaccine component gives rise to TNF-α induction.[42] In contrast, in vitro studies on $CD4^+$ and $CD8^+$ T-cell proliferation and cytokine production in HIV-infected people have shown that IL-10 hinders these processes and IL-10 has harmful effects during HIV infection by reducing IL-2 and IL-12 production.[46] In some HIV-infected people, IL-10 blockage ameliorated T-cell function.[47] Our results showed higher secretion of granzyme B in groups immunized with pcDNA-IFNγ-Nef and pcDNA-CD40L-Nef fusion constructs than groups receiving pcDNA-Nef + pcDNA-CD40L and pcDNA-Nef + pcDNA-IFNγ constructs. Moreover, IFNγ adjuvant was more potent in inducing Granzyme B with respect to Nef antigen compared to CD40L adjuvant suggesting a possible enhancement of antigen-specific CTL responses. The studies indicated that discharging Granzyme B by cytolytic lymphocytes was considered as an indicator of CTL activity.[48,49] Ceglia et al. showed that CD40L enhanced $CD8^+$ T cell responses in HIV-1^+ donor PBMCs by evaluating HIV5pep (a Gag, Nef, and Pol vaccine construct)-specific granzyme secretion.[50] Our study also demonstrated long-term secretion of antibodies (especially IgG2a), cytokines (IFN-γ and TNF-α), and Granzyme B in groups receiving the Nef DNA constructs

especially along with IFNγ or CD40L adjuvants suggesting the stability of immune responses directed toward Th1 response and CTL activity. Notably, Moureau et al. revealed that mice immunized with plasmid DNA encoding HIV-1 Nef represented considerable levels of long-lasting anti-Nef antibodies (>16 months).[51]

In the current study, mice lymphocytes were infected with safe SCR HIV-1 virions, and their ability was evaluated to secrete IFN-γ, IL-10 and TNF-α for detection of virions-specific T cell responses. The SCR virion is capable of eliciting potent immune responses in vaccine development as a key origin of HIV antigens.[17] Our findings showed that after virion exposure, IFN-γ and TNF-α secretion was significantly increased as compared to IL-10 secretion especially in groups immunized with pcDNA-CD40L-Nef and pcDNA-IFNγ-Nef indicating the importance of strong Th1 immune response compared to Th2 immune response. As a result of the in vivo viral challenge, it may suggest re-establishing responses that were lost early after vaccination, priming new T cell responses, or expanding already present T cells.[52] Some previous studies indicated that stimulation of dendritic cells with the non-replicating virus (i.e., AT-2 HIV-1) led to an increased T-cell response and improved HIV-specific immunity.[53–55] Dinter et al. reported that cross-presentation of HIV proteins by DCs enhanced specific CTL responses to dominant immune epitopes.[56] Generally, the linkage of IFNγ or CD40L as an adjuvant to Nef antigen (i.e., IFNγ-Nef or CD40L-Nef fusion construct) could significantly induce T-helper 1 pathway and activate cytotoxic T lymphocytes as compared to other regimens. Therefore, the fusion DNA constructs can be applied as a potential option for development of an efficient HIV-1 vaccine.

5. Conclusion

In summary, the DNA-based HIV-1 vaccine constructs harboring Nef antigen along with IFNγ cytokine or CD40L costimulatory molecule as an adjuvant were able to direct the immune responses towards cellular immunity. Furthermore, IFNγ or CD40L adjuvants could effectively induce Nef-specific long-term immune responses especially when adjuvants were linked to the N-terminal region of Nef antigen. Moreover, the fusion constructs could considerably stimulate the secretion of IFN-γ and TNF-α in the infected lymphocytes with safe virions in vitro. However, further studies are required to assess the adjuvant effects of IFNγ and CD40L in DNA vaccine design for reduction of HIV-1 replication in animal models (e.g., macaque).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

F.H.: investigation, formal analysis, project administration, writing-original draft preparation. A.B.: conceptualization, supervision, formal analysis, validation, writing-reviewing and editing. S.A.: methodology, formal analysis, writing-reviewing and editing. A.M.: investigation, data curation, project administration, writing-reviewing and editing. S.A. S.: formal analysis, writing-reviewing and editing.

Keywords

CD40L, DNA-based vaccine, HIV-1, IFNγ, Nef antigen, single-cycle replicable HIV-1 virion

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