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RESEARCH ARTICLE

Evaluation of HIV-1 Regulatory and Structural Proteins as Antigen Candidate in Mice and Humans

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ARTICLE HISTORY

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Abstract: ***Background*:** The diagnosis of HIV infection is important among different groups. Moreover, combination antiretroviral therapy is used to treat HIV-1, but it cannot eradicate the infection. Thus, the development of therapeutic vaccines, along with antiretroviral therapy, is recommended. This study evaluates the values of four HIV proteins as antigen candidates in therapeutic vaccine design as well as a possible diagnostic marker for HIV infection in humans.

***Methods*:** In this study, the HIV-1 Tat and Rev regulatory proteins and structural Gp120 and p24 proteins were generated in *E. coli* expression system. Their immunogenicity was evaluated in BALB/c mice using homologous and heterologous prime/boost strategies. Moreover, the detection of anti-HIV IgG antibodies against these recombinant proteins was assessed in untreated (Naïve/ HIV-infected), treated, and drug-resistant patients compared to the healthy (control) group as a possible diagnostic marker for HIV infection.

***Results*:** In humans, our results showed that among HIV-1 proteins, anti-Gp120 antibody was not detected in treated individuals compared to the healthy (control) group. The levels of anti-Gp120 antibody were significantly different between the treated group and Naïve as well as drug-resistant subjects. Moreover, the level of anti-p24 antibody was significantly lower in the treated group than the Naive group. In mice, the results of immunization indicated that the Rev antigen could significantly induce IgG2a, IgG2b, and IFN-γ secretion aimed at Th1 response as well as Granzyme B generation as CTL activity in comparison with other antigens. Furthermore, the heterologous DNA prime/ protein boost regimen was more potent than the homologous regimen for stimulation of cellular immunity.

***Conclusion*:** Briefly, the levels of both anti-Gp120 and anti-p24 antibodies can be considered for the diagnosis of the HIV-infected individuals in different groups compared to the healthy group. Moreover, among four recombinant proteins, Rev elicited Th1 cellular immunity and CTL activity in mice as an antigen candidate in therapeutic vaccine development.

Keywords: HIV-1, regulatory protein, structural protein, drug resistance, immune responses, diagnosis.

1. INTRODUCTION

The human immunodeficiency virus (HIV), causing acquired deficiency syndrome (AIDS), remains undetectable even after a long time. Thus, a variety of molecular and immunological techniques were designed to diagnose and detect HIV in the early stage of infection [1, 2]. The detection of HIV infection was performed by IgG antibody assay (approximately 6-12 weeks after infection), the use of recombinant antigens for HIV test, IgM detection (approximately 3 weeks post-infection), and p24 antigen/ antibody detection (up to 11-14 days after infection) [3-5]. The data indicated that p24 antigen was assessed even in patients with low viral load. Therefore, HIV p24 antigen capture assay was designed based on the enzyme-linked immunosorbent assay (ELISA) with a modification to identify antigen, not antibody [1]. However, it was observed that p24 antigen was not detected in 50% of individuals with a CD4 count of more than 1500 [6]. Moreover, the p24 antigen assay of plasma stored on filter paper was a steady approach for screening HIV-1 load [7]. The researchers used p24 protein as a model HIV antigen and increased its immunogenicity by different methods [8]. In general, the HIV genome encodes Env, Gag, Pol structural, Rev, Tat regulatory, and Nef, Vpr, Vif, and Vpu accessory proteins. HIV-1 Env or envelope protein is a trimer of glycoprotein 160 kDa (Gp160) that the cellular proteases cleave into glycoproteins 120 and 41 kDa. HIV-1 Rev

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protein helps the transportation of viral RNA without processing from nucleus to cytoplasm for the translation process [1, 9]. Anti-Rev antibodies were detected in HIV-1 patients by western blotting [10]. An HIV DNA vaccine consisting of a cocktail of seven plasmids encoding HIV-1 proteins (*i.e.,* Env, Rev, Gag B & Inactivated reverse transcriptase) delivered with a needle-free system was designed. The recombinant GM-CSF and imiquimod were delivered along with vaccine plasmids. GM-CSF could enhance cellular and humoral immunity substantially without inducing any antibody response to GM-CSF alone [11]. HIV-1 Tat protein is necessary for replication of virus and AIDS pathogenesis [1, 12-14]. The HIV-1 Tat and Rev proteins were suggested as promising target antigens for the design of HIV-1 vaccine candidates. However, their immunosuppressive effects may reduce the potent immune responses that need to be evaluated *in vivo* [15]. Recent studies showed that the $CD8^+$ cytotoxic T cell (CTL) activity against Tat and Rev proteins could control HIV-1 load [16, 17]. Moreover, the Tat therapeutic vaccine as a pathogenesis-driven intervention effectively intensifies combination antiretroviral therapy (cART), leading to a potent cure and virus eradication [18, 19]. Some studies focused on the effects of Tat expression plasmid (pTat) for regulating the immune responses induced by HIV DNA vaccines. For example, pTat could modulate immune responses to HIV Gag, Env, and Pol antigens [20]. Tat-based DNA vaccines could confer some levels of protection in animal models [21]. According to the reported results, it is important for immunological assay of HIV-1 regulatory and structural proteins as antigen candidates. In the current study, we focused on comparing immunogenic properties of Tat and Rev regulatory proteins, and P24 and Gp120 structural proteins in mice as antigen candidates in vaccine design. Moreover, the detection of anti-HIV IgG antibodies against these proteins was performed to determine a possible diagnostic marker for HIV infections in humans.

2. METHODS

2.1. HIV-1 Antigens

The *tat*, *rev*, *gp120* and *p24* genes were cloned from pNL4.3 (AF324493.2) into the *Xho*I/ *Nhe*I, *Bam*HI/*Hind*III, *Nhe*I/*Xho*I, and *Xho*I/ *Nhe*I cloning sites of pET24a, pET28a, pET23a, and pET24a vectors, respectively. For expression of the full-length Tat, Rev, Gp120 and p24 proteins, the *E. coli* Rosetta strain was transformed with the recombinant (r) pET24a-*tat*, pET28a-*rev,* pET23a-*gp120,* and pET24a-*p24*. The single clone was cultured in LB Broth (Quelab, Canada) for 16-18 h at 37˚C and then these cells were added to Ty2x medium (1% v/v; Quelab). When the culture absorbance was in the range of 0.6-0.7 (OD_{600}=0.6-0.7), isopropyl-β-D-1-thiogalactopyranoside (IPTG, 1 mM, Sigma) was added to induce protein expression. The protein expression was evaluated at different times after induction (*i.e.,* 2, 3, 4 & 16 h) using 12.5% SDS-PAGE. Next, the purification of the recombinant proteins was done by affinity chromatography (Ni-NTA agarose column) under both native and denaturing conditions based on the manufacturer's instructions (Machery-Nagel, Germany). The native procedure was performed by increasing imidazole concentration up to 250 mM at constant pH (= 8). The denaturing procedure was done by reducing pH up to 4.5 at a constant concentration of Urea (~ 8 M). After that, dialysis of the pure proteins was performed against PBS1X. Finally, the concentration of the recombinant proteins was determined by a NanoDrop spectrophotometer. On the other hand, their expression was assessed by western blot using peroxidase-conjugated anti-His antibody (Abcam) and 3,3'-Diaminobenzidine tetrahydrochloride hydrate (DAB/H_2O_2) as substrate (Sigma). Finally, endotoxin contamination with LPS was assessed by Limulus Amebocyte Lysate (LAL assay, QCL-1000) that was found to be less than 0.5 EU/mg for all proteins.

2.2. Molecular Cloning of HIV-1 Tat, Rev, gp120 and p24 Genes in a Eukaryotic Expression Vector

The *tat, rev, gp120 and p24* genes amplified from pNL4.3 (AF324493.2) were cloned into pcDNA3.1 (-) vector using *Nhe*I/ *Xho*I, *Nhe*I/*Hind*III, *Bam*HI/*Hind*III and *Nhe*I/ *Xho*I restriction enzymes. The pcDNA-*tat*, pcDNA-*rev*, pcDNA-*gp120*, and pcDNA-*p24* constructs were prepared by an endotoxin-free plasmid kit (MN, Germany) on a large scale. The purity and concentration of the recombinant plasmid DNAs were evaluated by a NanoDrop spectrophotometer.

2.3. Antibody Detection in Human

2.3.1. Population Study

The sera of untreated (naïve) and treated participants by antiretroviral drugs were provided from the Infectious Disease Department of Imam Khomeini Hospital (Tehran, Iran), and maintained in Biobank at Pasteur Institute of Iran. The specimens for antiretroviral drug resistance mutations were previously determined by Nested PCR based on the published manuscripts [22, 23]. The participants were older than 18 years of age. The study protocol was approved by the Ethical Committee of the Iranian Research Center for HIV/AIDS as well as the Ethical Committee of Pasteur Institute of Iran, and the consent was taken from all participants before blood collection. In this cross-sectional study, 141 individuals were enrolled. We considered about 71 treated (responded to treatment), 20 naïve HIV-infected (/untreated HIV-infected), 30 drug resistant [22, 23], 20 HIV uninfected (/HIV-negative) individuals in each group. The demographic data were previously prepared through physician interviews, as well.

2.3.2. Anti-HIV IgG ELISA

The HIV-1 Tat, Rev, Gp120, and p24-specific serum IgG levels were assessed by the ELISA technique. In brief, HIV-1 antigens (~ 3 µg/ ml) were individually coated to 96-well plates. After blocking with 1% bovine serum albumin (BSA, Sigma) in PBS1X for 2 h at 37°C, sera were added at a 1:100 dilution in blocking buffer containing 0.05% (v/v) Tween 20. After 2h of incubation at 37°C, horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody

(1:10000 v/v, Sigma, Germany) was added and incubated for 90 min. The 3, 3′, 5, 5'-Tetramethylbenzidine substrate (TMB, Sigma) was added and the absorbance was assessed at 450 nm. To determine a positive anti-protein response among groups, cut-off values were calculated and the SDs set mean+2 as the cut-off value, above which a response was considered positive [24].

2.4. Design of Constructs for Eliciting Immune Responses in Mice

2.4.1. Physicochemical Properties of the Tat, Rev, gp120, and p24 DNA/ MPG Nanoparticles

For the preparation of the non-covalent DNA/MPG complexes, the MPG amphipathic cell-penetrating peptide (MPG CPP: GALFLGFLGAAGSTMGAWSQPKKKRKV) was individually added to 1 µg of DNA at an N/P ratio of 10 and incubated for one hour at room temperature [25]. The formation of the nanoparticles was determined by gel retardation assay, and their stability was detected against DNA nuclease and serum protease based on the previous studies [25]. Moreover, the zeta potential and size of the nanoparticles were evaluated by the Zetasizer Nano ZS instrument and scanning electron microscope (SEM), respectively.

2.4.2. Physicochemical Properties of the Tat, Rev, Gp120 and p24 Protein/ CPP Nanoparticles

The LDP-NLS amphipathic CPP (KWRRKLKKLRP-KKKRKV) and CyLoP-1 cationic CPP (CRWRWKCCKK) were used to form the non-covalent complexes with the recombinant (r)Tat, rRev, rGp120, and rp24 proteins at a molar ratio of 1:10 (1 µg protein), and incubated for 60 minutes at room temperature [25, 26]. The zeta potential and size of the complexes were analyzed using Zetasizer and SEM, respectively.

2.4.3. Mice Immunization

Six to eight week old female BALB/c mice (n= 4 in each group) maintained under pathogen-free conditions were provided from breeding stock at Pasteur Institute of Iran. The whole experiments were done based on approved protocols for the care of laboratory animals at Pasteur Institute of Iran. Three immunization regimens were utilized, including DNA prime/DNA boost, protein prime/protein boost, and DNA prime/protein boost strategies for each protein, as shown in Table **1**. Mice were subcutaneously immunized at the footpad three times at two-week intervals.

Table 1. Immunization program in mice for HIV-1 Rev/ Tat/ P24/ Gp120 antigens.

Group	Vaccine Modality	Priming	Booster 1 (Two Weeks After Priming)	Booster 2 (Two Weeks After Booster 1)	Antibody, Cytokine and Granzyme B Assays (One Month After Booster 2)
G1	DNA/DNA/DNA	pcDNA-*rev*/*tat*/*p24*/*gp120*[a]	pcDNA-*rev*/*tat*/*p24*/*gp120*	pcDNA- *rev*/*tat*/*p24*/*gp120*	Done
G2	DNA/DNA/DNA	pcDNA- *rev*/*tat*/*p24*/*gp120* + MPG[b]	pcDNA- *rev*/*tat*/*p24*/*gp120* + MPG	pcDNA- *rev*/*tat*/*p24*/*gp120* + MPG	Done
G3	Protein/protein/protein	rRev/Tat/p24/Gp120 + Montanide[c]	rRev/Tat/p24/Gp120 + Montanide	rRev/Tat/P24/Gp120 + Montanide	Done
G4	Protein/protein/protein	rRev/Tat/p24/Gp120 + CyLop-1[d]	rRev/Tat/p24/Gp120 + CyLop-1	rRev/Tat/p24/Gp120 + CyLop-1	Done
G5	Protein/protein/protein	rRev/Tat/p24/Gp120 + LDP-NLS[e]	rRev/Tat/p24/Gp120 + LDP-NLS	rRev/Tat/p24/Gp120 + LDP-NLS	Done
G6	DNA/protein/protein	pcDNA- *rev*/*tat*/*p24*/*gp120* + MPG	rRev/Tat/p24/Gp120 + Montanide	rRev/Tat/p24/Gp120 + Montanide	Done
G7	DNA/protein/protein	pcDNA- *rev*/*tat*/*p24*/*gp120* + MPG	rRev/Tat/p24/Gp120 + CyLop-1	rRev/Tat/p24/Gp120 + CyLop-1	Done
G8	DNA/protein/protein	pcDNA- *rev*/*tat*/*p24*/*gp120* + MPG	rRev/Tat/p24/Gp120 + LDP-NLS	rRev/Tat/p24/Gp120 + LDP-NLS	Done
G9	control	pcDNA3.1	pcDNA3.1	pcDNA3.1	Done
G10	control	MPG	MPG	MPG	Done
G11	control	CyLop-1	CyLop-1	CyLop-1	Done
G12	control	LDP-NLS	LDP-NLS	LDP-NLS	Done
G13	control	Montanide	Montanide	Montanide	Done
G14	control	PBS	PBS	PBS	Done

[a]: Dose used: 50 µg.
[b]: Dose used: N/P= 10:1 (CPP: DNA); DNA: 5µg.
[c]: Dose used: 70: 30 (v/v; Montanide ISA720: protein); protein: 5µg.
[d]: Dose used: molar ratio = 10:1 (CPP: protein); protein: 5µg.
[e]: Dose used: molar ratio = 10:1 (CPP: protein); protein: 5µg.
*** HIV-1 Tat/ Rev/ Gp120/ p24 (/ means or): This program was individually performed for all proteins.**

2.4.4. Evaluation of Antibodies in Mice Sera

Briefly, the pooled sera were prepared for each group (n= 4) one month after the last immunization, and the levels of horseradish peroxidase-conjugated total IgG, IgG1, IgG2a, and IgG2b antibodies (1:10000 v/v, Sigma, Germany) were individually assessed in the sera using indirect ELISA against the recombinant Tat, Rev, Gp120 and p24 proteins (5 µg/ml) as coating antigens [27]. The absorbance was determined at 450 nm using TMB substrate.

2.4.5. Evaluation of IFN-γ and IL-5 Cytokines in the Splenocytes of Mice

All mice in each group (n= 4) were sacrificed one month after the second booster. The 2×10^6 red blood cell-depleted splenocytes pooled for each group were seeded in 48-well plates (SPL) in the presence of rTat, rRev, rGp120 and rp24 proteins (5 µg/ml), and also RPMI 5% and concanavalin A as negative and positive controls, respectively. After incubation of plates for 72 h at 37°C and 5% CO_2, the supernatants were collected to assess the levels of IFN-γ and IL-5 cytokines using a sandwich-based ELISA (Mabtech, Sweden). The lower detection limits were 4 pg/ ml for both cytokines. Moreover, MTT assay was used to determine the proliferation of lymphocytes calculated as the stimulation indices (SI). The rest of the splenocytes were used to assess the release of Granzyme B (GrB).

2.4.6. In vitro Evaluation of Granzyme B in Mice

The 2×10^4 SP2/0 target cells (T) cultured in 96-well plates were incubated with Tat, Rev, Gp120, and p24 antigens (~ 5 µg/ ml) at 37°C and 5% CO_2 for 24 h. The splenocytes as effector cells (E) were added to the target cells at a T: E ratio of 1:100 and incubated for 6 h. The wells containing effector cells were considered for possible spontaneous release of Granzyme B. Then, the microplates were centrifuged for 5 min at 4°C and 250×g. Finally, the supernatants were collected [28] to assess the concentration of Granzyme B (GrB) using an ELISA kit according to the manufacturer's instructions (eBioscience, USA).

2.5. Statistical Analysis

Prism 5.0 software (GraphPad, San Diego, CA) was used for statistical analysis. One-way ANOVA and Student's *t*-test determined the significant differences between groups (p-value < 0.05). The median described the data for Mann-Whitney U tests. The data were shown as mean ± SD.

3. RESULTS

3.1. Production of the Recombinant Tat, Rev, Gp120, and p24 Proteins

The recombinant Tat (pET24a/Rosetta system, OD_{600}: 0.6-0.7, 4 h after induction, 37ºC), Rev (pET28a/Rosetta system, OD_{600}: 0.6-0.7, 16 h after induction, 37ºC), Gp120 (pET23a/ Rosetta system, OD_{600}: 0.6-0.7, 16 h after induction, 37ºC), and p24 (pET24a/ Rosetta system, OD_{600}: 0.6-0.7, 3 h after induction, 37ºC) proteins were efficiently expressed in *E. coli*, and migrated as the clear bands of ~11, ~19, ~50 and ~24 kDa in SDS-PAGE, respectively. The purification of rTat, rRev, and rp24 proteins was done *via* the native method using imidazole buffer (250 mM, pH=8), and the purification of rGp120 protein was performed through the denaturing method using Urea buffer (8 M, pH=4.5). The concentrations of the recombinant proteins were in the range of 0.6-0.8 mg/ ml.

3.2. Preparation of DNA and Protein Nanoparticles

The highly pure endotoxin-free pcDNA-*tat*, pcDNA-*rev*, pcDNA-*gp120*, and pcDNA-*p24* were confirmed by PCR, enzyme digestion, and also sequencing. The *tat*, *rev*, *gp120,* and *p24* genes migrated as ~ 218, ~ 520, ~ 1527, and ~ 696 bp on an agarose gel, respectively, after digestion or PCR. Then, the DNA/MPG complexes and the protein/CyLop-1 or LDP-NLS complexes were formed at an N/P ratio of 10:1 and a molar ratio of 10: 1, respectively, as determined in our previous study [29]. The results of SEM and zeta potential for the DNA/MPG and protein/CyLop-1 or LDP-NLS nanoparticles are shown in Table **2**. As observed, the surface charges of nanoparticles indicated a positive charge as compared to DNA plasmids and proteins alone.

3.3. Detection of Tat, Rev, Gp120 and p24-Specific Antibodies in Human

Iranian subjects were divided into four groups: 20 Naïve HIV-infected individuals (or untreated HIV-infected subjects: G1), 71 treated individuals with ART (G2), 30 drug resistant individuals (G3), and 20 control individuals (healthy or HIV-uninfected/ HIV-negative subjects: G4). The frequency of antibodies to Tat, Rev, Gp120, and p24 proteins was assessed in sera of different groups. The mean absorbance values for Tat were 1.405 ± 0.505 (G1), 1.194 ± 0.465 (G2), 1.292 ± 0.560 (G3), and 0.528 ± 0.147 (G4), respectively. The mean absorbance values for Rev were 1.074 ± 0.426 (G1), 0.906 ± 0.362 (G2), 0.983 ± 0.564 (G3) and 0.531 ± 0.129 (G4), respectively. The mean absorbance values for Gp120 were 0.932 ± 0.545 (G1), 0.613 ± 0.268 (G2), 0.823 ± 0.486 (G3) and 0.499 ± 0.162 (G4), respectively. The mean absorbance values for p24 were 1.355 ± 0.353 (G1), 1.123 ± 0.401 (G2), 1.234 ± 0.327 (G3) and 0.317 ± 0.070 (G4), respectively. The levels of antibodies against all recombinant proteins in all groups (except for the level of anti-Gp120 antibody in treated group) were significantly higher than those in the control group ($p < 0.05$). Furthermore, the levels of antibodies against rTat and rRev proteins did not show any statistical differences in Naïve (G1), treated (G2), and drug resistance (G3) groups ($p > 0.05$). In contrast, the level of antibody against rGp120 in the treated group (G2) was lower than the Naive group (G1, $p < 0.01$) and also the drug resistance group (G3, $p < 0.05$). However, the level of anti-Gp120 antibody did not indicate any considerable difference between naïve and drug resistance groups ($p > 0.05$). Additionally, the results indicated that the p24 protein could be considered as a possible diagnostic marker for the determination of the Naïve group from the treated

Table 2. Size and charge of the nanoparticles.

Construct	Size (nm; SEM)	Charge (mV; Zetasizer)
pcDNA-*rev*	180-230	-17.7
pcDNA-*rev* + MPG	110-160	13.6
Rev protein	200-250	-8.84
Rev protein+CyLop	120-190	2.26
Rev protein+LDP-NLS	110-140	1.91
pcDNA-*tat*	150-200	-17.0
pcDNA-*tat*+MPG	150-200	19.4
Tat protein	170- 210	-8.96
Tat+LDP-NLS	130-200	5.52
Tat+CyLop	100-140	7.75
pcDNA-*p24*	240-280	-24.8
pcDNA-*p24*+MPG	150-200	23.4
P24 protein	250-300	-7.5
P24 + LDP-NLS	270-350	3.08
P24 + Cylope	110-190	2.88
pcDNA-*gp120*	120-160	-25.2
pcDNA-*gp120* + MPG	130-180	26.5
Gp120 protein	190-250	-25.9
Gp120 + LDP-NLS	100-150	5.69
Gp120 + Cylope	70-110	1.54

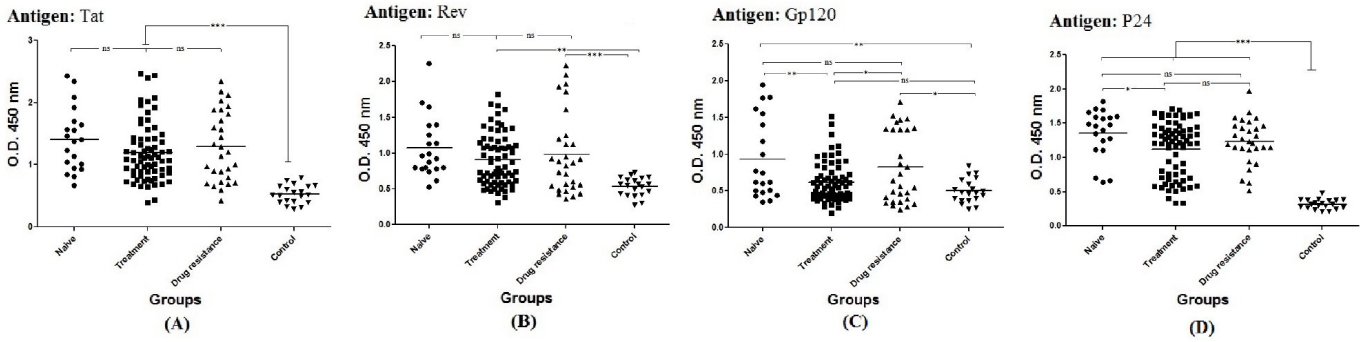

Fig. (1). Analysis of anti-Tat, anti-Rev, anti-Gp120 and anti-p24 antibody levels in the sera of untreated/Naïve, treated, and drug resistance groups compared to control/healthy group using ELISA. All analyses were performed in duplicate for each sample. The horizontal line represents the mean value of optical density in respect to each antigen; ns: non-significant, *: $p < 0.05$, **$p < 0.01$, ***: $p < 0.001$.

group but not from the drug resistance group. (Fig. **1**) demonstrates the differences between the seroreactivities in all groups against the rTat, rRev, rGp120, and rp24 proteins. In general, 90, 75, 40, and 100% of the naive sera recognized the recombinant Tat, Rev, Gp120, and p24 proteins, respectively. Moreover, 84, 55, 9, and 96% of the treated sera recognized the recombinant Tat, Rev, Gp120, and p24 proteins, respectively. On the other hand, 73, 56, 45, and 100% of the drug resistant sera recognized the recombinant Tat, Rev, Gp120, and p24 proteins, respectively.

3.4. Detection of Immune Responses in Mice

3.4.1. Antibody Responses Against HIV-1 Antigens

The levels of total IgG in groups immunized with the homologous Rev or Gp120 protein regimens (G3-G5) were considerably higher than other groups ($p < 0.05$; (Fig. **2A**). Moreover, the levels of total IgG in groups immunized with the homologous Tat or p24 protein regimens (G3-G5) were similar to those in the heterologous Tat or p24 protein regimens (G4-G8, $p > 0.05$; (Fig. **2A**). The levels of IgG1 in

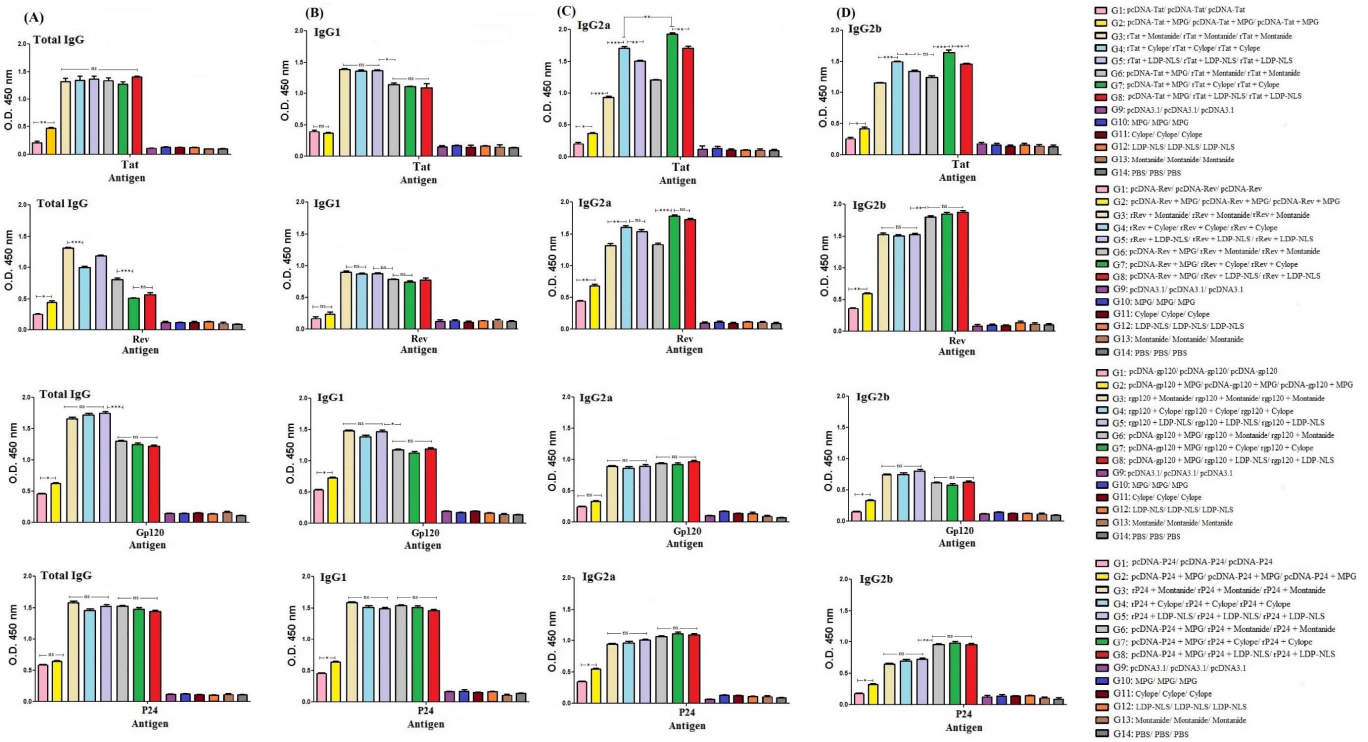

Fig. (2). Antibody responses (A: total IgG, B: IgG1, C: IgG2a and D: IgG2b) against the recombinant Tat, Rev, Gp120 and p24 proteins in different regimens: All analyses were performed in duplicate for each sample. The results were shown as mean absorbance at 450 nm ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: non-significant ($p > 0.05$). (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

groups administrated by Rev (G3-G8) were significantly less than groups immunized with other recombinant proteins (Fig. **2B**), $p < 0.05$). On the contrary, the levels of IgG2a and IgG2b in groups injected with Tat and Rev (G3-G8) were significantly higher than groups immunized with other recombinant proteins (Figs. **2C** & **D**), $p < 0.05$). As known, the ratios of IgG2a/IgG1 or IgG2b/IgG1 indicate Th1-directed responses [27, 30-32]. These ratios were higher in the heterologous and then homologous Rev and Tat regimens (G3-G8) as compared to those in Gp120 and p24 groups ($p < 0.05$). Indeed, the ratio of IgG2a/IgG1 was lower in the homologous and heterologous Gp120 and p24 regimens (G3-G8), indicating Th2-directed responses. The immunological effects of CyLop-1 and LDP-NLS as protein delivery systems were almost similar to Montanide adjuvant.

3.4.2. Evaluation of Cytokines

The levels of IFN-γ and IL-5 cytokines were evaluated for Th1 and Th2 immune responses in mice splenocytes, respectively. All groups immunized with different modalities significantly increased the levels of IFN-γ compared to control groups for all injections ($p < 0.05$, (Figs. **3A-D**). In addition, groups injected with the heterologous DNA prime/ protein boost regimens (G6-G8) could secrete Tat and Rev-specific IFN-γ in a significantly higher proportion than other groups. Our data showed that the levels of IFN-γ in groups immunized with different Gp120 and p24 regimens were significantly lower than groups injected with Rev and Tat regimens ($p < 0.05$, (Figs. **3A-D**). The levels of IL-5 were considerably higher in groups immunized with Gp120 and p24 regimens than groups immunized with Rev and Tat regimens ($p < 0.05$, (Figs. **3E-H**). Furthermore, the ratios of IFN-γ/ IL-5 were significantly higher in the heterologous and then homologous Tat and Rev regimens (G3-G8), indicating Th1-directed responses. In contrast, the ratio of IFN-gamma/ IL-5 was lower in the homologous and heterologous Gp120 and p24 regimens (G3-G8), indicating Th2-directed responses (Fig. **3**). In addition, CyLop-1 and LDP-NLS could induce cytokine secretion effectively, similar to Montanide adjuvant in all immunizations with different antigens ($p < 0.05$, Fig. **3**). MPG could significantly stimulate IFN-gamma response in DNA immunizations ($p < 0.05$, Figs. **3A-D**). The proliferation of splenocytes was significantly detected in groups injected with the heterologous Rev DNA prime/ Rev protein boost regimens (Fig. **4**), which confirmed the results of cytokine secretion. Other antigens showed the same lymphoproliferative responses between the heterologous DNA prime/ protein boost regimens and the homologous protein prime/ protein boost regimens (Fig. **4**; $p > 0.05$). However, these responses were higher than DNA-injected groups and controls ($p < 0.05$). In general, immunization with different regimens of Rev elicited antigen-specific Th-cell proliferative responses.

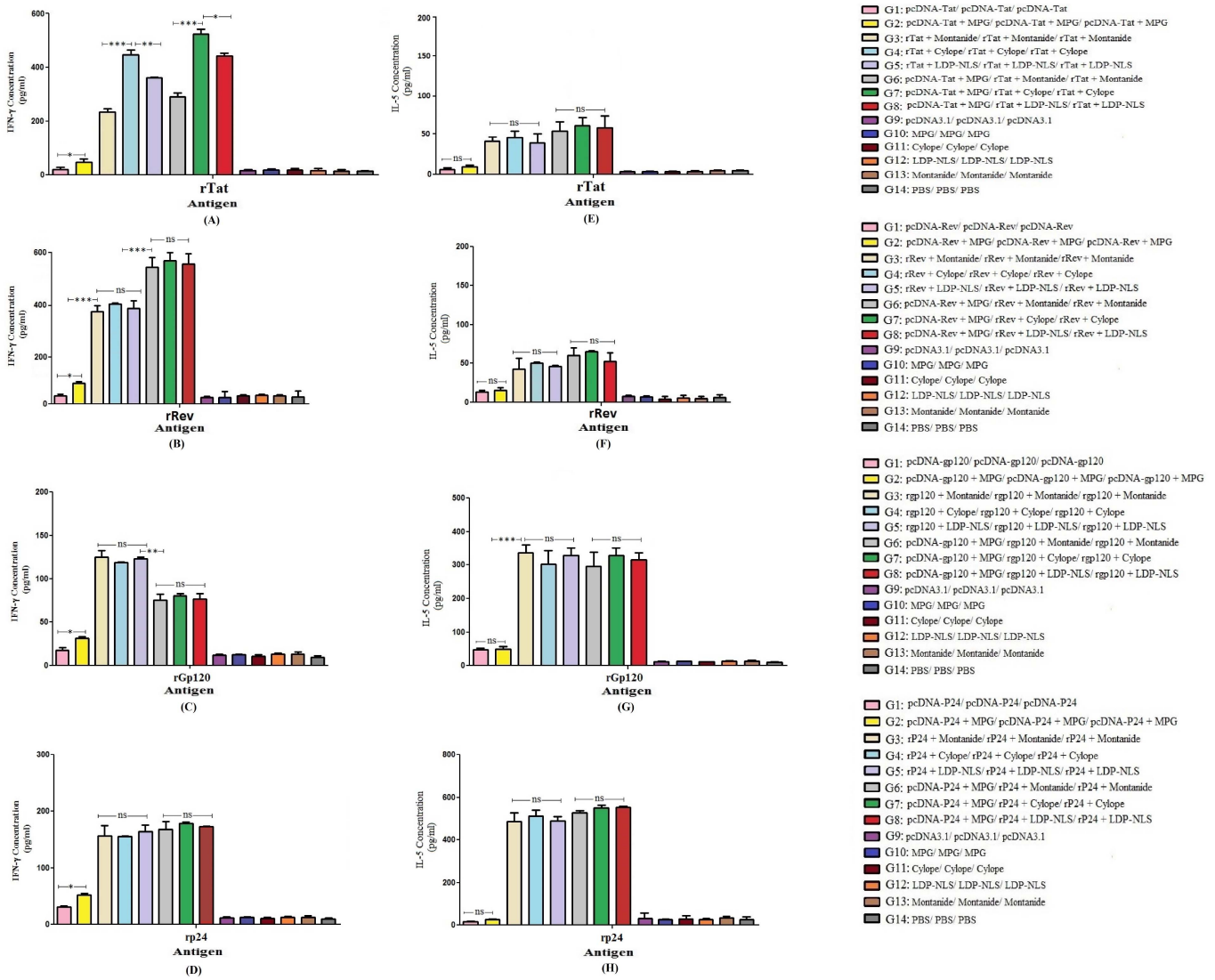

Fig. (3). The secretion of IFN-γ (A-D) and IL-5 (E-H) cytokines in immunized groups with the Tat (A & E), Rev (B & F), Gp120 (C & G) and p24 (D & H) antigens in various formulations: The levels of cytokines were determined by ELISA as mean absorbance at 450 nm ± SD for each set of samples. All analyses were performed in duplicate for each sample; ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

3.4.3. Generation of GrB

Generally, groups receiving the heterologous DNA prime/ protein boost (G6-G8) induced significantly higher levels of Granzyme B than other groups in all immunizations ($p < 0.05$, Fig. **5A-D**). Moreover, the levels of GrB generated by groups receiving Rev were higher than those receiving Tat, Gp120, and p24 ($p < 0.05$). The data showed that MPG peptide could only elicit higher Granzyme B secretion in the group injected with Rev DNA than the naked Rev DNA ($p < 0.05$). The CyLop-1 and LDP-NLS CPPs induced the secretion of Granzyme B, similar to Montanide adjuvant ($p > 0.05$).

4. DISCUSSION

Improvements in an effective and safe HIV vaccine preventing HIV infection have been challenging [33]. Virus-specific $CD4^+$ T cell responses can control HIV-1 replication. Thus, HIV-1 vaccine candidates must induce effective $CD4^+$ and $CD8^+$ T cell responses [34]. In the current study, we evaluated the immune responses of regulatory and structural proteins such as Tat, Rev, Gp120, and p24 in mice, as well as their role in HIV detection using the ELISA technique. It was shown that the HIV p66, p24, gp41 assay was a more effective approach to detect HIV antibodies than the p24 antigen test using ELISA [35]. The generation of antibodies

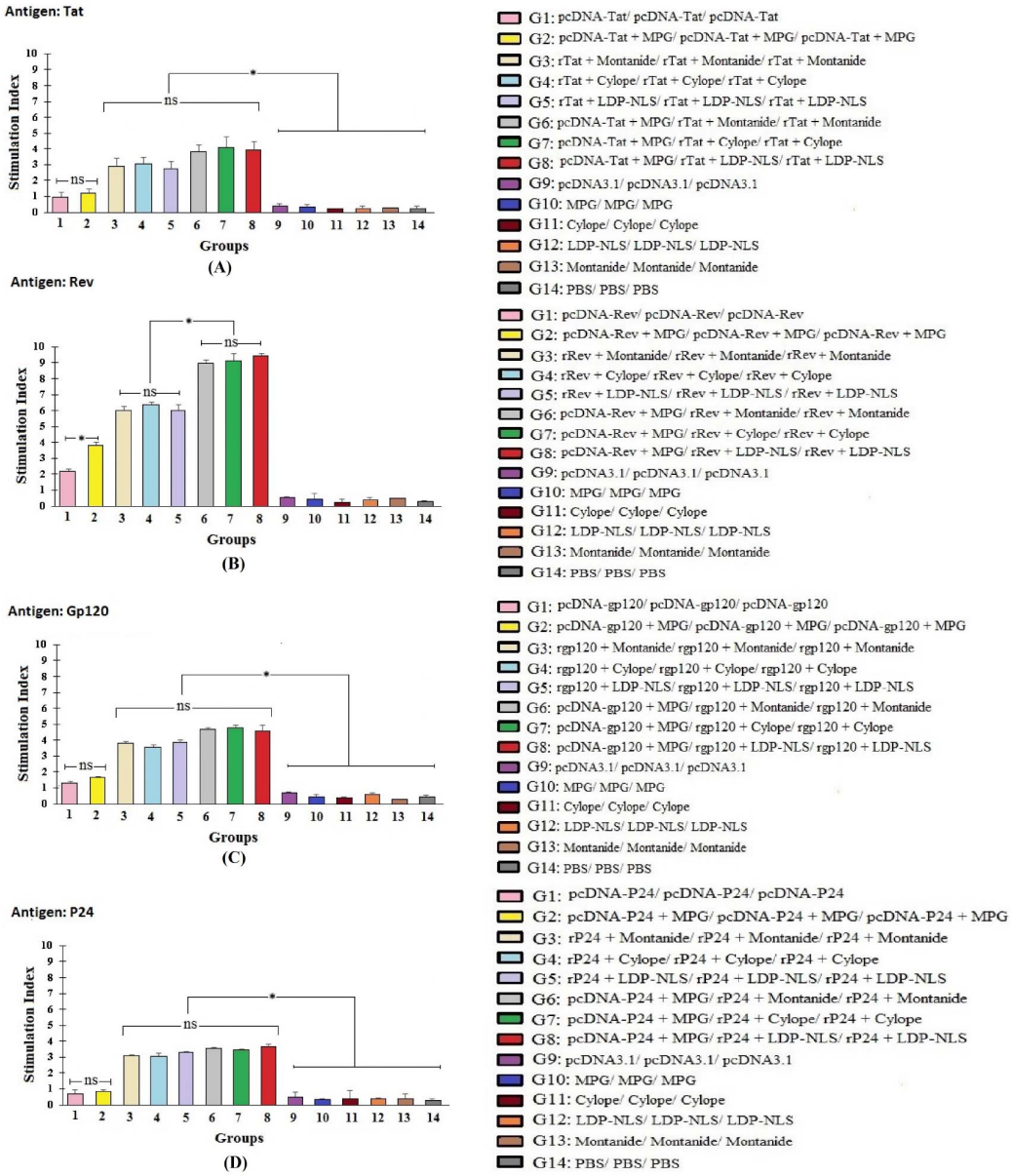

Fig. (4). The lymphocyte proliferation assay in immunized groups with the Tat (A), Rev (B), Gp120 (C), and p24 (D) antigens: The lymphocyte proliferation was performed by MTT assay. All analyses were performed in duplicate for each sample; ns: non-significant; * $p < 0.05$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

against six recombinant HIV-1 structural proteins (gp120, gp41, p66, p31, p24 and p17) in the sera of individuals with severe hemophilia (asymptomatic and symptomatic for AIDS) using dot blot technique showed high levels of antibodies to gp120, gp41, and p31 in all patients regardless of clinical conditions for 4-6 years [36]. On the contrary, the levels of anti-p66, anti-p17, and anti-p24 antibodies were gradually decreased in symptomatic patients and became undetectable, suggesting the first signals of rapid clinical progression [36]. It was observed that the permanent expression of Gp120 occurred in 33% of individuals with acute HIV infection, indicating its relationship with high levels of plasma TNF-α, IL-6, and IL-10 [37]. Moreover, detectable levels of serum anti-Tat antibody were observed in HIV-1-infected patients [38]. On the other hand, the progression to advanced HIV-2 AIDS was significantly lower in anti-Tat-positive individuals than in anti-Tat-negative individuals indicating its role as a prognostic marker. It is likely due to the lack of ability for inhibition of the Tat extracellular effects that are lethal to various functions of immune cells [39]. A Tat-neutralizing activity was significantly higher in anti-Tat-positive sera than healthy or anti-Tat-negative sera, suggesting the importance of anti-Tat responses in suppressing HIV infections, and was useful for the construction of more potent vaccines

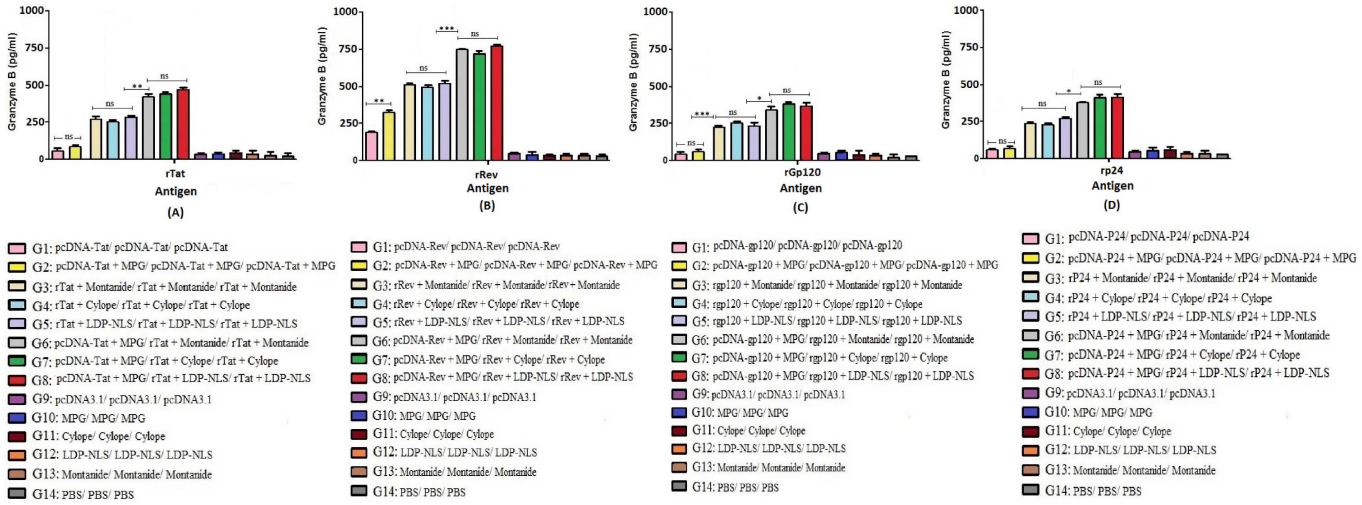

Fig. (5). The levels of Granzyme B (A-D) determined by ELISA as mean absorbance at 450 nm ± SD for each set of samples: All analyses were performed in duplicate for each sample. ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

[40]. In this study, anti-Tat, anti-Rev, anti-Gp120, and anti-p24 antibodies were detected in sera of all groups as compared to the healthy group. Moreover, anti-Gp120 antibody was not detected in treated individuals as compared to the healthy group. The levels of anti-Gp120 antibody showed a significant difference between the treated group with untreated and drug-resistant subjects. Moreover, the levels of anti-p24 antibody were lower in the treated group than the untreated group, but there was no significant difference between the untreated/ treated group with drug-resistant subjects.

On the other hand, different studies were performed to evaluate the efficiency of Tat, Rev, Gp120 and p24 antigens in vaccine design against HIV infections. For example, co-immunization with certain HIV-1 genes, including *nef, rev* and *tat* administered intramuscularly resulted in the suppression of specific immune responses in transgenic mice. Indeed, the T-cell responses against Nef and Rev were suppressed as compared to responses against Nef or Tat alone. This hindrance was not observed for the Tat co-immunization [41]. Moreover, the immunogenicity of DNA vectors harboring HIV-1 *nef*, *tat* and *rev* genes individually showed that the pBN-*nef* construct induced humoral and cellular immunity using intramuscular and intradermal delivery. In contrast, the pBN-*rev* construct only induced the CTL responses and the pBN-*tat* construct did not elicit any effective immune responses [42]. The CTL responses against Tat and Rev were detected in about 19 and 37% of HIV-1-infected individuals, respectively, indicating their importance as a target in HIV vaccine design [43]. Another study showed that the combination of Tat and Rev proteins in a vaccine harboring Env or Gag proteins was useful in the development of a prophylactic vaccine [16]. Furthermore, the DNA vectors encoding the HIV-1 Env and Rev proteins stimulated significant antigen-specific antibodies and suppressed HIV-1 infection in mice, rabbits or macaque [44]. Moreover, vaccination of seronegative individuals with an HIV-1 *env/rev* DNA vaccine led to the proliferation of lymphocytes and, subsequently, the generation of β-chemokines [45]. Vaccination of monkeys with HIV-1 Tat protein could stimulate cellular and humoral immunity, reduce simian-human immunodeficiency virus (SHIV89.6P) infection, and prevent a decrease in $CD4^+$ T cell [46]. Furthermore, DNA vaccination with pCV-*tat* and CpG motif could protect monkeys infected with SHIV89.6P [47].

We evaluated the immune responses against four antigens (Tat, Rev, Gp120 and p24) individually in BALB/c mice. Our results indicated that the Rev antigen could significantly induce IFN-gamma, IgG2a and IgG2b secretion directed toward Th1 response and GrB generation as CTL activity in comparison with other antigens. Also, the heterologous DNA prime/ protein boost regimens were more effective than the homologous protein prime/ protein boost regimens. DNA immunization showed lower potency in the stimulation of immune responses than DNA prime/ protein boost and protein prime/ protein boost immunizations. Indeed, Rev and Tat antigens could enhance Th1-directed immune responses, whereas Gp120 and p24 antigens could increase Th2-directed immune responses. These results indicated that T cell-mediated immunity was influenced by the nature of the antigen.

There is a significant emphasis on using adjuvants or delivery systems in vaccine design [48, 49-51]. For example, naked DNA should be injected several times in high doses without adjuvants or delivery systems for the induction of effective immune responses [52]. Thus, developing molecular strategies to optimize the immunogenicity of DNA vaccines is important for inducing strong immune responses [53]. Moreover, the heterologous intradermal priming/ intranasal boosting vaccination using Tat protein along with MALP-2 mucosal adjuvant induced significantly secretory IgA (sI-

gA), Th1, and CTL as compared to the homologous prime/ boost regimen [54]. The priming with the pCV-*tat* delivered by biocompatible core-shell cationic nanoparticles in a very low dose and boosting with Tat protein stimulated significantly humoral and cellular immunity directed toward Th1 responses and CTL activity against Tat antigen [55]. In this line, a subcutaneous injection with Tat (1-72)-coated anionic nanoparticle enhanced Th1-biased immunity in mice. This study showed that Tat (1-72) co-injected with ovalbumin was not immunosuppressive in mice [48]. Other results demonstrated that the heterologous prime-boost immunization with HIV-1 Tat DNA and the Tat protein significantly elicited the proliferation of lymphocyte, Th1 and CTL responses, and secretion of IgGs in comparison with the homologous immunization [56]. The results from phase II clinical trials in Italy (ISST-002) and South Africa (ISST-003) showed that vaccination with Tat was potent irrespective of the individual immunological status [57, 58].

Other experiments were performed to enhance immune responses against Gp120 and p24, as well. For instance, immunization with a bicistronic plasmid co-expressing gp120 antigen and GM-CSF adjuvant significantly enhanced $CD4^+$ T cell responses [34]. Moreover, intramuscular immunization with plasmid DNA encoding gp120 elicited IL-10 and IgG1 directed toward Th2 responses [59]. In contrast, the plasmid DNA encoding gp120 protein fused to monocyte chemoattractant protein-3 could elicit T-cell proliferation, IFN-γ and IL-6 secretion directed toward Th1 responses [60]. Furthermore, a recombinant gp120/p24 fusion protein linked to multiple α-gal epitopes (gp120α_{gal}/p24 vaccine) induced higher immune responses (~10-30-fold) compared to gp120/p24 fusion protein alone in mice [61]. Also, small animals were intramuscularly vaccinated with plasmid DNA encoding gp120, followed by a recombinant gp120 protein mixed with MF59 adjuvant. This DNA prime/protein boost could induce gp120-specific antibodies and CTL immune responses [62]. On the other hand, gp120 DNA fused to IFN-γ DNA or TNF-α DNA resulted in high IgG2a responses and strong Th1 responses [63, 64]. A DNA vaccine encoding a mouse Ig Fc*γ*2a fragment fused to gp120 (gp120-Ig) also significantly elicited high $CD8^+$ CTL activity and decreased the viral load after being challenged with gp160-expressing *vaccinia* virus [65]. Additionally, the p24-coated poly (D, L-lactide) nanoparticles stimulated higher antibody, CTL, and Th1-oriented responses than the p24 protein emulsified with Freund's adjuvant [66]. Heat shock proteins (HSPs) as an adjuvant enhanced antiviral immunity as well. For example, mice immunization using p24 antigen mixed with gp96 or its N-terminal fragment (N336) as an adjuvant and also fused to N336 significantly increased anti-p24 antibody titer and cellular immunity [67]. Also, the C- or N-terminal fragments of murine Hsp70 fused to p24 protein (Hsp70-p24 fusion protein) could induce IFN-γ and IgG2b secretion, leading to Th1-oriented responses and CTL activity [68]. Liposome-encapsulated p24 could induce memory $CD8^+$ T-cells and effector $CD4^+$ T-cells, indicating the role of the liposomal lipid A as an effective adjuvant [69]. Targeting of p24 by fusion to CCL3 or XCL1 DNA significantly increased p24-reactive $CD8^+$ T cell responses in mice [70].

Our study showed that MPG cell-penetrating peptide could only induce higher Granzyme B secretion in the group injected with Rev DNA than the naked Rev DNA, but it could significantly stimulate IFN-gamma response in all DNA immunizations. In addition, the CyLop-1 and LDP-NLS CPPs effectively induced the secretion of Granzyme B, IFN-gamma, and antibodies similar to Montanide adjuvant. It was previously shown that the cationic CPPs importantly deliver therapeutic molecules [71]. Saleh *et al*. showed that delivery of DNA using MPG (N/P ratio of 10) elicited strong Th1 immune responses in mice [72]. However, delivery systems reduced the used dose of antigen in vaccines [73]. In this study, we only used 5 µg of the DNA complexed with MPG peptide in comparison with 50 µg of the naked DNA. Moreover, CyLoP-1 and LDP-NLS CPPs, and Montanide 720 adjuvant significantly increased immune responses against various antigens. Based on the reports, CyLoP-1 and LDP-NLS efficiently delivered proteins into mammalian cells [74, 75]. Generally, the HIV-1 Rev and Tat antigens could induce Th1-directed immune responses as compared to HIV-1 Gp120 and p24 antigens. However, Rev antigen was more effective than other antigens for the secretion of IgG2a, IgG2b, IFN-gamma, and Granzyme B in mice. Indeed, Rev antigen elicited Th1 cellular immunity and CTL activity. On the other hand, p24 and Gp120 antigens were more effective in eliciting Th2 immune responses. Moreover, the use of protein delivery systems could significantly increase antigen-specific immune responses similar to commercial Montanide adjuvant.

CONCLUSION

In conclusion, the identification of potent antigens and novel immunization strategies can accelerate HIV vaccine development. Our data showed that the Rev regulatory protein was an antigen candidate for the improvement of the HIV vaccine. Different formulations of Rev, especially the heterologous DNA prime/ protein boost, demonstrated higher stimulation index and IFN-γ production with a significant increase in IgG2a/IgG1 and IFN-gamma/IL-5 ratios, suggesting the stimulation of cellular immunity towards Th1 responses, and Granzyme B generation as CTL activity. Tat antigen was also effective for stimulation of Th1-directed immune responses, but the responses were lower than Rev antigen in BALB/c mice. In contrast, Gp120 and p24 antigens stimulated Th2-directed immune responses in mice. In humans, the secretion of anti-Tat, anti-Rev, anti-Gp120, and anti-p24 antibodies was significantly higher in the sera of Naïve, treated, and drug-resistant individuals than the healthy group. It was observed that the levels of both anti-Gp120 and anti-p24 antibodies could be considered as possible diagnostic markers in HIV-infected subjects. Indeed, the detection of both anti-Gp120 and anti-p24 antibodies in human sera is important to differentiate the treated group from the Naïve group. Generally, this study indicated the values of four HIV proteins as antigen candidates in therapeutic vaccine design as well as a possible diagnostic marker in humans. However, further studies are required to evaluate the effects of antigens alone or together upon reducing viral

load in a mouse model or monkey. Moreover, it is needed to assess the concentration of the recombinant proteins in sera as a possible diagnostic marker in larger human populations.

STANDARD OF REPORTING

The study conforms to the STROBE guidelines.

CONTRIBUTION STATEMENT

NFK, MF, SHS, and AM performed the experiments and wrote the manuscript draft. KB assisted with patient identification and sample collection. AB conceived the study, performed all statistical analysis, edited, and contributed to the final draft.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval of this study was obtained from the Pasteur Institute of Iran, Tehran, Iran (a local approval Committee; Approval No: IR.PII.REC.1397.024).

HUMAN AND ANIMAL RIGHTS

All humans research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931). The whole mice experiments were done based on approved protocols for the care of laboratory animals at Pasteur Institute of Iran (ethical code No: IR.PII.REC.1397.024).

CONSENT FOR PUBLICATION

The studied participants were informed about the present research, and a written consent form was taken from all of them before their enrollment.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the results and findings of this study are available within the article.

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CONFLICT OF INTEREST

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

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