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RESEARCH ARTICLE

HIV-1 Accessory Proteins: Which one is Potentially Effective in Diagnosis and Vaccine Development?

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Abstract: ***Background*:** The combination antiretroviral therapy (cART) could increase the number of circulating naive CD4 T lymphocytes, but was not able to eradicate human immunodeficiency virus-1 (HIV-1) infection.

***Objective*:** Thus, induction of strong immune responses is important for control of HIV-1 infection. Furthermore, a simple and perfect serological method is required to detect virus in untreated-, treated- and drug resistant- HIV-1 infected individuals.

***Methods*:** This study was conducted to assess and compare immunogenic properties of Nef, Vif, Vpr and Vpu accessory proteins as an antigen candidate in mice and their diagnostic importance in human as a biomarker.

***Results*:** Our data showed that in mice, all heterologous prime/ boost regimens were more potent than homologous prime/ boost regimens in eliciting Th1 response and Granzyme B secretion as CTL activity. Moreover, the Nef, Vpu and Vif proteins could significantly increase Th1 immune response. In contrast, the Vpr protein could considerably induce Th2 immune response. On the other hand, among four accessory proteins, HIV-1 Vpu could significantly detect treated group from untreated group as a possible biomarker in human.

***Conclusion*:** Generally, among accessory proteins, Nef, Vpu and Vif antigens were potentially more suitable vaccine antigen candidates than Vpr antigen. Human antibodies against all these proteins were higher in HIV-1 different groups than healthy group. Among them, Vpu was known as a potent antigen in diagnosis of treated from untreated individuals. The potency of accessory proteins as an antigen candidate in an animal model and a human cohort study are underway.

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1. INTRODUCTION

Human immunodeficiency virus (HIV) is a retrovirus that infects the immune cells. HIV is a major reason of death in the world that is not detected for a long time [1]. The diagnosis of HIV is performed by different molecular and immunological techniques. Among them, the enzyme-linked immunosorbent assay (ELISA) is a normal and easy approach of HIV screening [1]. The HIV genome encodes the structural, regulatory and accessory proteins. The accessory proteins include Nef, Vpr, Vpu and Vif proteins. Negative regulatory factor (Nef) reduces the activity of T cells leading to HIV infection. Virus protein R (Vpr) plays an important role in the cell cycle arrest and T cells' apoptosis [1-3]. Virus protein U (Vpu) down-regulates the CD4 receptors and increases the release of new virions. Viral infectivity factor (Vif) is necessary for assembling and maturation of the virions similar to Nef protein [1, 4-6].

The HIV infection was usually diagnosed by detection of antibodies in the sera against HIV structural proteins. However, some data represented that the serum antibodies were detected against accessory proteins such as Nef in HIV infection before the generation of antibodies against HIV-1 structural proteins [7]. Thus, HIV-1 accessory proteins can be considered for diagnosis of HIV infection. On the other hand, humoral and cellular immunity are important for control of HIV infection [8]. The effects of anti-retroviral drugs (combination antiretroviral therapy: cART) could increase the number of circulating CD4 T cells, and restore the response to memory antigens; but however, the cART treatment was not able to eradicate the infection because the cytotoxic T cells (CTL) activity was decreased and thus, the

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latent HIV-1 (as integrated proviral DNA) was not eliminated [8].

The HIV vaccine studies indicated that $CD4^+$ T cells are necessary to elicit antibody responses and CTL activity against HIV-1 infection [9-12]. However, subunit vaccines had low immunogenicity in primates and humans. This disadvantage could be overcome using delivery systems such as cell penetrating peptides (CPPs) [13-17]. For example, MPG CPP was used to deliver DNA, and CyLoP-1 and LDP-NLS CPPs were applied to deliver protein into the cells [18-20]. Another approach is the use of adjuvants and prime-boost vaccination strategy for inducing potent immunity versus viral infections [21].

Regarding the published reports, there are few studies about comparison of HIV accessory proteins as an antigen candidate in vaccine design. Among them, Nef was further studied and known as an antigen candidate. In addition, few studies were performed on detection of antibodies against accessory proteins in patients infected with HIV-1. Indeed, there are no reports on detection of anti-accessory proteins antibodies in different HIV groups, *i.e.,* patients infected with HIV, patients treated with drugs, and patients with drug resistance after treatment. Thus, it is important for immunological assay of HIV-1 accessory proteins as antigen candidates in therapeutic vaccine design *in vivo*. A variety of strategies were used to obtain the best and most effective immune responses in mice (*i.e.,* DNA prime/DNA boost, Protein prime/Protein boost & DNA prime/Protein boost). Moreover, the anti-HIV IgG antibodies against these proteins were detected to find a possible diagnostic marker for HIV infections in human. Briefly, in this study, we carried out more detailed analyses to better understand immunogenic properties of Nef, Vif, Vpr and Vpu accessory proteins in mice and their diagnostic importance in human.

2. METHODS

2.1. Population Study

In a cross-sectional study, untreated and treated participants were collected from Infectious Disease Department of Imam Khomeini Hospital (Tehran, Iran), and maintained in Biobank at Pasteur Institute of Iran. They were older than 18 years of age and include untreated (naïve) and treated by antiretroviral drugs (one NNRTI or one PI combined with two nucleoside reverse transcriptase inhibitors (NRTI) for at least one year). The specimens for antiretroviral drug resistance mutations were previously screened by Nested PCR based on the published manuscripts [22, 23]. 141 participants were enrolled in this study. We considered about 71 treated, 20 naïve HIV-infected (/untreated HIV-infected), 30 drug resistance, 20 HIV uninfected (/HIV-negative) individuals in each group. Human sera were provided and stored at -70°C for the next process (Supplementary Table).

2.2. Expression and Purification of HIV-1 Nef, Vpr, Vpu and Vif Proteins in *E. coli*

The recombinant (r) Nef protein was generated based on our previous study [24]. Briefly, *Escherichia coli* Rosetta strains harboring pET-23a-*nef* were grown in Ty2x medium to an optical density of 0.7-0.8 at a wavelength of 600 nm. Then, the Nef protein was expressed by 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma) at 37°C and 16 hours after induction. Finally, Nef protein was purified by affinity chromatography under native conditions [24].

In order to prepare the recombinant Vpr, Vpu and Vif proteins, the *vpu, vif and vpr* genes were cloned in pET expression vectors. At first, the DNA constructs were amplified from pNL4.3 (AF324493.2) by PCR using the designed primers as shown in Table **1**. The amplified genes were digested by the restriction enzymes, and ligated to the linearized pET-23a (+)/24a (+)/28a (+) vectors using T4 DNA ligase. Following the *E. coli* DH5α transformation with the recombinant plasmids, pET23a-*vif*, pET24a-*vpu* and pET28a-*vpr* were purified using plasmid extraction mini kit (FavorGen, Taiwan) and confirmed by PCR, digestion, and sequencing.

For expression of the recombinant (r) Vif, Vpu and Vpr proteins, two *E. coli* strains including BL21 (DE3) and Rosetta were transformed with the recombinant pET23a-*vif*, pET24a-*vpu* and pET28a-*vpr* using heat shock. Then, single clone on LB agar was cultured in LB Broth for 16 hours at 37°C. Precultured cells were added to Ty2x medium at a concentration of 1% (v/v), and induced by IPTG, when OD_{600} reached 0.5-0.7. The expression of the recombinant proteins was optimized at different incubation times (*i.e.,* 2, 3, 4 and 16 hours) after induction at 37°C and analyzed by 12.5% SDS-PAGE. The recombinant proteins were purified by affinity chromatography using Ni-NTA agarose column under both denaturing (*i.e.,* 8 M Urea buffer and pH = 4.5) and native (*i.e.,* 250 mM imidazole buffer, pH=8) conditions (Machery-Nagel, Germany) and dialyzed against phosphate buffer saline 1X (PBS1X). The concentrations of all proteins were determined by NanoDrop spectrophotometer. Finally, the expression of the recombinant proteins was analyzed by western blotting using anti-His antibody conjugated to peroxidase (Abcam, UK). Moreover, the endotoxin contamination with LPS measured by Limulus Amebocyte Lysate (QCL-1000) was less than 0.4 EU/mg for each protein.

2.3. Detection of Seroantibodies Against the Recombinant Nef, Vpr, Vif and Vpu Proteins in Human

The detection of anti-Vpr IgG, anti-Vpu IgG, anti-Vif IgG, and anti-Nef IgG was performed by an indirect ELISA protocol [25]. The concentrations of the recombinant proteins as coated antigens were approximately 3 µg/ml, individually. The anti-Vpr IgG, anti-Vpu IgG, anti-Vif IgG, and anti-Nef IgG antibodies were detected by goat anti-human IgG conjugated to horseradish peroxidase (HRP, Sigma, 1:10000) and Tetramethylbenzidine (TMB) substrate. To show a positive anti-protein response between

groups, cutoff values were calculated and the SDs set mean+2 as the cutoff value, above which a response was considered positive.

2.4. Cloning *nef, vpr, vpu, vif* Genes in Eukaryotic Vectors

The pcDNA-Nef was prepared in our previous studies [24, 26]. The cloning *vif, vpu and vpr* genes amplified from pNL4.3 were performed in pcDNA3.1 (-) vector using *Nhe*I/ *Xho*I restriction enzymes. Next, pcDNA3.1-*vif*, pcDNA3.1-*vpu* and pcDNA3.1-*vpr* constructs were purified by Endo-free plasmid Mega kit (MN, Germany), and their concentration were assessed by NanoDrop spectrophotometer.

2.5. Physicochemical Properties of the Nef, Vpr, Vpu, Vif DNA/ MPG Complexes

The MPG amphipathic peptide was synthesized by Biomatik Corporation (Canada) [24]. The formation of the non-covalent DNA/MPG complexes at an N/P ratio of 10 was confirmed by gel retardation assay, scanning electron microscope (ESEM) and Zetasizer as our previous report [24].

2.6. Physicochemical Properties of the Vpr, Vpu and Vif Proteins Complexed with CyLoP-1 or LDP-NLS CPPs

The LDP-NLS amphipathic peptide and CyLoP-1 cationic peptide were synthesized by Biomatik Corporation (Canada) [24, 26]. The formation of the non-covalent recombinant proteins (rNef, rVpr, rVif and rVpu)/ CyLoP-1 (or LDP-NLS) complexes with molar ratio of 1:10 (protein: CPP) was confirmed by scanning electron microscope and Zetasizer as our previous reports [24, 26].

2.7. Mice Immunization

Five to seven week old female BALB/c mice (n= 4 per group) were maintained under pathogen-free conditions at Pasteur Institute of Iran. Whole process was performed according to care protocols of laboratory animals approved at Pasteur Institute of Iran. Immunization program was shown in Table **2**. Mice were subcutaneously immunized three times. The used doses for DNA and protein were 5 microgram. The N: P ratios of DNA: CPP and the molar ratios of protein: CPP were 1:10. The ratio of recombinant proteins to Montanide ISA720 was 30:70 (*v*/*v*).

2.8. Assessment of Total IgG and its Isotypes in Mice

Four weeks after third immunization, the sera of mice were collected and pooled for each group (n= 4). The levels of total IgG, IgG1, IgG2a and IgG2b conjugated to peroxidase (1:10000 v/v) were assessed by indirect ELISA against the recombinant Nef, Vif, Vpu and Vpr proteins (5 µg/ml) using TMB substrate [27].

2.9. Assessment of IFN-Gamma and IL-5 Cytokines in Mice

Four mice from each group (n= 4 per group) were sacrificed four weeks after third injection. The red blood cell-depleted splenocytes pooled for each group (2×10^6 cells/ml) were seeded in 48-well plates along with the recombinant Nef, Vpr, Vif or Vpu proteins (5 µg/ml), concanavalin A (5 µg/ml, positive control) and RPMI 5% (negative control), and incubated for 72 h at 37°C. The supernatants were applied to assess the levels of IL-5 and IFN-γ cytokines using sandwich-based ELISA (Mabtech). The lymphocyte proliferation was assessed by MTT and shown as stimulation index (SI) [28].

Table 1. The designed primers for gene amplification.

Gene	Primers	Sequence	Enzyme in Bold	$T_{annealing}$	Length
vif	Forward	GA**GCTAGC**GGTACCA TGGAAAACAGATG	*Nhe*I	61° C	~579 bp
	Reverse	CTAAAA**AAGCTT**GTGTCCGTTCA TTGTATGGCTCCC	*Hind*III		
vpu	Forward	CC**GCTAGC**GGTACCATGGAA CCTATAATAG	*Nhe*I	61° C	~ 246 bp
	Reverse	CC**AAGCTT**CAGAGCATCAAT ATCCCAAGG	*Hind*III		
vpr	Forward	GC**GCTAGC**GGTACCATGGAACAAGC-CCC	*Nhe*I	61° C	~291 bp
	Reverse	GG**AAGCTT**TCTACTGGCTCCATTTCTT-GCTC	*Hind*III		

Table 2. Immunization program in mice for HIV-1 Nef/ Vpr/ Vpu/ Vif *

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-Nef/ Vpr/ Vpu/ Vif [a]	pcDNA-Nef/ Vpr/ Vpu/ Vif	pcDNA-Nef/ Vpr/ Vpu/ Vif	Done
G2	DNA/DNA/DNA	pcDNA-Nef/ Vpr/ Vpu/ Vif + MPG[b]	pcDNA-Nef/ Vpr/ Vpu/ Vif + MPG	pcDNA-Nef/ Vpr/ Vpu/ Vif + MPG	Done
G3	Protein/protein/protein	rNef/ Vpr/ Vpu/ Vif + Montanide[c]	rNef/ Vpr/ Vpu/ Vif + Montanide	rNef/ Vpr/ Vpu/ Vif + Montanide	Done
G4	Protein/protein/protein	rNef/ Vpr/ Vpu/ Vif + CyLop-1[d]	rNef/ Vpr/ Vpu/ Vif + CyLop-1	rNef/ Vpr/ Vpu/ Vif + CyLop-1	Done
G5	Protein/protein/protein	rNef/ Vpr/ Vpu/ Vif + LDP-NLS[e]	rNef/ Vpr/ Vpu/ Vif + LDP-NLS	rNef/ Vpr/ Vpu/ Vif + LDP-NLS	Done
G6	DNA/protein/protein	pcDNA-Nef/ Vpr/ Vpu/ Vif + MPG	rNef/ Vpr/ Vpu/ Vif + Montanide	rNef/ Vpr/ Vpu/ Vif + Montanide	Done
G7	DNA/protein/protein	pcDNA-Nef/ Vpr/ Vpu/ Vif + MPG	rNef/ Vpr/ Vpu/ Vif + CyLop-1	rNef/ Vpr/ Vpu/ Vif + CyLop-1	Done
G8	DNA/protein/protein	pcDNA-Nef/ Vpr/ Vpu/ Vif + MPG	rNef/ Vpr/ Vpu/ Vif + LDP-NLS	rNef/ Vpr/ Vpu/ Vif + 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: 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 Nef/ Vpr/ Vpu/ Vif (/ means or): This program was individually performed for all proteins.

2.10. Granzyme B Assay in Mice

SP2/0 target cells (T) were cultured in 96-well plates (2×10^4 cells/ well) along with Nef, Vpr, Vpu and Vif antigens (~ 5 µg/ ml) for a day. The splenocytes (E: Effector cells) were added to the target cells at E: T ratio of 100:1 and incubated for 6 h. The supernatants were collected to assess Granzyme B using ELISA kit (eBioscience) [28].

2.11. Statistical Analysis

Statistical analysis was done by Prism 5.0 software (GraphPad, San Diego, CA) using One-way ANOVA and Student's *t*-test. The *p*-value < 0.05 was considered statistically significant. Data were represented as mean ± standard deviation (SD).

3. RESULTS

3.1. Study Population

Iranian subjects were divided into four groups: 20 naïve HIV-infected individuals (or untreated HIV-infected subjects: group 1), 71 treated individuals with ART (group 2), 30 drug resistant individuals (group 3), and 20 control individuals (or HIV-uninfected/ HIV-negative subjects: group 4).

3.2. Generation of the Recombinant Nef, Vpr, Vpu and Vif Proteins

The recombinant Nef (pET23a/Rosetta system, OD_{600}: 0.7-0.8, 16 h after induction, 37ºC), Vpr (pET28a/Rosetta system, OD_{600}: 0.6-0.7, 2 h after induction, 37ºC), Vpu (pET24a/BL21 system, OD_{600}: 0.7-0.8, 16 h after induction, 37ºC) and Vif (pET23a/BL21 system, OD_{600}: 0.5, 16 h after induction, 37ºC) proteins were successfully expressed in *E. coli* strains, and migrated as the clear bands of ~30, ~14, ~16 and ~23 kDa in SDS-PAGE, respectively (data not shown). Their purification was performed using affinity chromatography under native conditions for Nef and Vpr proteins, and denaturing conditions for Vpu and Vif proteins. The purified proteins were identified by western blotting and showed a concentration range (0.5-0.8 mg/ml) by the NanoDrop spectrophotometer.

3.3. Generation of DNA Constructs

The recombinant endotoxin-free pcDNA-*vif*, pcDNA-*vpu*, pcDNA-*vpr*, and pcDNA-*nef* were provided in large scale. Each four gene migrated as ~579, ~246, ~291, and ~618 bp on agarose gel, respectively after enzyme digestion. The DNA/MPG complexes were formed at N/P ratio of 10:1 as determined in our previous study [29]. The results of nanoparticles formation were shown using SEM and Zetasizer in Table **3**. A positive charge was obtained for nanoparticles compared to plasmids and proteins, alone.

Table 3. Size and charge of the nanoparticles.

Construct	Size (nm; SEM)	Charge (mV; Zeta-sizer)
pcDNA-Vpr	200-250	-28.8
pcDNA-Vpr + MPG	130-190	25.6
Vpr protein	230-280	-14.3
Vpr protein+CyLop	120-190	2.23
Vpr protein+LDP-NLS	110-140	2.3
pcDNA-Nef	150-250	-11.7
pcDNA-Nef+MPG	100-200	21.6
Nef protein	250-350	-24.9
Nef+LDP	130-200	5.5
Nef+CyLop	100-150	4.35
pcDNA-Vif	240-300	-35.7
pcDNA-Vif+MPG	140-200	21.8
Vif protein	210-270	-8.29
Vif + LDP	100-140	5.46
Vif + Cylope	80-120	12.3
pcDNA-Vpu	190-230	-18.8
pcDNA-Vpu + MPG	90-130	28.7
Vpu protein	250-300	-28
Vpu + LDP	100-150	3.76
Vpu + Cylope	80-110	12.5

3.4. Detection of Nef, Vpr, Vif and Vpu-Specific Antibodies in Human Subjects

The frequency of antibodies to Nef, Vpr, Vpu and Vif proteins was evalauted in sera of untreated/Naïve (G1), treated (G2), drug resistant (G3) and control (G4) groups. The mean absorbance values for Nef were 1.155 ± 0.398 (G1), 0.941 ± 0.164 (G2), 0.951 ± 0.410 (G3) and 0.371 ± 0.057 (G4), respectively. The mean absorbance values for Vif were 1.027 ± 0.165 (G1), 1.046 ± 0.186 (G2), 0.986 ± 0.225 (G3) and 0.574 ± 0.049 (G4), respectively. The mean absorbance values for Vpu were 1.304 ± 0.520 (G1), 0.979 ± 0.406 (G2), 1.084 ± 0.509 (G3) and 0.490 ± 0.102 (G4), respectively. The mean absorbance values for Vpr were 0.904 ± 0.402 (G1), 0.737 ± 0.336 (G2), 0.767 ± 0.425 (G3) and 0.348 ± 0.074 (G4), respectively. All groups showed higher levels of antibodies than control group against all recombinant proteins ($p < 0.001$). The data indicated that the levels of antibodies against rNef, rVpr and rVif proteins showed no statistically differences in Naïve (G1), treated (G2) and drug resistance (G3) groups ($p > 0.05$). In contrast, the levels of antibody against the rVpu in naïve group (G1) was higher than treated group (G2, $p < 0.05$), but not against drug resistance group (G3, $p > 0.05$). The results demonstrated that the Vpu protein can be used as a possible biomarker for diagnosis of Naïve from treated group. The differences between the seroreactivities in all groups against the recombinant Nef, Vif, Vpu and Vpr proteins were shown in Figure **1**. Using calculation of a cut-off value, 100, 100, 86 and 90% of the naive sera recognized the recombinant Nef, Vif, Vpu and Vpr proteins, respectively. Moreover, 95, 98, 76 and 75% of the treated sera recognized the recombinant Nef, Vif, Vpu and Vpr proteins, respectively. On the other hand, 93, 90, 77 and 70% of the drug resistant sera recognized the recombinant Nef, Vif, Vpu and Vpr proteins.

3.5. Antibody Assay in Mice

The levels of Nef, Vpr, Vpu and Vif-specific total IgG and their isotypes were evaluated in mice sera. In general, the levels of total IgG in mice immunized with the homologous protein regimens in all injections with rNef, rVpr, rVpu and rVif (G3-G5) were significantly higher than other groups ($p < 0.05$; Figure **2A**). It was interesting that the levels of IgG1 in groups injected by rVpu (G3-G8) were significantly less than groups immunized with other recombinant proteins (Figures **2B**, $p < 0.05$). In contrast, the level of IgG1 was higher in groups immunized with rVpr (G3-G8) than other groups (Figure **2B**). The ratios of IgG2a/IgG1 were higher in the heterologous and then homologous rNef, rVpu and rVif regimens (G3-G8) indicating Th1-directed responses. The ratio of IgG2a/IgG1 was lower in the homologous and heterologous rVpr regimens (G3-G8) indicating Th2-directed responses. The results of humoral immune responses indicated that there were no significant differences in the levels of total IgG, and their isotypes between mice immunized with the CyLop-1 and LDP-NLS formulations ($p > 0.05$, Figure **2**). It was interesting that the immunological effects of CyLop-1 and LDP-NLS were almost similar to Montanide. In addition, MPG peptide could significantly increase antibody responses in groups immunized with Nef, Vpu, Vif and Vpr DNA (G2) compared to the naked DNA (G1; $p < 0.05$, Figure **2**).

3.6. Cytokine Assay in Mice

The levels of IFN-γ and IL-5 cytokines were evaluated by ELISA to determine the Th1 and Th2 immune responses in mice splenocytes, respectively. Our results showed that all groups immunized with different modalities could significantly increase the levels of IFN-γ and IL-5 secretion in comparison with control groups for all immunizations ($p < 0.05$, Figure **3**). Moreover, groups immunized with the heterologous DNA prime/ protein boost regimens (G6-G8) could secrete Nef, Vpr, Vif and Vpu-specific IFN-γ significantly higher than other groups. Our data indicated that the levels of IFN-γ in groups injected with various Vpr regimens were significantly lower than groups injected with

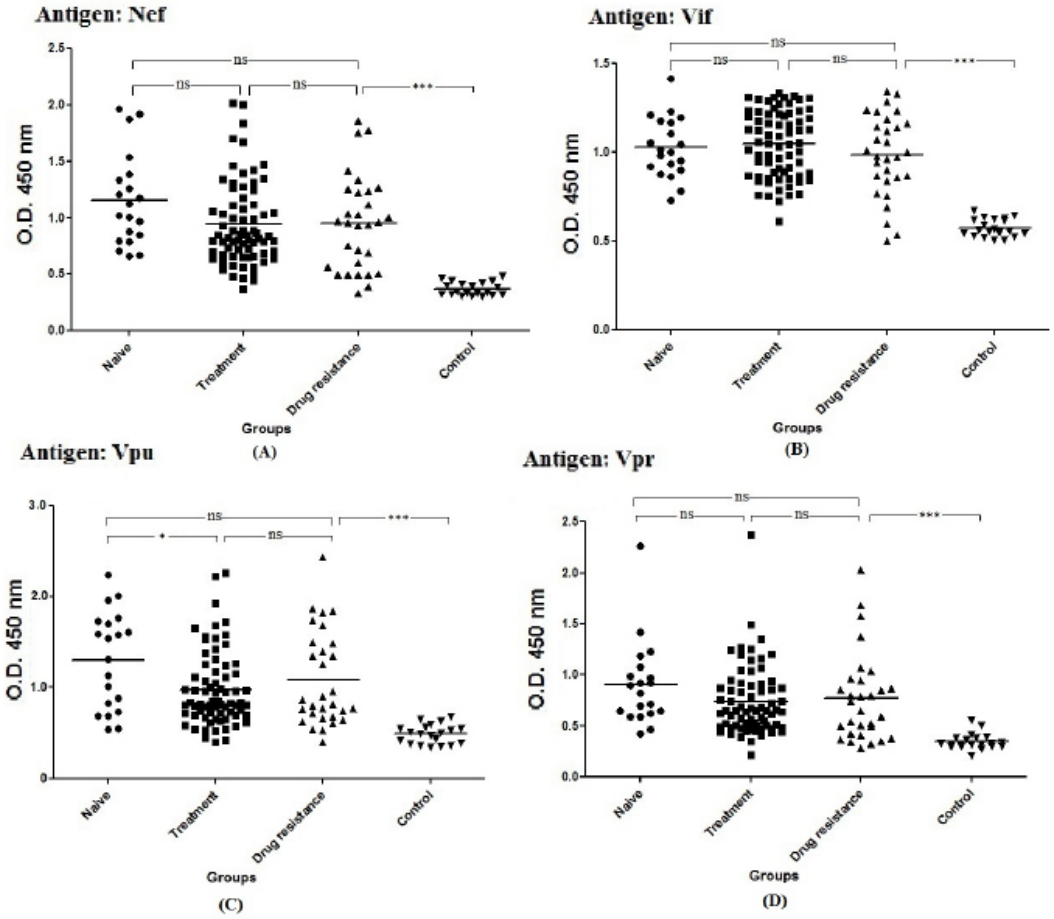

Figure 1. Analysis of IgG antibody levels with respect to Nef (A), Vif (B), Vpu (C), and Vpr (D) coating antigens in untreated/Naïve (G1), treated (G2), and drug resistant (G3) groups compared to control group (G4) using ELISA. All analyses were performed in duplicate for each sample. The horizontal line represents the mean value of optical density in respect to each antigen; ns: non-significant, *: $p < 0.05$, ***: $p < 0.001$. The data showed that Vpu is a potent antigen in diagnosis of treated from untreated (Naïve) individuals.

Nef, Vif and Vpu regimens ($p < 0.05$, Figure **3A-D**). The level of IL-5 was significantly higher in groups immunized with Vpr regimens than groups immunized with Vpu, Vif and Nef regimens ($p < 0.05$, Figure **3E-H**). Moreover, the ratios of IFN-gamma/ IL-5 were importantly higher in the heterologous and then homologous Nef, Vpu and Vif regimens (G3-G8) indicating Th1-directed responses. In contrast, the ratio of IFN-gamma/ IL-5 was lower in the homologous and heterologous Vpr regimens (G3-G8) indicating Th2-directed responses (Figure **3**). Furthermore, CyLop-1 and LDP-NLS could induce IFN-gamma secretion more than Montanide in all immunizations with different antigens ($p < 0.05$, Figure **3A-D**). MPG significantly stimulated IFN-γ response in DNA immunization ($p < 0.05$, Figure **3A-D**). On the other hand, the highest lymhoproliferative responses were determined in groups receiving the heterologous DNA prime/ protein boost regimens for all antigens as shown in Figure **4**. In addition, the stimulation index against the Vpr recombinant protein was significantly less than rNef, rVpu and rVif proteins ($p < 0.05$). Generally, immunization with the heterologous DNA prime/ protein boost regimens induced antigen-specific Th-cell proliferative responses.

3.7. Secretion of Granzyme B (GrB) in Mice

Our data showed that there were no significant differences in Granzyme B secretion between groups receiving the homologous protein regimens ($p > 0.05$) as well as the heterologous DNA/protein regimens in all immunizations ($p > 0.05$; Figure **5A-D** However, higher levels of Granzyme B were observed in the heterologous DNA prime/ protein boost (G6-G8) than other groups ($p < 0.001$, Figure **5**). Moreover, the levels of Granzyme B in all regimens of Vpr antigen were less than all regimens of Nef, Vpu and Vif antigens ($p < 0.05$). In addition, the MPG/DNA nanoparticles induced higher levels of Granzyme B than DNA alone ($p < 0.05$). The CyLop-1 and LDP-NLS CPPs were similar to Montanide for increasing the secretion of Granzyme B ($p > 0.05$).

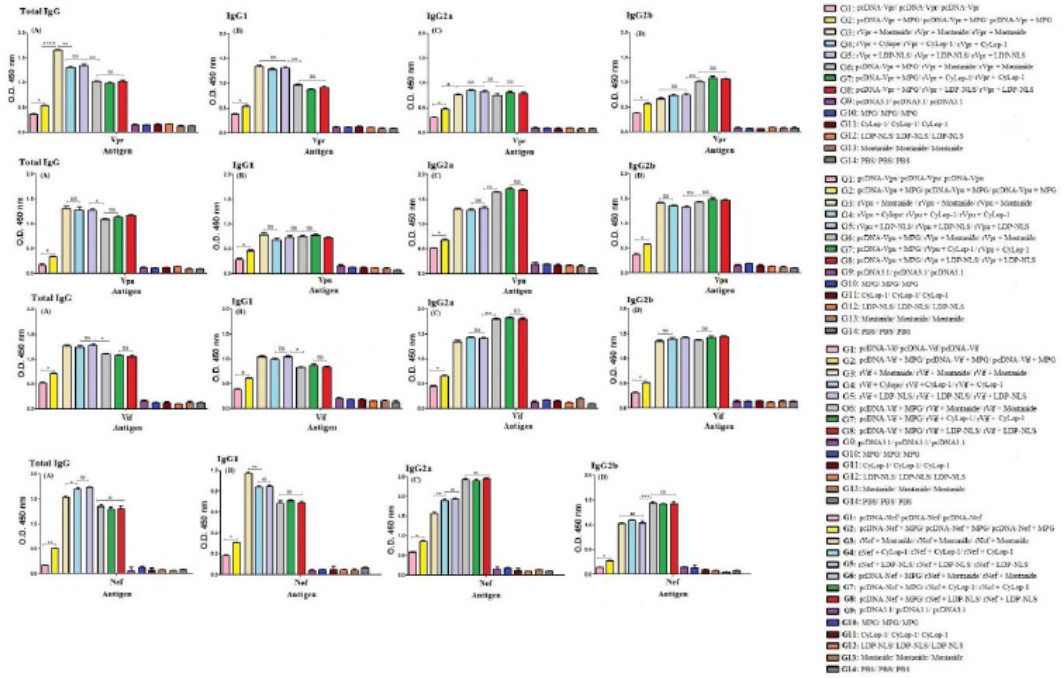

Figure 2. Antibody responses (A: total IgG, B: IgG1, C: IgG2a and D: IgG2b) against the recombinant Vpr, Vpu, Vif and Nef proteins in different regimens: All analyses were performed in duplicate for each sample. The results were shown as mean absorbance at 450 nm ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns: non-significant ($p > 0.05$). The data indicated that the ratios of IgG2a/IgG1 were higher in the heterologous and then homologous rNef, rVpu and rVif regimens (G3-G8) indicating Th1-directed responses in mice. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

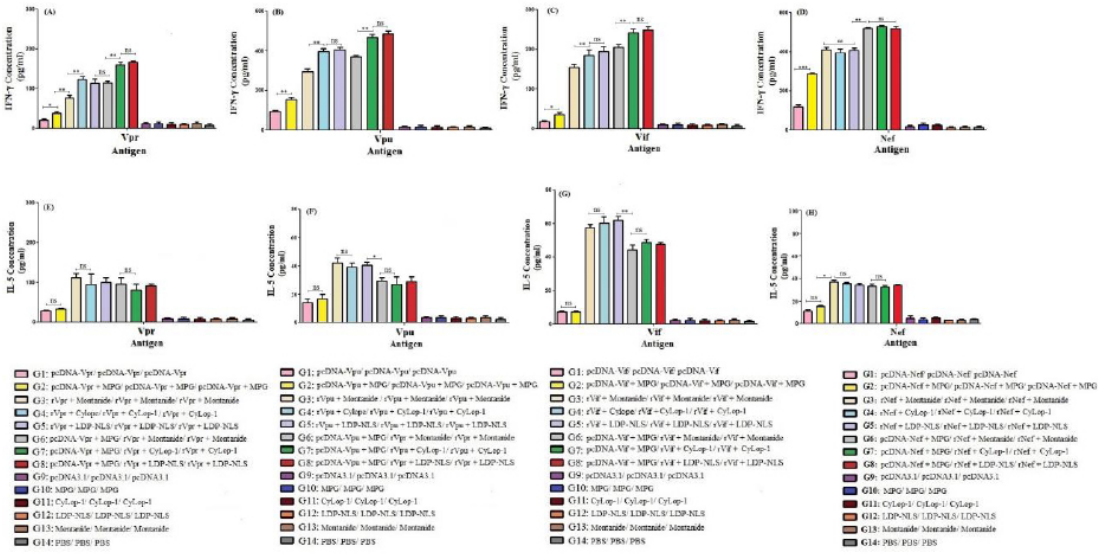

Figure 3. The secretion of IFN-γ (**A-D**) and IL-5 (**E-H**) cytokines in immunized groups with the Vpr (A & E), Vpu (B & F), Vif (C & G), Nef (D & H) antigens in various formulations: The levels of cytokines were determined by ELISA as mean absorbance at 450 nm ± SD for each set of samples. All analyses were performed in duplicate for each sample; ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The data showed that the ratios of IFN-gamma/ IL-5 were importantly higher in the heterologous and then homologous Nef, Vpu and Vif regimens (G3-G8) indicating Th1-directed responses in mice. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

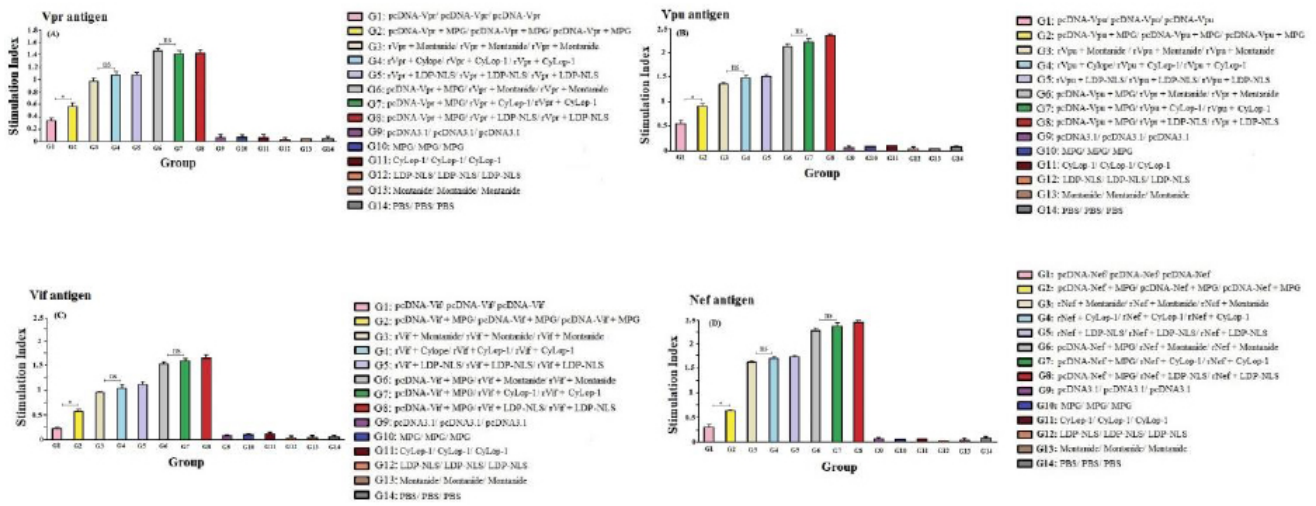

Figure 4. The lymphocyte proliferation assay in immunized groups with the Vpr **(A)**, Vpu **(B)**, Vif **(C)**, Nef **(D)** antigens in various formulations: The lymphocyte proliferation was performed by MTT assay. All analyses were performed in duplicate for each sample; ns: non-significant; * $p < 0.05$. The data showed that the stimulation index against the Vpr recombinant protein was significantly less than rNef, rVpu and rVif proteins. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

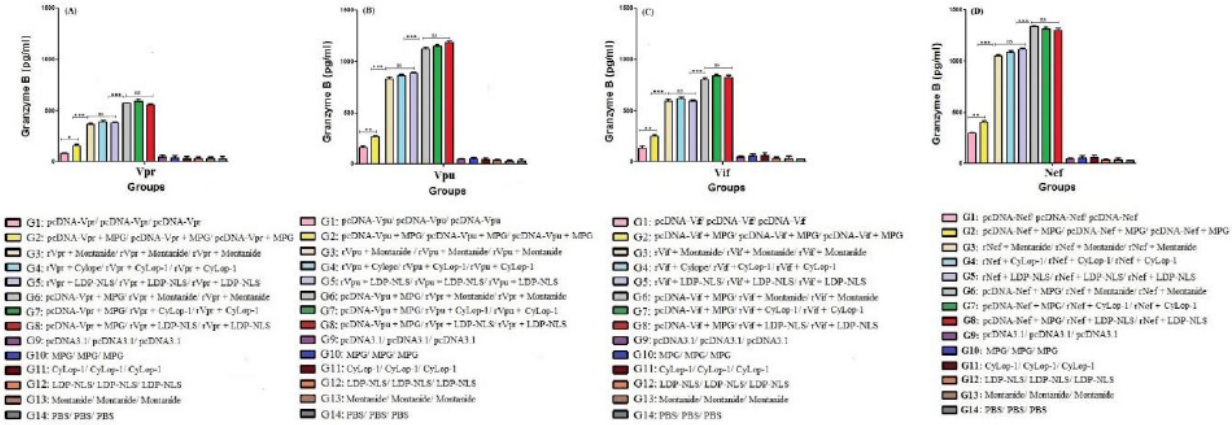

Figure 5. The levels of Granzyme B **(A-D)** determined by ELISA as mean absorbance at 450 nm ± SD for each set of samples: All analyses were performed in duplicate for each sample. ns: non-significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The data showed that the levels of Granzyme B in all regimens of Vpr antigen were less than all regimens of Nef, Vpu and Vif antigens. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

4. DISCUSSION

In the current study, we showed that Nef, Vpu and Vif accessory proteins are more effective than Vpr accessory protein for inducing immune responses in mice. In addition, Nef and Vpu stimulated higher cellular immune responses than Vif in mice. Our study demonstrated that among four accessory proteins, Vpu protein can be effective in diagnosis of untreated patients from treated patients.

The studies indicated that HIV virological tests were used to diagnose HIV infection in HIV-exposed subjects [30]. ELISA was the most common immunoassay used to detect HIV infection. For instance, the reports showed detection of Nef-containing exosomes in suppressed HIV-infected individuals [31-33]. Moreover, antibodies secreted against the full-length Nef protein and also synthetic peptides of the Nef protein (6-23 mers) were detected in HIV-1-infected individuals using ELISA [34]. Accordingly, it seems that a simple and exact assay of viral proteins in human sera is required to diagnose HIV infection in untreated, treated and drug-resistant individuals. In our study, the serum levels of HIV accessory proteins were

detected in untreated/Naïve, treated and drug-resistant subjects compared to HIV-uninfected subjects (control group) using ELISA. Our data indicated that the levels of antibodies against all recombinant proteins were significantly higher in all groups than those in control group. The levels of antibodies against rNef, rVpr and rVif proteins showed no significant differences in Naïve, treated and drug resistant groups. In contrast, the level of antibody against rVpu in Naïve was higher than that in treated group, but not in drug resistant group. Thus, Vpu can be used as a possible biomarker for diagnosis of Naïve from treated group.

Wieland *et al*. showed that the prevalence of antibodies against HIV-1 Vif and Nef expressed as MS-2 fusion proteins in bacterial system had high levels of anti-Nef antibody in all stages of disease in HIV infection. In contrast, anti-Vif antibodies were detected only in progression of disease to AIDS [35]. In the present study, we produced four accessory proteins without tag fusion in *E. coli* for the presence of antibodies in untreated, treated and drug resistant subjects. Reiss *et al*. evaluated antibody responses against the HIV-1 Nef protein in asymptomatic HIV-1-seropositive individuals. Nef-specific antibodies were stably detected in the majority of men (~ 67.6%) at early stages of infection [36]. Moreover, antibody-dependent cellular cytotoxicity (ADCC) against HIV-1 Vpu peptide (13-mer) was observed in Elite controllers [37]. Another study showed that HIV-1 Vpr was detected in the plasma of HIV-1-positive patients [38]. Furthermore, antibodies to the recombinant HIV-1 Vpr and Vpu proteins generated by *E. coli* were detected by ELISA in men sera within 12 months of seroconversion for antibodies to structural proteins [39]. Our study showed that a significant Vpu-specific antibody response was observed between untreated and treated subjects.

In the next process, we evaluated immunogenicity of the recombinant Vpr, Vif, Vpu and Nef proteins in mice. It was interesting that among four accessory proteins, Vpr showed lower immunogenicity than other proteins. Moreover, it seems that HIV-1 Vpr led to the direction of responses toward Th2 response due to higher IgG1 and IL-5, and lower IgG2a and IFN-gamma than other proteins. The level of Granzyme B was lower in groups immunized with rVpr than that in groups immunized with other proteins. In this line, a study indicated that Vpr reduced antigen-specific cytotoxic T lymphocyte activity, induced lower levels of IFN-gamma, and inhibited Th1 immune responses. In the presence of Vpr, there was a strong shift towards a Th2 response [40]. Our data about immunogenicity of Vpr was similar to this study. Another study showed that DNA vector containing HIV-1 *nef* gene (pBN-Nef) was immunogenic in mice [41]. Du *et al*. demonstrated that pcDNA-vif induced significantly antibody secretion and T-cell activity in mice especially in combination with pcDNA-LIGHT as an adjuvant [42]. Our study indicated that pcDNA-Nef, pcDNA-Vpr, pcDNA-Vpu and pcDNA-Vif delivered by MPG induced higher levels of IFN-gamma, IgG2a and Granzyme B than the naked DNA plasmids. The efficiency of the cationic CPPs was proved to deliver biomolecules. For example, the MPG/DNA nanoparticles induced a strong Th1 immune response in tumor mouse model [43, 44]. The CPPs could reduce the effective dose of vaccine [45]. In our study, 5 μg of the MPG/DNA nanoparticles was used compared to 50 μg of the naked DNA. Furthermore, the CyLoP-1 or LDP-NLS/ protein nanoparticles at a certain molar ratio of 10:1 were used to increase immune responses against a variety of antigens. These CPPs could potently deliver proteins in mammalian cells [19, 20]. Also, the size and charge of nanoparticles were important issues for entry the cells (/ cellular uptake) [46, 47]. In the current study, the small size of nanoparticles and their positive charges had a main role for their cellular uptake with respect to effective immune responses induced *in vivo*. One study in Argentina described whether three doses of priming with a plasmid harboring Nef and DNAs expressing IL-12 and/or GM-CSF cytokines followed by boosting with a dose of modified *vaccinia virus Ankara* (MVA) Nef could improve cellular immunity in mice. The data indicated that IL-12 significantly induced a higher T-cell activity as compared to other cytokine. Moreover, simultaneous administration of both cytokines led to the deletion of anti-Nef responses in mice [48]. Indeed, type of adjuvant influences the stimulation of cellular immunity. In the recent study, we used an adjuvant approved in human study named as Montanide 720 that could induce cellular immunity in mice. Our study showed that the order of antigens for induction of cellular immunity and high immunogenicity was: Nef> Vpu> Vif> Vpr. Casella *et al.* previously showed that Vpu increased sensitivity to Fas-induced death in HIV-infected cells [49]. Thus, Vpu may be considered as a vaccine candidate, as well.

The heterologous immunization strategy led to higher immunogenicity of HIV-1 vaccine candidates than homologous immunization in primates. The importance of priming agents was determined in heterologous prime-boost vaccination. For example, the priming with DNA vaccine harboring Gag-Pol-Nef followed by the boosting with *vaccinia virus* strain NYVAC and/or protein increased effective immune responses [50]. Our results also showed that heterologous DNA prime/ protein boost strategy was more potent than homologous protein prime/ protein boost strategy in IFN-gamma, IgG2a and IgG2b secretion directed toward Th1 response as well as Granzyme B secretion as CTL activity. In addition, homologous protein prime/ protein boost showed higher immunogenicity than homologous DNA prime/ DNA boost strategy. In 1996, the first report of inducing HIV-1 Nef-specific CTLs by DNA vaccine was published [51]. However, our data showed that Nef protein prime/ Nef protein boost, and Nef DNA prime/ Nef protein boost regimens were more effective than Nef DNA prime/ Nef DNA boost regimen for induction of Granzyme B secretion as well as Th1 response.

CONCLUSION

Generally, in mice immunization, the Nef, Vpu and Vif proteins could significantly increase Th1 immune response. In contrast, the Vpr protein could importantly induce Th2

immune response. Indeed, Vpr showed lower immunogenicity than other accessory proteins. Moreover, the Nef and Vpu proteins stimulated more effective cellular immunity than the Vif protein. Among different immunization strategies, the heterologous DNA/ protein modality was the best regimen for increasing the levels of IgG2a, IgG2b, IFN-γ and Granzyme B in mice. The CPPs could significantly enhance cellular immunity in DNA and protein immunizations, as well. On the other hand, among four accessory proteins, anti-Vpu antibodies were significantly detected to diagnose treated subjects from untreated (Naïve) patients. However, the success of these proteins for eradication of virus in mouse model and also HIV diagnosis in larger human populations remain to be further evaluated in near Future.

AUTHOR's CONTRIBUTION

A.B. and A.M. designed the experiments and wrote the manuscript. Experiments were performed and analyzed by A.M., K.B., E.A., G.M. and M.A. All authors reviewed the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol was approved by National Institute for Medical Research Development, Iran (ethical code: IR.NIMAD.REC.1398.329). The experiments were done based on approved protocols for care of laboratory animals at Pasteur Institute of Iran (ethical code: IR.PII.REC.1397.024).

HUMAN AND ANIMAL RIGHTS

No Humans were used in this research. The reported experiments on animals were performed in accordance with the institutional guidelines of the Pasture Institute of Iran.

CONSENT FOR PUBLICATION

The consent was taken from all participants before blood collection.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

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

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