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Engineered ClearColi™-derived outer membrane vesicles as functional carriers for development of HIV-1 therapeutic vaccine candidate

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ABSTRACT

Bacteria-derived outer membrane vesicles (OMVs) can be engineered to incorporate foreign antigens. This study explored the potential of ClearColi™-derived OMVs as a natural adjuvant and a carrier (recombinant OMVs or rOMVs) for development of an innovative therapeutic vaccine candidate harboring HIV-1 Nef and Nef-Tat antigens. Herein, the rOMVs containing CytolysinA (ClyA)-Nef and ClyA-Nef-Tat fusion proteins were isolated from ClearColi™ strain. The presence of Nef and Nef-Tat proteins on their surface ($rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$) was confirmed by western blotting after proteinase K treatment. Immune responses induced by Nef and Nef-Tat proteins emulsified with Montanide® ISA720 or mixed with OMVs, and also $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$ were investigated in BALB/c mice. Additionally, the potency of splenocytes exposed to single-cycle replicable (SCR) HIV-1 virions was assessed for the secretion of cytokines *in vitro*. Our findings showed that the rOMVs as an antigen carrier ($rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$) induced higher levels of IgG2a, IFN-γ and granzyme B compared to OMVs as an adjuvant (Nef + OMV and Nef-Tat + OMV), and also Montanide® ISA720 (Nef + Montanide and Nef-Tat + Montanide). Moreover, IFN-γ level in splenocytes isolated from mice immunized with $rOMV_{Nef\text{-}Tat}$ was higher than other regimens after exposure to SCR virions. Generally, ClearColi™-derived rOMVs can serve as potent carriers for developing effective vaccines against HIV-1 infection.

1. Introduction

According to the United Nations Programme on HIV/AIDS (UNAIDS), about 38.4 million individuals were living with human immunodeficiency virus (HIV) by the end of 2021, and 650,000 people died of acquired immune deficiency syndrome (AIDS)-related diseases during the same period [1]. Although, combination antiretroviral therapy (cART) serves for HIV treatment, but it is not accessible for all HIV-infected individuals in the world [2] and obtaining cure or eradication is impossible [3,4]. On the other hand, up to now, no effective vaccine has been approved against HIV infections [2]. Developing a universal HIV vaccine poses significant challenges due to the need for immunogens that can elicit effective responses against diverse strains [5]. Subunit vaccines, classified as second-generation vaccines, utilize specific components of pathogens such as protein antigens, synthetic peptides, or polysaccharides to elicit immune responses. Main limitation of this type of vaccines is their low immunogenicity which can be solved by effective adjuvants [6]. Thus, finding a potent antigen and adjuvant is critical to overcome HIV infection [7]. Among HIV-1 proteins, HIV-1 Tat and Nef proteins were known as ideal targets in vaccine design due to their early expression after infection, even before provirus integration. These proteins have high conservancy in their immunogenic epitopes among HIV clades. They play main roles in the HIV life cycle and in the pathogenesis of AIDS. On the other hand, in HIV natural infection, humoral and cellular responses directed toward Tat and Nef antigens correlate with the nonprogression to AIDS [8,9]. Several pre-clinical and clinical vaccine studies were conducted using HIV-1 or Simian immunodeficiency virus (SIV) Nef and Tat antigens in various strategies such as protein, peptide, DNA or expression vector regimens. These vaccine candidates were safe and capable of eliciting immune responses in mice,

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monkeys, and even HIV-1 negative or infected individuals during human trials [10]. HIV-1 Tat protein is effectively internalized by monocyte-derived dendritic cells (MDDC) leading to their maturation and promoting T helper type 1 (Th1) response [9,11]. Moreover, a small region of Tat protein was considered as a cationic cell penetrating peptide (CPP) in the recent years [12,13].

Outer membrane vesicles (OMVs) are small spherical lipid nanoparticles (20–300 nm) derived from the outer membrane of gram-negative bacteria. The presence of pathogen-associated molecular patterns (PAMPs) on OMVs, which bind to the pattern recognition receptors (PRR) of the antigen presenting cells (APCs) and activate naïve T cells, makes them proper adjuvants resulting in immune system stimulation. Furthermore, OMVs promote the uptake of antigens by APCs to induce potent immune responses [14,15]. Therefore, native OMVs have been studied as vaccine candidates against bacterial infections (*e.g., Neisseria meningitidis*, *Acinetobacter baumannii*, *Porphyromonas gingivalis* and *Vibrio cholera*), viral infections, and various cancers [16,17]. Moreover, Bexsero™, the only OMV-based vaccine licensed by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), could be protective against 66–91 % of the MenB strains in the world [18]. On the other hand, OMVs were utilized as natural adjuvants in different vaccination platforms including live, inactivated and subunit vaccines [14,16]. However, to overcome the issue of triggering endotoxic responses induced by lipopolysaccharide (LPS) of OMVs, endotoxin removal methods during OMV isolation from bacterial cells or genetic manipulation have been applied [19,20]. Accordingly, many studies isolated OMVs from ClearColi™, an engineered *Escherichia coli* BL21 (DE3) strain with a genetically modified LPS for vaccine design [21]. To strengthen the immune-stimulating effects of OMVs, the recombinant OMVs (rOMVs) displaying different antigens were constructed. Decreased price of antigen purification compared to conventional subunit vaccines is one of the considerable benefits of rOMV-based vaccines [22]. Heterologous antigens can be displayed on the surface of OMVs through a variety of fusion proteins such as ClyA, fHbp and OprI. Cytolysin A (ClyA) is a 34-kDa α-pore-forming toxin (α-PFT) that is produced by some bacteria from the *Enterobacteriaceae* family including *E. coli* and *Salmonella enterica* [23,24]. For the first time, Chen et al. reported the generation of rOMVs displaying green fluorescent protein (GFP) through fusion of GFP to ClyA of *E. coli* that could induce strong GFP-specific antibodies without any adjuvant [25]. Up to now, the engineered ClyA-OMV system has been applied to improve vaccines against various pathogens (*e.g.,* SARS-CoV-2) [26].

The goal of this study was to evaluate the immunostimulatory effects of ClearColi™ -derived OMVs serving as either an adjuvant or as antigen carrier against HIV-1 Nef and Nef-Tat antigen candidates in BALB/c mice. For the first time, we aimed to compare two unique strategies utilizing *E. coli*-derived OMVs including 1) rOMVs displaying Nef and Nef-Tat antigen and 2) ClearColi™-derived OMVs as adjuvants in combination with Nef and Nef-Tat antigens. Furthermore, the secretion of cytokines from the pooled splenocytes was investigated after exposure to single-cycle replicable (SCR) HIV-1 virions. The SCR virions as a controlled *in vitro* model provide valuable insights into viral pathogenesis and serve as a vital tool in virology research and vaccine development, bridging the gap between *in vitro* and *in vivo* studies [27].

2. Material and methods

2.1. Generation of the recombinant DNA constructs

In order to prepare Nef-Tat DNA fusion constructs, the full length of *nef* and first exon of *tat* gene were determined from HIV-1 pNL4-3 vector (Accession No: AF324493.2). Then, gene cloning was designed in different vectors using SnapGene software. Next, the *nef-tat* fusion gene was synthesized in pUC57 vector by BioMatik Company (Canada). After that, the *nef-tat* fusion gene was subcloned from pUC57-*nef-tat* into the pET-24a (+) prokaryotic expression vector (Novagen) using *Nhe*I/*Sal*I restriction enzymes (Thermo Scientific, USA) to generate the recombinant Nef-Tat protein. It should be mentioned that the *nef* gene was previously cloned into the pET-23a prokaryotic expression vector (Novagen) by our group. Finally, the gene sequences of *nef* and *nef-tat* were cloned from pUC57-*nef-tat* into our previously constructed pET-22 (ClyA- CT26-polyneoepitopes) vector [28] (Novagen) for production of the recombinant OMV_{Nef} and $OMV_{Nef\text{-}Tat}$ in *Escherichia coli* (*E. coli*) system. For preparation of pET-22b-*clyA-nef* and pET-22b-*clyA-nef-tat*, the *nef* and *nef-tat* genes were amplified from pUC57-*nef-tat* by polymerase chain reaction (PCR) using the designed primers (Table 1) under standard conditions (94 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min: 30 cycles). The PCR products were detected using DNA agarose gel electrophoresis. Then, the amplified *nef* and *nef-tat* fragments were cloned into the pET-22 (ClyA-CT26-polyneoepitopes) [28] vector using *Nco*I/*Hind*III and *Eco*RI/*Xho*I (Thermo Scientific, USA) restriction enzymes, respectively. Finally, the recombinant DNA constructs was confirmed using enzymatic digestion, cloning PCR, and sequencing. NanoDrop spectrophotometer was used to determine the purity and concentration of DNA constructs.

2.2. Preparation of the recombinant proteins

In our previous studies, Nef protein was expressed [29,30] and detected by Western blot analysis using anti-Nef antibody [30]. Expression of the recombinant Nef-Tat protein was performed in *E. coli* using the Isopropyl thiogalactopyranoside (IPTG, Sigma, Germany) inducer. Briefly, *E. coli* Rosetta and BL21 strains were transformed by pET-24a (+) vector harboring the *nef-tat* gene. Kanamycin-resistant recombinant clones were chosen on LB-agar (Sigma, Germany) and then cultured in Ty2x medium (Sigma, Germany) until reaching an OD_{600} of 0.6–0.7. The expression of the recombinant protein was induced by adding 1 mM IPTG at 37 °C, and evaluated in different incubation times (*i.e.,* 2, 4 and 16 h) after induction using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) technique. Moreover, the expression of HIV-1 Nef protein was performed in *E. coli* as previously performed by our group [30]. For generation of the recombinant ClyA-Nef and ClyA-Nef-Tat proteins, the cultures of ClearColi™ carrying pET-22b-*clyA-nef* and pET-22b-*clyA-nef-tat* were grown in LB broth Miller medium until reaching an OD_{600} of 0.6–0.7. The expression of ClyA-Nef and ClyA-Nef-Tat protein was done by adding 1 mM IPTG inducer at 37 °C and incubation times of 2, 4 and 16 h. Then, the expression of the recombinant proteins was evaluated by SDS-PAGE and also western blotting using anti-Nef monoclonal antibody (Abcam, USA; 1:10000 *v/v* in blocking buffer) conjugated to horseradish peroxidase (HRP) and diaminobenzidine (DAB) substrate (Sigma, Germany). Moreover, the recombinant Nef and Nef-Tat proteins were purified by affinity chromatography using Ni-NTA column under denaturing conditions (Urea buffer, pH = 4.5) according to the manufacturer's guidelines (Qiagen, Germany). After purification, the recombinant proteins were dialyzed against phosphate-buffered saline 1X (PBS1X), and their concentrations were determined using NanoDrop spectrophotometry. The protein contamination with endotoxin was analyzed by the LAL assay (QCL-1000, Lonza, UK), and the results indicated that it was below 0.4 EU/mg of proteins.

Table 1
The primer sequences used for amplification of HIV-1 *nef* and *nef-tat* genes.

Primer	Sequence (5′-3′)
Forward-*nef*	GG CCATGGGTATGGGTTGTAAGTGGTC
Reverse-*nef*	GG AAGCTTGCAGTTCTTGAAGTACTCCG
Forward-*nef-tat*	GG GAATTCGTATGGGTTGTAAGTGGTC
Reverse-*nef-tat*	GG CTCGAG CTTTGATAGAGATGCCTGATG

2.3. Preparation of OMVs as an adjuvant

For the preparation of empty OMVs, the ClearColi™ strain was cultured in 15 mL of LB broth Miller (Sigma, Germany) at 37 °C with shaking at 180 rpm. The culture was then subcultured in 500 mL of LB broth Miller with an initial OD_{600} of 0.1. After 16 h incubation time, the bacteria were harvested by centrifugation at 5000×*g* for 15 min at 4 °C, and the supernatant was filtered by a 0.22 μm vacuum filter (Millipore). To isolate OMVs, ammonium sulfate at 70 % saturation (Merck, Germany) was added to the supernatant, and centrifuged at 10,000×*g* for 30 min at 4 °C. The resulting pellet was then resuspended in PBS, and the supernatant was filtered by a 0.45 μm vacuum filter. The filtered supernatant containing OMVs was then concentrated using an Amicon Ultra centrifugal filter device (Merck, Germany) with a 50 kDa cut-off membrane (Merck, Germany). Finally, the concentrated OMVs were stored at −20 °C until further use. To confirm OMV purification, OmpA porin that is expressed at high levels in the bacterial cell walls, was detected by 12.5 % SDS-PAGE, and also western blotting using HRP-conjugated anti-OmpA antibody (Biocompare, USA, 1:2000 *v/v*) and DAB substrate. The morphology and size of OMVs were characterized by scanning electron microscopy (SEM, KYKY-EM3200).

2.4. Preparation of rOMVs as a carrier

For the preparation of ClearColi™-derived rOMVs (*i.e.,* $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$), the transformed ClearColi™ strains with pET-22b-*clyA-nef* and pET-22b-*clyA-nef-tat* were induced by IPTG in LB broth Miller at 37 °C with shaking at 180 rpm overnight (according to above section: Preparation of the recombinant proteins). The OMV isolation method was performed according to section 2.3. The isolated rOMVs were analyzed by 12.5 % SDS-PAGE, and also western blotting using HRP-conjugated anti-Nef monoclonal antibody (1: 2000 *v/v* in blocking buffer) and DAB substrate. Moreover, for evaluating the distribution of the recombinant proteins on the surface, OMVs were digested with proteinase K and analyzed by SDS-PAGE and western blotting. The morphology and size of OMVs were characterized by transmission electron microscopy (TEM, PHILIPS, EM208S, 100 KV). The surface charge of recombinant OMVs was measured using dynamic light scattering (DLS) at 25 °C with the nano-zetasizer ZEN3600 (Malvern Instrument, UK) and Zetasizer Software (version 7.03).

2.5. Mice immunization

Different regimens were subcutaneously injected in the right footpad of the 6–8 old female BALB/c mice (n = 7 per group) three times with a 2-week interval (Table 2). The animals were maintained under specific pathogen-free conditions based on the guidelines of the Pasteur Institute of Iran. The study was approved by the local ethical committee (ethical code: IR.PII.REC.1400.009) for scientific purposes. Mice were immunized with 5 μg of protein (G1: Nef protein and G2: Nef-Tat protein emulsified with Montanide® ISA720 at the ratio of 70:30 v/v (oil: aqueous phase)), and 20 μg of OMV-based regimens (G3: Nef protein + OMV, G4: Nef-Tat protein + OMV, G5: $rOMV_{Nef}$ and G6: $rOMV_{Nef\text{-}Tat}$).

2.6. Monitoring antibody responses

For evaluating antibody response, the pooled sera from each group were collected 3 weeks (short term) and 12 weeks (long term) after the last immunization. The levels of anti-mouse total IgG, IgG1, IgG2a antibodies were determined in the pooled sera of each group (diluted 1:100 v/v in 1 % BSA/PBS-Tween) using indirect enzyme-linked immunosorbent assays (ELISA) against the Nef and Nef-Tat coated antigens, as previously described [31]. Briefly, 100 μL of the purified Nef and Nef-Tat (5 μg/mL) diluted in PBS 1X were coated in 96-well flat-bottom ELISA plates (SPL, Korea) and then the plates were incubated overnight at 4 °C. After three rounds of washing with washing buffer (0.05 % (v/v) Tween 20 in PBS), 100 μL of blocking buffer (1 % (w/v) bovine serum albumin (BSA)/PBS) was added to each well and the plates were incubated for 2 h at 37 °C. Then, the diluted sera at ratio of 1:100 (diluted in dilution buffer: PBS containing 0.05 % (v/v) Tween 20 and 1 % (w/v) BSA) were added to the plates. Then, the plates were incubated for 2 h at 37 °C and washed 3 times. In the next step, 100 μL of gout anti-mouse total IgG, IgG1 and IgG2a HRP conjugate antibody (Sigma, USA), (diluted 1:10,000 in dilution buffer) was added and then the plates were incubated for 2 h at 37 °C and washed 3 times. 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB, Sigma, USA) was added as substrate to each well and incubated for 15 min at room temperature away from light. Finally, the reaction was terminated by adding 100 μL H_2SO_4 and the optical absorption of each sample at a wavelength of 450 nm was measured by an ELISA reader (Biotek, USA).

2.7. Cytokine assay

The secretion of cytokines was assessed in the pooled splenocytes of four mice for short-term and also three mice for long-term. Briefly, the pooled red blood cell-depleted splenocytes (2×10^6 cells/mL) of each group were restimulated in 48-well plates with the recombinant Nef or Nef-Tat proteins (5 μg/mL) in complete RPMI culture medium (*i.e.*, RPMI-1640 supplemented with 10 % heat-inactivated fetal bovine serum (FBS)), and incubated at 37 °C and 5 % CO_2 under humidified conditions for 72 h. The unstimulated splenocytes and stimulated splenocytes with concanavalin A (5 μg/mL) served as the negative and positive controls, respectively. Sandwich-based ELISA kit (Mabtech, Swedish Biotech Company) was used to measure the secretion of IFN-γ and IL-5 cytokines in the supernatants according to the manufacturer's instructions.

2.8. Granzyme B (GrB) assay

SP2/0 target cells (2×10^4 cells/well) were seeded into 96-well plates containing complete RPMI-1640 medium in the presence of the recombinant Nef or Nef-Tat proteins (5 μg/mL) for 24 h. The pooled effector splenocytes were counted using trypan blue and co-cultured with the target cells at an effector-to-target ratio of 100:1 for 6 h. After this time, the supernatants were collected by centrifugation (250 g, 5 min, 4 °C), and the levels of granzyme B were determined using ELISA (eBioscience, USA) according to the manufacturer's instructions.

Table 2
Immunization program in BALB/c mice.

Group	First injection (Day 0)	Second injection (Day 14)	Third injection (Day 28)
G1	Nef protein + Montanide®	Nef protein + Montanide®	Nef protein + Montanide®
G2	Nef-Tat protein + Montanide®	Nef-Tat protein + Montanide®	Nef-Tat protein + Montanide®
G3	Nef protein + OMV	Nef protein + OMV	Nef protein + OMV
G4	Nef-Tat protein + OMV	Nef-Tat protein + OMV	Nef-Tat protein + OMV
G5	$rOMV_{Nef}$	$rOMV_{Nef}$	$rOMV_{Nef}$
G6	$rOMV_{Nef\text{-}Tat}$	$rOMV_{Nef\text{-}Tat}$	$rOMV_{Nef\text{-}Tat}$
G7 (control)	PBS	PBS	PBS

2.9. Generation of SCR virions and in vitro virion-specific cytokine assay

Production of the safe SCR virions was performed according to our previous study [32]. In brief, the human embryonic kidney (HEK 293T) cells were co-transfected with pmzNL4.3, psPAX2, and pMD2G plasmids [27] using TurboFect reagent (Fermentas, Germany). After 72 h, culture supernatants were collected, and recombinant virions were isolated by high-speed centrifugation. Their quantity was assessed using a p24 ELISA assay kit (rETRO-TEK, USA). After virion preparation, the secretion of IFN-γ and IL-5 cytokines was assessed in the culture supernatants of the pooled splenocytes (2×10^6 cells/mL for each group) at 72 h after exposure to SCR virions (50 μg of p24 antigen) using a sandwich-based ELISA kit (Mabtech, Swedish Biotech Company).

2.10. Statistical analysis

Statistical analysis was assessed by prism 8 (GraphPad Software) using one-way ANOVA. The data were shown as mean ± SD for each group. The *p*-value <0.05 was considered statistically significant (*$p < 0.05$, * *$p < 0.01$, * * *$p < 0.001$, * * * *$p < 0.0001$). Moreover, the experiments were independently performed twice.

3. Results

3.1. Confirmation of the recombinant DNA constructs

The recombinant pET-24a-*nef-tat* was confirmed as a clear band of ~911 bp for the *nef-tat* gene on agarose gel after enzymatic digestion. Moreover, the recombinant pET-22b-*clyA-nef* and pET–22b-*clyA-nef-tat* were confirmed by digestion with *Nco*I/*Hind*III and *Eco*RI/*Xho*I restriction enzymes as the clear bands of ~648 bp and ~911 bp for the *nef* and *nef-tat* genes on agarose gel, respectively (Fig. 1). The accuracy of the cloning was confirmed by cloning PCR and sequencing, as well.

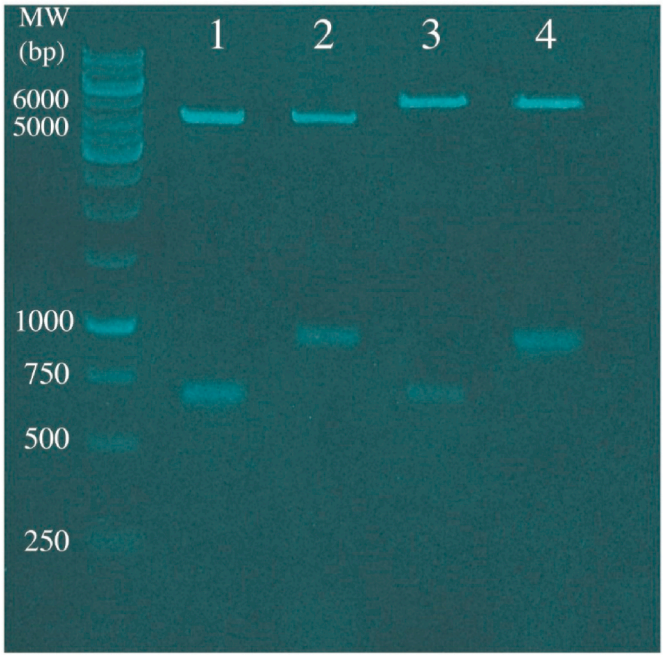

Fig. 1. Confirmation of the recombinant plasmids using double-digestion in agarose gel electrophoresis. Lanes 1 and 2 denote the double digested products using *Eco*RI/*Sal*I for pET-23a-*nef* (~648 bp: *nef*) and *Nhe*I/*Sal*I for pET-24a-*nef-tat* (~911 bp: *nef-tat*), respectively. Lanes 3 and 4 represent the double digested products using *Nco*I/*Hind*III for pET-22b-*clyA-nef* (~648 bp: *nef*) and *Eco*RI/*Xho*I for pET-22b-*clyA-nef-tat* (~911 bp: *nef-tat*). DNA ladder is 1 kb (Fermentas).

3.2. Expression and purification of the recombinant proteins

The expression of the recombinant Nef-Tat protein was evaluated in two bacterial systems including pET-24a/Rosetta or pET-24a/BL21 systems. Our results showed that the highest level of Nef-Tat expression was obtained in Rosetta strain 16 h after induction (Suppl. Fig. 1), which was confirmed by observing a clear band of ~35 kDa on 12.5 % SDS-PAGE and also western blotting. Also, the results indicated that Nef-Tat protein can be successfully purified by affinity chromatography under denaturing conditions. Moreover, the recombinant Nef (~30 kDa) protein was expressed in Rosetta strain 4 h after induction and purified under denaturing conditions according to the previous results of our group (Fig. 2). The concentrations of Nef and Nef-Tat proteins assessed by NanoDrop Spectrophotometry were 1.2 and 0.98 mg/mL, respectively. On the other hand, the expression of ClyA-Nef and ClyA-Nef-Tat proteins in the ClearColi™ strain was detected on 12.5 % SDS-PAGE as clear bands of ~60 and ~65 kDa, respectively. The results indicated successful expression of ClyA-Nef and ClyA-Nef-Tat at 2, 4 and 16 h after induction with 1 mM IPTG (Fig. 3A & B). The optimal expression time was considered 2 h post-induction for the next experiments. The recombinant ClyA-Nef and ClyA-Nef-Tat proteins were identified by Western blot analysis using an anti-Nef antibody (Fig. 3C).

3.3. Isolation of OMVs and rOMVs from ClearColi™

Western blot analysis was performed using anti-OmpA and anti-Nef antibodies to confirm the isolated ClearColi™ OMV and rOMVs ($rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$), respectively. The isolated OMV was analyzed by the presence of ~35 kDa protein band in SDS-PAGE analysis, which refer to the most common OMV protein, OmpA (Fig. 4A). Moreover, Western blot analysis confirmed the isolated ClearColi™ OMV by the presence of a clear band of ~35 kDa using peroxidase-conjugated anti-OmpA antibody (Fig. 4B). In order to confirm the presence of recombinant proteins on the surface of rOMVs ($rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$), they were digested by proteinase K. As proteinase K cannot penetrate the membrane, only proteins expressed on the exterior of OMVs are degraded while the proteins in the lumen of OMVs are protected from proteolysis [33]. Immunoblotting analysis using anti-Nef antibody showed that after hydrolysis of surface proteins by proteinase K, the samples lost reactivity with the anti-Nef antibody. The results indicated that only proteins exposed on the surface of rOMV reacted with specific antibody. For unhydrolyzed $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$, the clear bands of ~60 and ~65 kDa were observed in western blotting, respectively indicating successful expression and presentation of the ClyA-Nef and ClyA-Nef-Tat fusion protein on the rOMV surface (Fig. 4C). The concentrations of OMV, $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$ obtained by BCA kit were 2.52 mg/mL, 2.38 mg/mL and 2.55 mg/mL respectively.

3.4. Characterization of OMVs

In this study, DLS (Suppl. Fig. 2A) and electron microscopy (Fig. 5 and Suppl. Fig. 3) were used to determine the size of ClearColi™-derived OMVs. Electron microscopy showed that the size of OMV (Fig. 5A), $rOMV_{Nef}$ (Fig. 5B) and $rOMV_{Nef\text{-}Tat}$ (Fig. 5C) was about 30–50 nm. Consistent with our previous study [16], as DLS and SEM measure the size of hydrodynamic diameter and the size of the dense core without solvation layer, respectively, the size of ClearColi™-derived OMVs determined by SEM was smaller than that by DLS. The zeta potential or surface charge of $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$ was −13.9 mV and −11.8 mV, respectively (Suppl. Figs. 2B and C). This data indicated that the rOMVs ($rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$) retained the structure of natural vesicles and also their negative charge.

3.5. Antibody responses in immunized mice

Antibody responses (total IgG and the related subclasses (IgG1 and

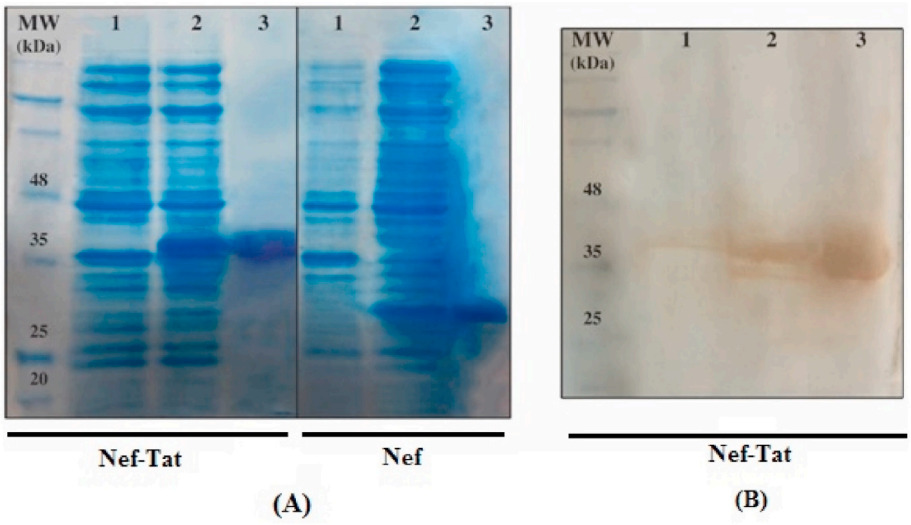

Fig. 2. Expression and purification of the recombinant Nef and Nef-Tat proteins in *E. coli* Rosetta strain: **A.** SDS-PAGE analysis; Lane 1: before induction, Lane 2: after induction, Lane 3: the purified protein. **B.** Identification of the recombinant Nef-Tat protein by western blotting using anti-Nef monoclonal antibody; Lane 1: before induction, Lane 2: after induction, Lane 3: the purified protein; MW is molecular weight marker (10–180 kDa, Fermentas).

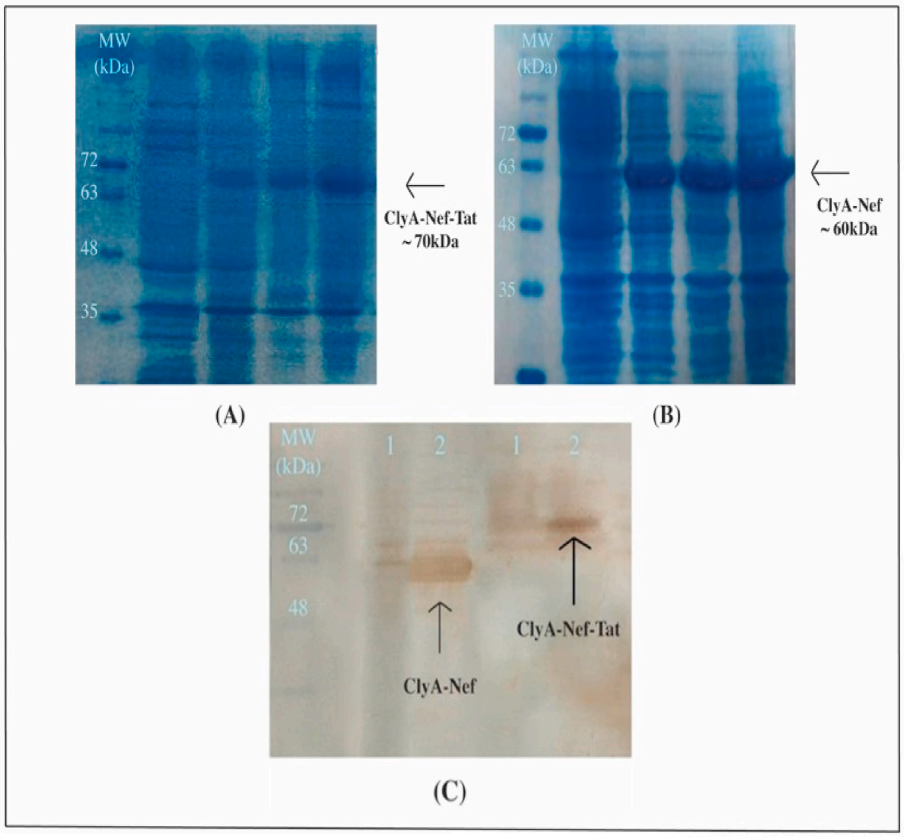

Fig. 3. The expression of ClyA-Nef and ClyA-Nef-Tat proteins in ClearColi™ strain expression system. **A.** The expression of ClyA-Nef-Tat protein: Lane 1: Before induction, Lanes 2–4: 2 h, 4 h and 16 h after induction, respectively; **B.** The expression of ClyA-Nef protein: Lane 1: Before induction, Lanes 2–4: 2 h, 4 h and 16 h after induction; **C.** Identification of ClyA-Nef and ClyA-Nef-Tat proteins by western blotting using anti-Nef monoclonal antibody: Lane 1: Before induction; Lane 2: 16 h after induction; MW is molecular weight marker (10–180 kDa, Fermentas).

IgG2a)) against Nef and Nef-Tat antigens were measured in the sera of immunized mice using indirect ELISA at 3 and 12 weeks after final immunization. The levels of total IgG against Nef-Tat antigen in groups that received Nef-Tat protein (G2, G4 and G6) were significantly higher than those received Nef protein (G1, G3 and G5) (Fig. 6A and B). In contrast, the levels of total IgG against Nef antigen in groups immunized with Nef and Nef-Tat proteins were not statistically significant ($p > 0.05$). Also, the levels of total IgG in groups that received $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$ (G5 & G6) were higher than groups that received the recombinant Nef and Nef-Tat protein (G1& G2; $p < 0.05$). The levels of total IgG against

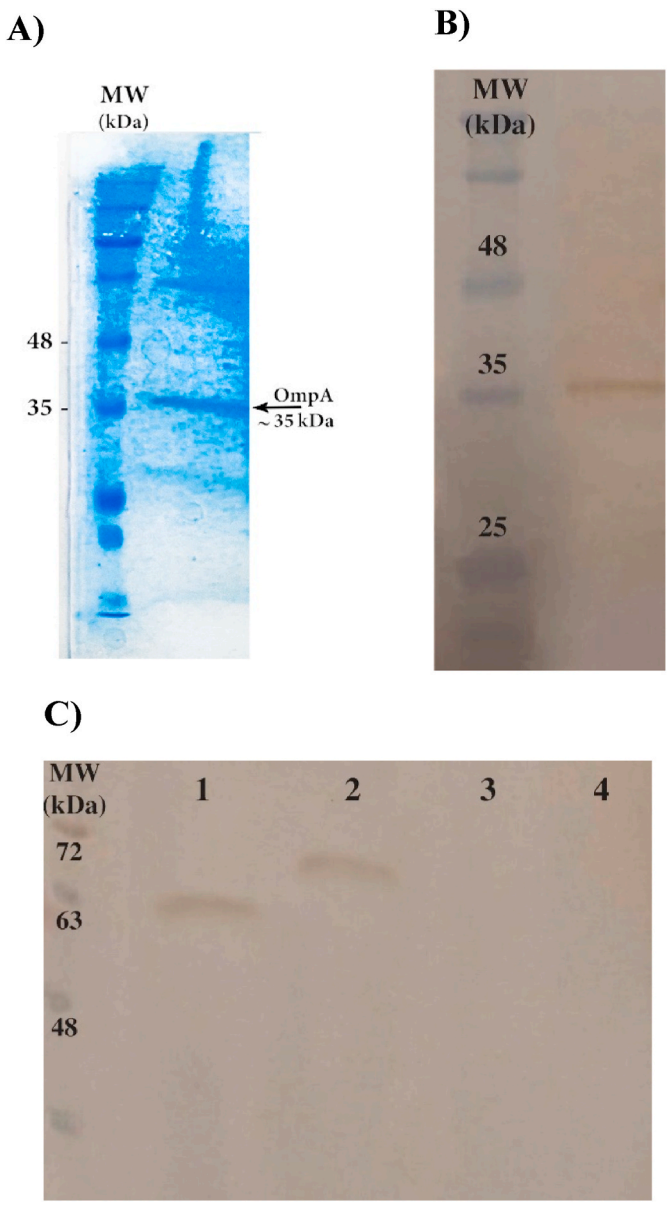

Fig. 4. Confirmation of the isolated ClearColi™ OMV and ClearColi™ rOMVs. **A.** Expression of OmpA in OMV using SDS-PAGE. **B.** Identification of OmpA protein in OMV by western blotting using anti-OmpA antibody. **C.** Confirmation of the ClyA-Nef and ClyA-Nef-Tat proteins on the surface of rOMVs ($rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$) using western blotting: Lane 1: The presence of ClyA-Nef protein on the surface of rOMVs; Lane 2: The presence of ClyA-Nef-Tat protein on the surface of rOMVs; Lane 3: Lack of ClyA-Nef in the presence of proteinase K; Lane 4: Lack of ClyA-Nef-Tat in the presence of proteinase K.

Nef-Tat antigen in $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$ regimens (G5 & G6) were higher than OMV + Nef and OMV + Nef-Tat regimens (G3& G4) at 3 and 12 weeks after the last immunization ($p < 0.05$; Fig. 6A and B). On the other hand, the secretion of IgG1 against Nef-Tat antigen in group immunized with Nef protein (G1) was significantly higher than that immunized with Nef-Tat protein (G2) (Fig. 6C and D). Moreover, serum IgG1 responses against Nef-Tat antigen in group receiving Nef protein (G1) was significantly higher than groups receiving Nef protein + OMV (G3) and $rOMV_{Nef}$ (G4; $p < 0.05$; Fig. 6C and D). Additionally, the secretion of IgG2a against Nef-Tat antigen in group immunized with Nef-Tat protein (G2) was significantly higher than that immunized with Nef protein (G1) Fig. 6E and F). The highest level of IgG2a against Nef-Tat antigen was observed in group receiving $rOMV_{Nef\text{-}Tat}$ (G6). The levels of IgG2a in groups receiving $rOMV_{Nef}$ (G5) and $rOMV_{Nef\text{-}Tat}$ (G6) were significantly higher than groups receiving Nef + OMV (G3) and Nef-Tat + OMV (G4) at 12 weeks after the last immunization ($p < 0.05$; Fig. 6E and F). Generally, higher IgG2a/IgG1 ratio (Table 3) in Nef-Tat groups (G2, G4 and G6) compared to Nef groups (G1, G3 and G5) demonstrated that addition of Tat to Nef protein can direct the induced immunity towards Th1 cellular immunity. Furthermore, higher mean ratios of IgG2a/IgG1 (Table 3) in groups immunized with $rOMV_{Nef\text{-}Tat}$ (G6) and Nef-Tat + OMV (G4) against both Nef and Nef-Tat antigens than other groups indicated a possible shift in the immune response towards Th1 cellular immunity.

3.6. Evaluation of IFN-γ and IL-5 cytokines

The analysis of cytokine secretion was performed for the pooled splenocytes of mice at 3 and 12 weeks after the last immunization. All mice immunization with different modalities effectively enhanced the levels of IFN-γ as compared to control group ($p < 0.0001$). The results showed that Nef-Tat protein (G2) was more effective than Nef protein (G1) in eliciting IFN-γ responses (Fig. 7A and B). The levels of IFN-γ in mice immunized with $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$ (G5 & G6) were significantly higher than mice immunized with Nef or Nef-Tat protein regimens (G1& G2; $p < 0.0001$). Moreover, OMV could increase the secretion of IFN-γ in groups immunized with Nef + OMV (G3) and Nef-Tat + OMV (G4) as compared to groups immunized with Nef protein (G1) and Nef-Tat protein (G2), respectively ($p < 0.05$; Fig. 7A and B). Generally, the levels of IFN-γ secretion were almost constant at 12 weeks after the last immunization. On the other hand, the levels of IL-5 cytokine in mice immunized with the recombinant Nef and Nef-Tat proteins (G1 & G2) were significantly higher than mice immunized with OMV + protein (G3 & G4; $p < 0.05$) and rOMV (G5 & G6; $p < 0.0001$) at 3 and 12 weeks after the last immunization (Fig. 7C and D). The highest mean ratio of IFN-γ to IL-5 was observed in $rOMV_{Nef\text{-}Tat}$ group (G6) indicating that $rOMV_{Nef\text{-}Tat}$ regimen can regulate Th1 cellular immunity development. According to the results of IFN-γ/IL-5 ratio, Nef-Tat-containing regimens (G2, G4 and G6) were superior to Nef-containing regimens (G1, G3 and G5) in stimulating Th1 cell response (Table 4).

3.7. Evaluation of granzyme B secretion

It was reported that the secretion of granzyme B as a key cytotoxic mediator in target cell destruction, can be assessed to establish an attractive alternative to ^{51}Cr-release assays for evaluating of antigen-specific CTL responses [34]. In this study, granzyme B secretion was assessed for each group at 3 and 12 weeks after the last immunization. Our data showed that the immunized group with $rOMV_{Nef\text{-}Tat}$ (G6) produced higher concentration of Granzyme B than all other groups at 3 week after final immunization ($p < 0.05$). Moreover, mice immunized with rOMV regimens (G5 & G6) showed higher levels of Granzyme B than groups immunized with protein regimens (G1 & G2; $p < 0.0001$). A significant decrease in Granzyme B secretion was observed in mice sacrificed at 12 weeks after the last immunization compared to mice sacrificed at 3 weeks after the last immunization ($p < 0.05$). Interestingly, the secretion of granzyme B in groups received OMV as an adjuvant (G3 & G4) was significantly higher compared to groups received montanide adjuvant (G1 & G2). Consistent with the ratio of IFN-γ/IL-5, our results indicated that mice immunized with $rOMV_{Nef\text{-}Tat}$ exhibited the highest granzyme B secretion after restimulation with Nef and Nef-Tat antigens, indicating that this regimen is more potent in inducing cellular immune responses especially CTL activity. Furthermore, the regimens containing Nef-Tat protein (G2,G4 and G6) were superior in stimulating the secretion of granzyme B over regimens containing Nef protein (G1,G3 and G5) (Fig. 8A and B).

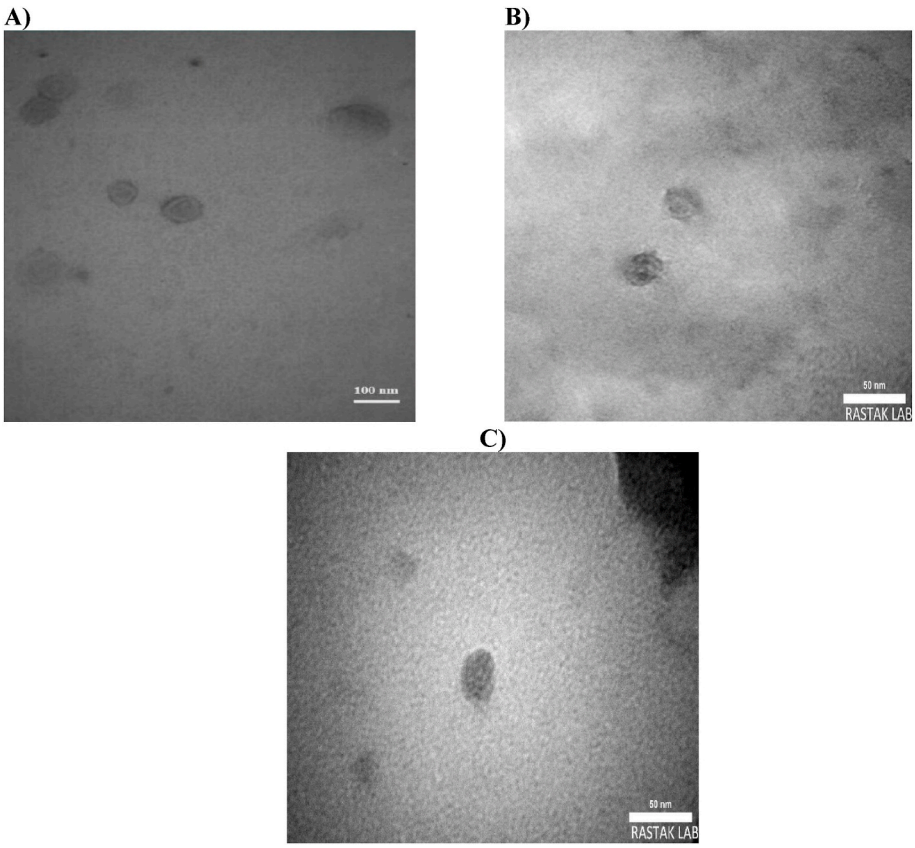

Fig. 5. Morphology and size of OMVs using electron microscopy. TEM analysis of **A.** ClearColi™ OMVs, **B.** $rOMV_{Nef}$, and **C.** $rOMV_{Nef\text{-}Tat}$.

3.8. Cytokine secretion in splenocytes exposed to SCR virions

The pooled splenocytes exposed to SCR virions in all test groups could considerably secrete IFN-γ cytokine in comparison with control group ($p < 0.0001$). Furthermore, our study revealed that the levels of IFN-γ secretion were significantly higher than the levels of IL-5 secretion in all immunized groups ($p < 0.0001$). The rOMV regimens (G5 & G6) exhibited a significant difference in IFN-γ secretion as compared to other groups ($p < 0.001$). In contrast, the level of IL-5 secretion didn't show any significant difference between different groups ($p > 0.05$; Fig. 9).

4. Discussion

Outer membrane vesicles (OMVs) have been recently used as bio-engineered tools in medical applications [35]. Different studies have aimed to apply OMVs as nanovaccine delivery systems [36,37]. For example, two OMV-based vaccine formulations against *Neisseria meningitidis* serogroup b (Bexsero® and VA-MENGOC-BC®) were approved in human [37]. OMVs can safely transport toxins, enzymes and DNA to eukaryotic cells [38]. They are stable at room temperature and thus cost-effective for using in developing countries [39].

The present study marks the initial exploration of *E. coli*-released outer membrane vesicles (OMVs) as immunogenic carriers for delivering HIV-1 Nef and HIV-1 Nef-Tat antigens, while also serving as an adjuvant in the development of an HIV therapeutic candidate. This innovative approach investigates the potential of OMVs to enhance the immune response against HIV antigens, opening new avenues for therapeutic vaccine development against HIV-1 infections. In this regard, the immunogenicity of the OMV regimens as an adjuvant (*i.e.,* OMV + Nef protein and OMV + Nef-Tat protein) was compared with the recombinant OMV regimens as a carrier (*i.e.,* OMV_{Nef} and $OMV_{Nef\text{-}Tat}$). Moreover, the immunostimulatory effects of all OMV regimens were compared with the recombinant proteins in mice. Importantly, *in vitro* cytokine secretion was investigated in mice splenocytes exposed to the safe HIV-1 virions.

The selection of an optimal immunogen is crucial for development of an effective HIV-1 vaccine. Among the HIV-1 proteins, Tat protein is encoded by two exons that are separated by about 2300 nucleotides. Tat activity is often related to the first exon including core and domains rich in proline, cysteine, arginine and glutamine, individually [40]. Moreover, the significance of cell penetrating region of Tat comprising 10 amino acids (aa 48–57) was determined for its entry into the cells [41]. In this study, the first exon of Tat protein was used to increase the immunogenicity of HIV-1 Nef as a main antigen candidate (*i.e.,* a fusion Nef-Tat construct). The studies of Tat-based vaccines showed the capability of Tat for induction of Th-1 type immune responses and CTL activity [42]. Moreover, some studies have focused on HIV-1 Nef, a highly conserved and immunogenic regulatory protein in vaccine design. HVTN 505, HVTN 502, HVTN 503, HVTN 111, IAVI are some clinical trials which have used Nef protein as an immunogen [43]. The reports showed that multicomponent subunit vaccines (*e.g.,* combination of Nef, Tat and gp120 proteins in macaque; or DNA plasmid encoding Env, Nef and Tat in mice) could reduce viral load and protect the animals from a decline in CD4-positive cells [44,45].

In the current study, Nef-Tat was expressed in Rosetta host due to high level of expression and acceptable level of endotoxin similar to many other studies [46–49]. In agreement with our results, in the study of Nesrini et al. the best performance with intense bands was achieved in Rosetta™ stain [50]. Indeed, the HIV-1 Nef-Tat protein could induce higher level of total IgG and IFN-γ in comparison with HIV-1 Nef protein in short-term and long-term analyses. In addition, the Nef-Tat protein induced lower level of IL-5 than the Nef protein. Overall, our results also demonstrated that the linkage of Tat to Nef could confer an advantage in stimulation of immune responses in different vaccination regimens.

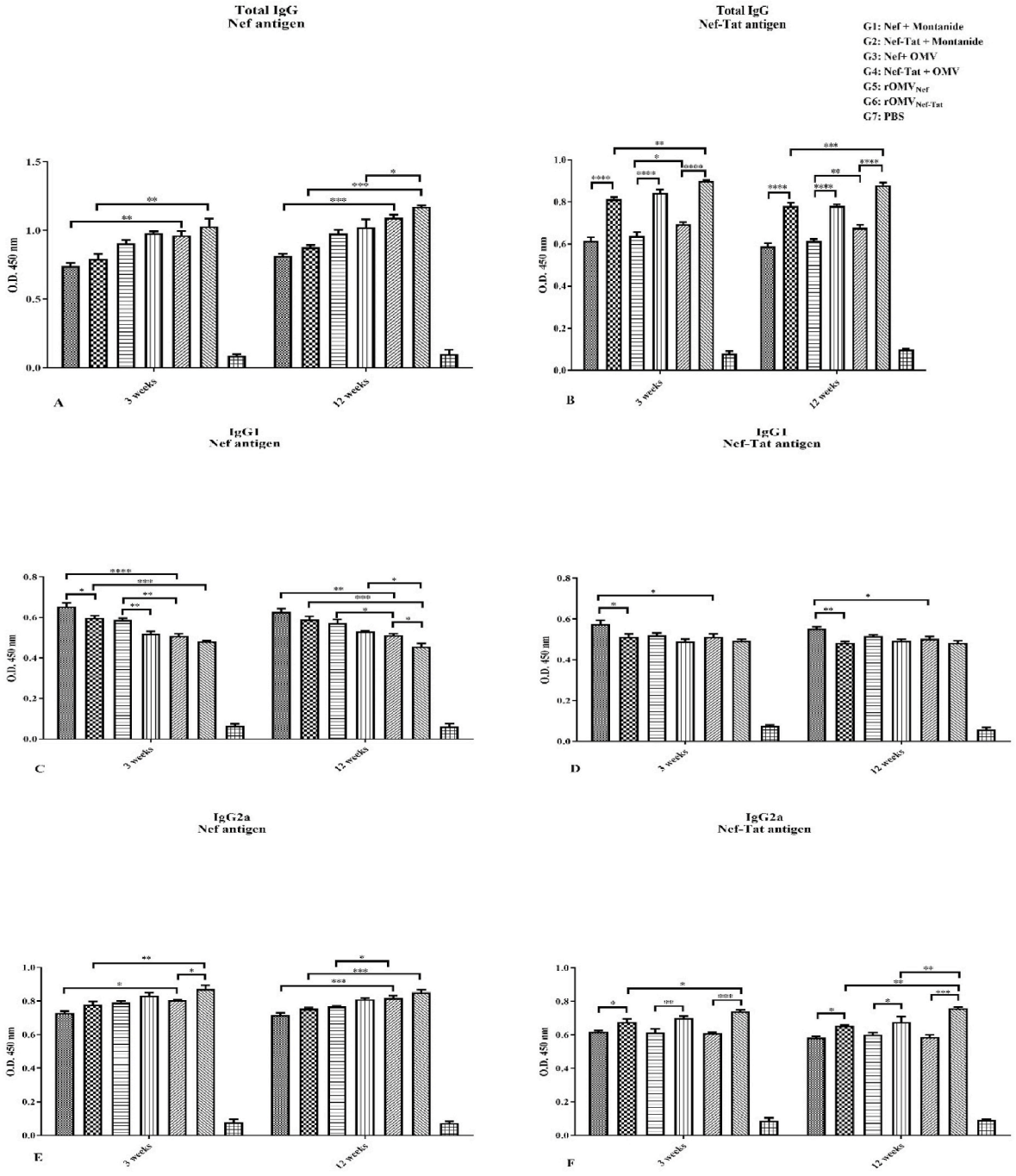

Fig. 6. Evaluation of antibody responses (total IgG, IgG1 and IgG2a) against Nef and Nef-Tat antigens in different regimens. **A.** Total IgG against Nef protein. **B.** Total IgG against Nef-Tat protein. **C.** IgG1 isotype against Nef protein. **D.** IgG1 isotype against Nef-Tat protein. **E.** IgG2a isotype against Nef protein. **F.** IgG2a isotype against Nef-Tat protein. Mice pooled sera were prepared from the whole blood samples of each group at 3 and 12 weeks after the last immunization. All analyses were performed in duplicate for each sample. The results from the 1:100 sera dilutions were shown as mean absorbance at 450 nm ± standard deviation (SD). Significant differences were shown by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, and non-significant difference was shown by ns ($p > 0.05$).

On the other hand, adjuvants activate the innate immune system and provide key signals that regulate the acquired immune response for stimulation of antigen-specific T helper cells [51]. In this study, the Montanide® ISA-720 and OMV were used as an adjuvant. OMV-based vaccines hold great promise. For instance, there are ongoing trials exploring their potential for vaccine development against SARS-CoV-2 infection [52,53]. Moreover, OMVs act as a carrier for the antigen of pathogens and thus elicit immune system. OMVs stimulate the host innate immune system via the activation of toll-like receptors (TLRs) and NOD-like receptors (NLRs) as they contain various PAMPs such as lipoproteins, LPS, and pathogenic DNA fragments [54]. Bioengineered OMV-based vaccines can be developed to treat specific diseases, and these vaccines can be decorated with homologous and heterologous antigens to increase their immunogenicity [55]. In this regard, two OMV-based protein vaccine candidates are currently undergoing in pre-clinical or phase I clinical trials conducted by Quadram Institute Biosciences. Additionally, another OMV-based protein vaccine with a peptide adjuvant is being trialed by Intravacc/Epivax [56]. In a recent study, a novel COVID-19 vaccine platform was designed using rRBD from the S protein of SARS-CoV-2, OMVs from *N. meningitidis*, and

Table 3
The mean ratio of IgG2a/IgG1 in immunized groups at 3 and 12 weeks after the last immunization.

Groups	3 weeks after the last immunization (against Nef)	12 weeks after the last immunization (against Nef)	3 weeks after the last immunization (against Nef-Tat)	12 weeks after the last immunization (against Nef-Tat)
G1	1.11	1.14	1.07	1.05
G2	1.3	1.27	1.31	1.35
G3	1.34	1.33	1.18	1.16
G4	1.6	1.52	1.43	1.37
G5	1.58	1.59	1.19	1.16
G6	1.81	1.86	1.49	1.56

aluminum hydroxide as an adjuvant. Animals immunized with this formulation exhibited an increased IgG and IgA production in sera after 37 days [57].

OMVs act as an adjuvant through the LPS complex, but LPS toxicity concerns require LPS content reduction. Post-production endotoxin removal is necessary, but deficient LPS complexes may reduce immunogenicity. So, balancing low toxicity and high immunogenicity is crucial [58]. In our study, we used ClearColi™, a safe modified strain engineered to eliminate the adverse effects of LPS. ClearColi™ competent cells have a genetically modified LPS (*i.e.,* the incorporation of seven genetic deletions) that does not cause an endotoxic response in human. One additional compensating mutation (msbA148) enables viability in the presence of lipid IV_A [59]. Herein, in accordance with the study of Wei et al. [60], $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$ were isolated using Amicon Ultra centrifugal filter units. Furthermore, in the current study rOMVs and OMVs were isolated 16 h after bacterial incubation. We have previously demonstrated that the bacterial phase and isolation methods affects the size of isolated OMVs [21].

In this study, our results showed that using OMVs as an adjuvant (Nef + OMV: G3 & Nef-Tat + OMV: G4) increased total IgG compared to the Montanide® as an adjuvant (G1: Nef + Montanide® & G2: Nef-Tat + Montanide®). Moreover, OMVs caused a decrease in the level of IgG1 and an increase in IgG2a as compared to the Montanide®. The ratio of

Table 4
The mean ratio of IFN-γ/IL-5 in immunized groups at 3 and 12 weeks after the last immunization.

Groups	3 weeks after the last immunization (restimulated with Nef)	12 weeks after the last immunization (restimulated with Nef)	3 weeks after the last immunization (restimulated with Nef-Tat)	12 weeks after the last immunization (restimulated with Nef-Tat)
G1	4	4.47	4.89	5.03
G2	5.63	6.46	7.15	8.41
G3	6.76	7.69	7.15	9.57
G4	9.08	9.97	21.1	12.98
G5	15.59	17.56	13.24	12.89
G6	21.29	23.42	23.82	23.02

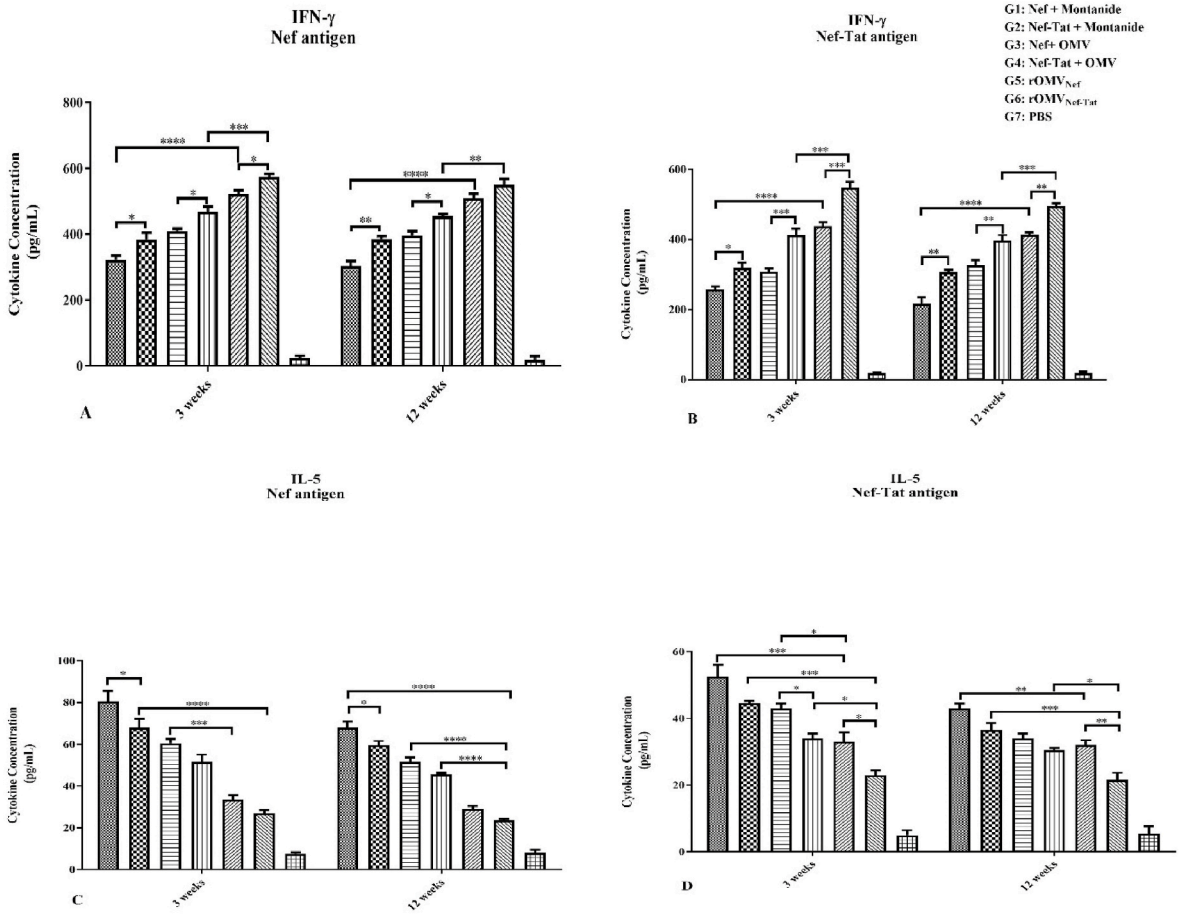

Fig. 7. Evaluation of cytokine secretion in mice immunized with protein, OMV + protein and recombinant OMV in different regimens. **A.** The levels of IFN-γ in splenocytes restimulated with Nef antigen. **B.** The levels of IFN-γ in splenocytes restimulated with Nef-Tat antigen. **C.** The levels of IL-5 in splenocytes restimulated with Nef antigen. **D.** The levels of IL-5 in splenocytes restimulated with Nef-Tat antigen. The levels of cytokines were determined in the cell supernatants using ELISA as mean absorbance at 450 nm ± SD for each set of samples. All analyses were performed in duplicate for each sample. Significant differences were shown by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

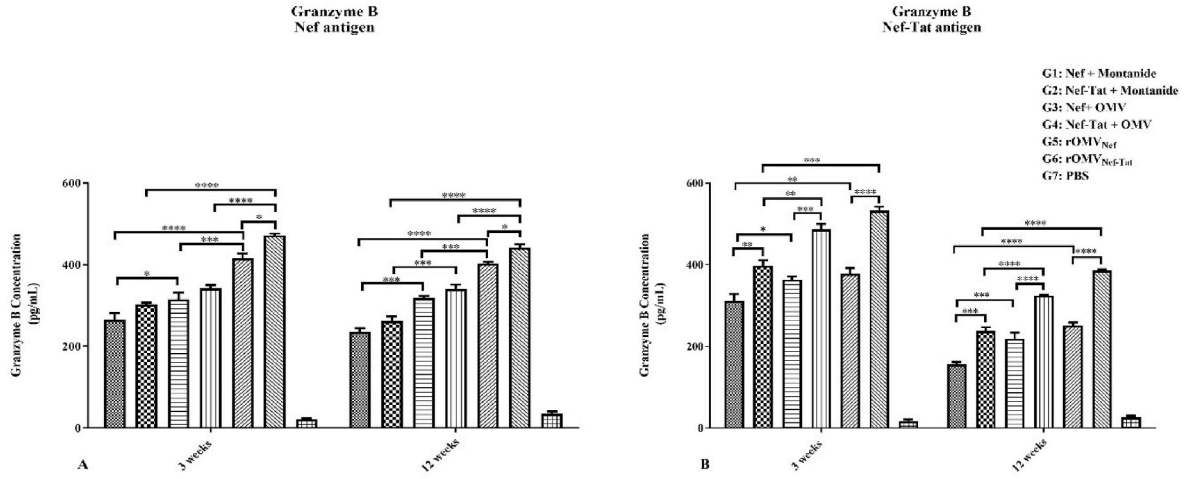

Fig. 8. Evaluation of Granzyme B concentration in mice immunized with protein, OMV + protein and rOMV in different regimens. **A.** The levels of Granzyme B after restimulation with Nef antigen. **B.** The levels of Granzyme B after restimulation with Nef-Tat antigen. The levels of Granzyme B was determined in the supernatants using ELISA as mean absorbance at 450 nm ± SD for each set of samples. All analyses were performed in duplicate for each sample. Significant differences were shown by $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

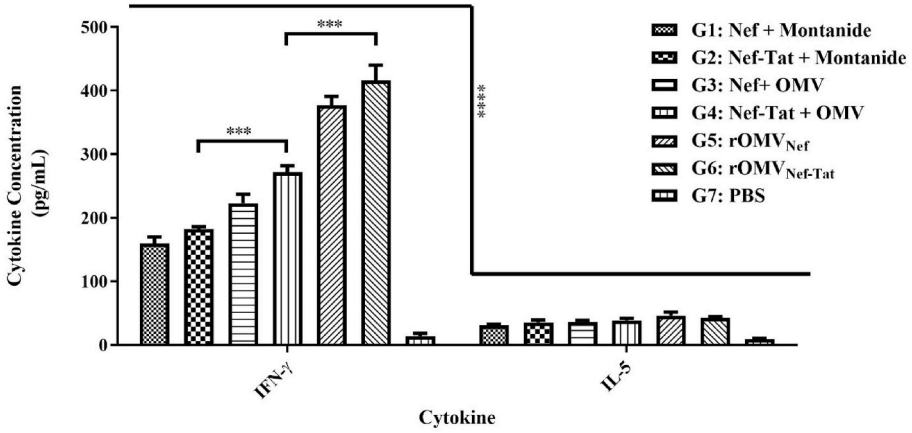

Fig. 9. Evaluation of cytokines secretion by SCR HIV-1-infected lymphocytes in different groups. Significant differences were shown by $^{***}p < 0.001$, $^{****}p < 0.0001$ and non-significant difference was shown by ns ($p > 0.05$).

IgG2a/IgG1 showed that Montanide®-based groups (G1 & G2) were more powerful in inducing Th2 immune responses. In contrast, OMV-based groups (G3-G6) acted as a Th1 inducer. In a recent study, a promising COVID-19 vaccine candidate utilized SARS-CoV-2 dimeric rRBD combined with *Neisseria meningitidis* OMVs adsorbed on alum. The results of immunogenicity in mice showed that RBD-d/OMV/alum regimen was more effective than RBD-d/alum regimen in eliciting IgG2a [61]. Our data indicated that the using OMVs as an adjuvant could increase IFN-γ cytokine compared to Montanide®. In alignment with our findings, higher levels of IgG and IFN-γ were observed when SARS-CoV-2 rRBD was incorporated with OMVs [57].

Lately, researchers have favored the genetic fusion of ClyA [62] that acts as a leading sequence for localization and presentation of functional proteins on the OMV surface [63]. For example, the successful expression and localization of ClyA-M2e fusion protein in OMVs could significantly induce IgG secretion with high IgG2a:IgG1 ratios in BALB/c mice and protect them against a lethal dose of H1N1 influenza A virus [64]. In our study, heterologous antigens were successfully expressed in fusion with ClyA in ClearColi™ host, which reduced endotoxin level by 95 % compared to BL21-expressed protein [65], and on the OMV surface as confirmed by immunoblotting.

Overall, our results showed that the highest secretion of total IgG, IgG2a, IFN-γ and granzyme B was observed in rOMV groups ($rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$) in comparison with other groups. These responses were stable even at 12 weeks after the last immunization. Among rOMV groups, $rOMV_{Nef\text{-}Tat}$ regimen was significantly more efficient in eliciting total IgG, IgG2a, IFN-γ and granzyme B than $rOMV_{Nef}$ regimen indicating the potency of Tat protein. Indeed, $rOMV_{Nef\text{-}Tat}$ regimen could regulate Th1 cellular immunity development and CTL activity (*i.e.,* cellular immune responses). Herein, directing cellular immunity towards Th1 immunity by $rOMV_{Nef\text{-}Tat}$ was determined by evaluating IFN-γ and IL-5, as one of the most characteristic Th1 and Th2 cytokines, respectively. However, it is highly recommended to further investigate the quality of the induced immune responses generated by rOMVs by evaluating the frequency of more antigen-specific cytokine responses in splenocytes of the immunized mice. The analysis of cytokine secretion in splenocytes exposed to SCR virions provided valuable insights into immune responses elicited by different immunization groups, particularly in HIV vaccine research where animal models may not be available. SCR virions, engineered for virology research and HIV vaccine development, undergo only one round of replication, reducing risks compared to wild-type viruses. To generate SCR virions, specific plasmids are transfected into host cells, and resulting recombinant virions are harvested and quantified, providing controlled conditions for *in vitro* experiments [27].

In this study, similar to many studies [32,47,66–69], we employed SCR HIV-1 virions as a model to investigate the immunogenicity and potential therapeutic applications. Our results demonstrated that all immunized groups exhibited a significant increase in the secretion of IFN-γ cytokine compared to the control group. Notably, splenocytes from mice immunized with rOMV regimens (G5 & G6) showed a substantial difference in IFN-γ secretion when exposed to SCR virions as compared to other groups. In contrast, the levels of IL-5 secretion did not exhibit any significant differences between different immunization groups. This data suggested that the immunization strategies employed in this study did not have a notable impact on the production of IL-5, a cytokine associated with Th2 responses. These findings highlight the potency of $rOMV_{Nef\text{-}Tat}$ in inducing cellular immune responses. However, further investigation into the specific mechanisms underlying these immune responses will be valuable for developing more effective therapeutic vaccines against HIV-1 and other infections. It is also essential to study the safety and immunogenicity of this HIV-1 therapeutic vaccine candidate in animal models and evaluate the induced immune responses. It is also highly suggested to evaluate the efficacy of this HIV-1 therapeutic vaccine candidate against HIV challenge in humanized mouse models [70] or macaques [71] in future studies.

In summary, our data showed that the incorporation of HIV-1 Tat protein significantly enhanced both cellular and humoral immune responses. Furthermore, the rOMVs as an antigen carrier ($rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$) induced more robust humoral and cellular immune responses than the OMVs as an adjuvant (Nef protein + OMV and Nef-Tat protein + OMV) and the recombinant proteins emulsified with Montanide® 720 as an adjuvant (Nef + Montanide® and Nef-Tat + Montanide®). Moreover, the potency of the rOMVs regimens was higher than other groups after exposure to safe virions by high IFN-γ secretion. Generally, the rOMVs could effectively present antigens on their surface resulting in significantly higher antigen-specific immune responses than antigens mixed with OMVs as an adjuvant. However, as ClyA can enhance the antigen immunogenicity [25], it is highly suggested to compare $rOMV_{Nef}$ and $rOMV_{Nef\text{-}Tat}$ with Nef-ClyA and Nef-Tat-ClyA fusion proteins or their mixture with OMVs in our future studies. Our promising findings show the potential of recombinant OMVs as a viable candidate for development of an HIV-1 therapeutic vaccine.

Ethics approval and consent to participate

The animals were maintained under specific pathogen-free conditions based on the guidelines of the Pasteur Institute of Iran. The study was approved by the local ethical committee (ethical code: IR.PII.REC.1400.009) for scientific purposes.

Consent for publication

Not applicable.

Consent to participate

Not applicable.

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Data availability

All data are available in the manuscript.

CRediT authorship contribution statement

Leila Sadeghi: Writing – original draft, Project administration, Methodology, Investigation. **Azam Bolhassani:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Elham Mohit:** Writing – review & editing, Resources, Project administration, Conceptualization. **Kazem Baesi:** Formal analysis, Data curation. **Mohammad Reza Aghasadeghi:** Formal analysis, Data curation. **Alireza Milani:** Software, Formal analysis, Data curation. **Elnaz Agi:** Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2024.106749.

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