Evaluation of Antibody and Cytokines Responses in Intranasally and Intramuscularly Administrated BALB/C Mice With Influenza Virus-Like Particle

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Abstract- We previously developed an influenza virus like particle with HA, M, and NA proteins using Bac-to-Bac expression system and SF9 cell line. To evaluate the immunogenicity of our construct, we assessed the humoral, cytokine induced by H1N1-VLP in BALB/c mice immunized intranasally and intramuscularly. Enzyme-linked immunosorbent assay and Relative quantitative real-time PCR were used to evaluate the antibody (IgG and IgA) and mRNA levels of IL-6, IL-4, IL-10 and IFN-g in PBMCs. Our results showed that VLP was capable of intranasal (I.N.) and intramuscular (I.M.) induction of serum IgG and IgA responses. Interestingly, I.N. route induced higher IgG and IgA titer than I.M. route, which was statistically significant. Moreover, mRNA levels of IL-6 (4.2-4.5 folds), IFN-g (5.5-5.7 folds), and the anti-inflammatory cytokine IL-10 (2.5-3 folds) and IL-4 (2.4-2.8 folds) were significantly elevated in mice immunized I.N. and I.M. with H1N1-VLP compared to the control group. Our findings indicated that a non-infectious genome-less VLP approach mimics parenteral virus with multiple viral antigens and epitopes that stimulate a diverse set of immune responses such as innate immunity, specific serum IgG antibody, cell-mediated immunity, and local antibodies.

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Introduction

Influenza A virus, a major causative pathogen of acute respiratory diseases worldwide, are estimated to cause more than 36000 annual death and over 200000 hospitalizations particularly in high-risk groups such as the elderly (1-2). Vaccination represents a highly effective approach to prevent the seasonal or pandemic outbreak of influenza (3-4). However, lessons learned from the 2009 H1N1 pandemic indicated that conventional vaccination seems to have considerable delay in controlling the recent pandemic spreads as result of the emerged strains produced by natural reassortment (1,5-8) and due to poor growth in embryonated chicken egg substrates compared to those observed with seasonal vaccines (1). Novel approaches are, indeed, needed to develop an effective influenza vaccine that can be rapidly produced on a large scale and with a low production cost (9). Recombinant non-infectious virus-like particles (VLPs), structurally naive and immunologically relevant viral antigens, seems to be a promising approach to produce bulk, safe, and low-cost influenza virus vaccines (4). Indeed, influenza VLP vaccines were shown to be more immunogenic and to provide better protection than a commercial split vaccine in ferrets (10) or a soluble hemagglutinin (HA) protein vaccine (11), indicating the capability of influenza VLPs as a new vaccine platform (12-13). Administration of VLPs by different routes, intranasal (I.N.), intramuscular and parenteral has been investigated to induced cellular and humoral immune responses. (4,14-19). Unlike parenteral immunization which stimulates systemic immune responses, mucosal vaccination has been shown to promote both local and systemic immunity. Given the fact that the respiratory mucosa is the initial line of defense against influenza, intranasal immunization offers an attractive route for

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vaccination against this pathogen (20). Several important groups of immune molecules, e.g., cytokines, chemokines and adhesion molecules involved in immune response after influenza virus infection and also the administration of commercial flu vaccines. Until now, few data are available regarding the expression of immune biomarkers induced by Influenza virus VLPs. In this study, we investigated the immunogenicity, protective efficacy, and immune biomarkers profile following influenza VLPs vaccination of mice.

Materials and Methods

VLP production and characterization

Construction of recombinant baculoviruses (rBVs) expressing H1N1 (PR8) VLP and VLPs characterization determined, as previously described (Rezaei *et al*., 2013). In brief, to produce VLPs containing influenza H1, N1, and M1, the Sf9 insect cells were seeded at a density of 2×10^7 per flask and infected with rBV expressing the H1, N1, and M1 proteins at multiplicity of infection (MOI) 3. Three days post-infection, supernatants containing the VLPs were harvested, clarified by low-speed centrifugation (2000 rpm for 20 min at 4° C), and then purified by ultracentrifugation sedimentation through 25% sucrose cushion at 27,000 rpm for 75 min. The Purified VLPs were finally resuspended in PBS at 4° C overnight. The HA incorporated into VLPs was initially determined by Western blot analysis and Electron Microscope as described elsewhere (Rezaei *et al*., 2013).

Hemagglutination assay

Suspensions of purified H1N1 VLPs and influenza virus A/PR8 with concentration of 1 mg/ml were prepared in PBS. Two-fold Serial dilutions of 1 µg of each were prepared in 96-well plates and incubated at room temperature for 30-60 min with 0.5% guinea pig red blood cells. The HA titer was finally inspected visually, and the highest dilution capable of agglutinating red blood cells was determined.

Immunization, challenges, and evaluation of humoral immune responses

Female BALB/c mice (12 per group) aged 6-8 weeks were used in this study. 12 female BALB/c mice per group were immunized intranasally (I.N.) and intramuscularly (I.M.) with 10 ug of H1N1 VLP per dose two-times on days 0 and 14. Control group of BALB/c mice only received PBS. Blood samples were taken before the primary and after booster inoculations by retro-orbital plexus puncture at days 0 and 28. Then, sera were collected and serum antibodies were measured using HAI and ELISA. Before virus challenge, serum samples were collected and tested by ELISA for the presence of anti HA-specific IgG antibody. To evaluate the IgA antibody and viral load in the lung, the bronchoalveolar lavage (BAL) of vaccinated mice was collected 14 days after booster inoculation. For challenge studies, immunized mice were isoflurane-anesthetized and infected intranasally with a lethal dose (10 LD50) of A/PR/8 in 50ul of PBS. Mice were daily monitored to record body weight changes as well as mortality. On day 14 post-challenge, mice were injected with 100 mg/kg intraperitoneal ketamine and sacrificed. (All experiments were approved and performed according to guidelines of the Animal Care and Use Committee from the Tehran University of Medical Sciences).

Hemagglutination inhibition assays

Serum samples were treated with receptor destroying enzyme (RDE) prior to being tested at a final dilution of 1:10. Sera were serially diluted 2-fold in microtiter plates. An equal volume of A/PR8 and A/HK viruses adjusted to approximately 4 HAU/25 ul were added to each well, separately. The plates were incubated at room temperature for 60 min before adding 0.5% guinea pig erythrocytes in PBS. Then, they were mixed by agitation and left for 1 h at 25˚ C to allow the RBCs settle. The reciprocal of the last dilution which contained non-agglutinated RBC was considered as HAI titer. Positive and negative serum controls were included in each plate.

Real-time PCR gene expression assay

RNA was purified from PBMCs using TRIzol (Invitrogen Cat. No. 15596026) and converted to cDNA using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Target mRNA was relatively quantified (RQ) by real-time PCR assay using specific primer sets and the Universal PCR Master Mix system (Applied Biosystems). Table 1 indicates the primer sets for mouse mRNA targets (IL-6, IL-4, IL-10, and IFN-g). All primers were designed to span exons and to avoid reaction with genomic DNA. Samples were assayed with the Stepone plus Real-Time PCR System (Applied Biosystems) and analyzed using LinRegPCR (www.hartfaalcentrum.nl), Ct values were normalized to β-actin mRNA and expressed as fold change relative to the mean Ct value for the control samples using the Ct method.

Table 1. Primer sequences of target genes and reference gene used for relative quantitative real-time RT-PCR

Target genes	Primer sequence	Amplicon size (bp)
IL-6	Forward:5'-TTCCATCCAGTTGCCTTCTTG-3' Reverse:5'-GGGAGTGGTATCCTCTGTGAAGTC-3'	99
IFN-g	Forward:5'-CCTGCGGCCTAGCTCTGA-3' Reverse:5'-TGGCAGTAACAGCCAGAAACA-3'	91
IL-10	Forward:5'-GGCAGCCTTGCAGAAAAGAG-3' Reverse:5'-GCTGATCCTCATGCCAGTCA-3'	86
IL-4	Forward:5'-GCCACCATGAGAAGGACACT-3' Reverse:5'-ACTCTGGTTGGCTTCCTTCA-3'	153
Reference gene		
β-actin	Forward:5'-GCTCTGGCTCCTAGCACCAT-3' Reverse:5'-GCCACCGATCCACACAGAGT-3'	76

Statistical analysis

GraphPad Prism software (V7.0) was used to compare and analyze data. Statistical analysis of data was performed using the one way-ANOVA. Data are expressed as mean±SD, and *P* less than 0.05 were considered to be statistically significant.

Results

Hemagglutination assay

Presence and functionality of HA protein in VLP were evaluated by hemagglutination assay. The HA titers were 512 and 1024 (HA unit) per 1 µg of VLPs and homologous virus, respectively. We found that the HA titer of VLP was approximately two-fold lower than homologous influenza virion. As indicated in previously published data, the content of HA in influenza virion is estimated to be 29% of total protein. Accordingly, influenza virus contains 2.9 ug HA per 10 ug total protein virion; therefore, our generated VLPs were estimated to contain 1.8 ug HA per 10 ug of total VLPs protein (data are not shown) (4,19,21-22).

Hemagglutination inhibition assay

For influenza vaccines, the induction of antibodies with hemagglutination-inhibition (HI) capacity correlates with their protective efficacy (Table 2). Antibodies elicited by the VLP vaccine were evaluated for the ability of preventing virus-induced agglutination of guinea pig RBCs. We determined the HI activity of immunized mouse sera against A/PR8 virus. As shown in Figure 1, the HI responses to A/PR8 virus in intramuscularly (IM) immunized mice were at a mean titer of 160, while the responses to the intranasally (I.N) immunized group was 453. The HI responses to A/PR8 virus at PBS-control were negative (HAI≤10). The mean titer of HI responses to A/PR8 in the intranasally immunized group was also significantly higher than that of intramuscularly immunized group ($P \leq 0.005$).

Table 2. Mouse mortality following H1N1-VLP vaccination and lethal H1N1 (A/PR8) virus challenge

Group	No. of mice	No. that survived virus challenge (% mortality)	MTD [b] (days post-challenge)	Serum HI antibody GMT prior to virus challenge
VLP I.N.	4	4 (0)	NA[a]	453
VLP I.M.	4	4 (0)	NA[a]	160
Infection control	4	0 (100)	8	< 20

[a] NA, not applicable
[b] Mean Time to Death

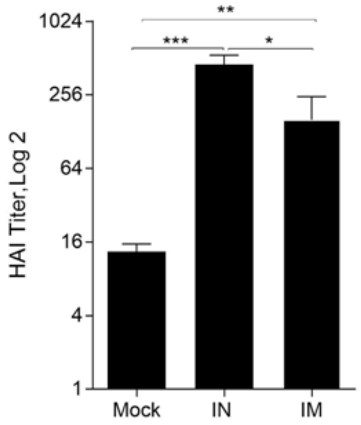

Figure 1. Hemagglutination inhibition (HI) titers against A/PR8 virus. HI, titers of immune sera were determined as the capacity of sera to inhibit virus hemagglutination of guinea pig red blood cells. Representative data are the geometric mean±S.D

Influenza VLPs elicit high levels of systemic and mucosal antibody responses

To determine if the immunization with influenza VLPs induced enhanced humoral responses, we used ELISA to evaluate the endpoint titers of influenza-specific IgG in sera and IgA in BAL of vaccinated mice. As indicated in (Figure 2), Both intramuscularly and intranasally immunized mice groups that received influenza VLPs showed high levels of IgG specific to A/PR8 virus (*P≤0.05* and *P≤0.0005*) compared to PBS-control group. However, looking at IgA antibodies, we also found a significant increase (*P≤0.0001*) in I.N. vaccinated mice than I.M. group. We aimed to determine if the immunization routes induced different level of antibodies. Indeed, higher levels of specific IgG and IgA antibodies (as shown in Figure 2), were found to be induced in the mice immunized intranasally than those immunized intramuscularly (*P≤0.05 and P≤0.0001*).

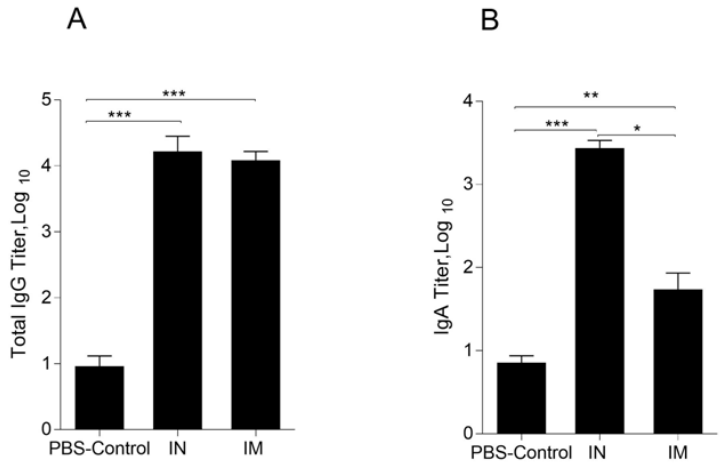

Figure 2. Serum IgG and mucosal IgA endpoint titers. Endpoint titers of immune serum IgG (A) and mucosal IgA (B) specific to A/PR8 were determined using ELISA as described in Materials and Methods

H1N1-VLP elicits protection against lethal virus challenge

To determine whether immunized mice are protected against a lethal challenge, all group were challenged with lethal doses (10 LD50) of A/PR8 except PBS-control group. As illustrated in (Figure 3B), all immunized mice

were protected and survived from lethal virus challenges with A/PR8, in as much as none of them showed signs of illness or weight loss. By contrast, marked signs of infection and illness were found in control group at 3 days post-challenge infection. Concurrently mice in this group showed significant weight loss ($P \leq 0.05$), and by day eight they lost approximately 25% of their original body weight.

Lung viral loads are shown to be important measures of decreased morbidity and mortality after infection. Therefore, on day three and following challenge with the PR8 virus, we determined the virus loads in the lungs of immunized mice. As shown in (Figure 3A), H1N1-VLPs in all immunized mice were significantly effective ($P \leq 0.0001$) in clearance of A/PR8 virus in comparison to infection control group. In addition, there was no significant difference between I.N. and I.M. immunized group in terms of virus clearance ($P \geq 0.05$).

Two groups of mice were vaccinated by the i.n. or i.m. routes with a total amount of 10 ug of H1N1-VLP per dose two times on days 0 and 14. PBS, and infection control groups received the same volume of PBS i.n. Twenty-eight days following the administration of two doses of H1N1VLPs or PBS, all mice except PBS-control group were challenged i.n. with 10 LD50 of the A/PR8 (H1N1) virus and monitored daily for signs of illness. The mice were weighed for 14 days (B), and virus titers were determined after lethal virus challenge (A). Infection control mice all succumbed to infection by day 8 post-challenge. Differences in body weight loss and virus titers between vaccination groups were analyzed by ANOVA ($P \leq 0.05$ and $P \leq 0.0001$).

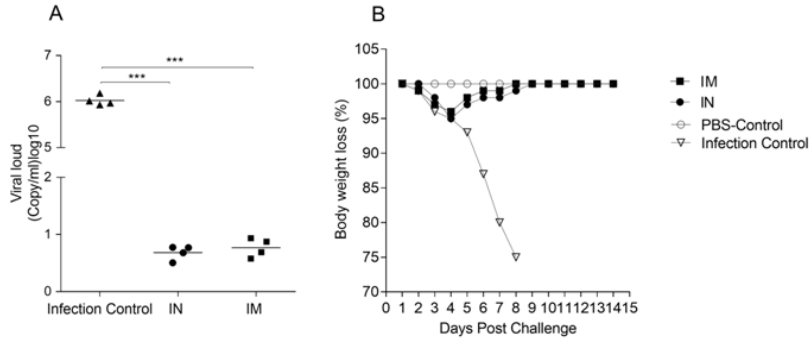

Figure 3. Mouse morbidity following H1N1-VLP vaccination and a H1N1 (A/PR8) virus challenge. Forty-eight mice were divided into four experimental groups (12 mice per group)

Immune biomarkers relative mRNA level after VLP administration

Three days after booster immunization, four mice were sacrificed. Then PBMCs were used for biomarkers profile investigation. As previously described, prior to assessing the relative expression levels of target genes the linearity and accuracy of the Real-Time PCR were confirmed. For the purpose of this study, we assessed the mRNA expression levels of a panel of immune biomarkers, including IL-6, IL-4, IL-10, and IFN-g. As seen in (Figure 4), relative mRNA expression levels of proinflammatory cytokines (IL-6, mean 4.2-4.5-folds, $P \leq 0.0005$; IFN-g, 5.5-5.7 folds, $P \leq 0.0005$), anti-inflammatory cytokine IL-10 (2.5-3 folds, $P \leq 0.05$) and IL4 (2.4-2.8 folds, $P \leq 0.05$ and 0.005) were significantly upregulated in I.N. and I.M. groups following immunization in comparison with PBS-control group.

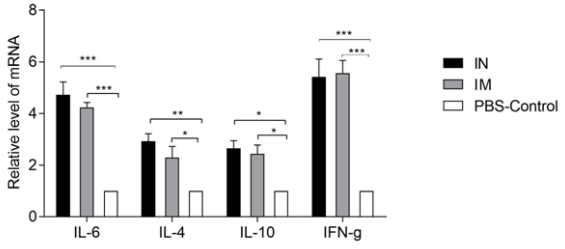

Figure 4. The gene expression levels of cytokines from PBMC. The bars (+SEM) shows the fold increase in gene expression in the I.N. (black), I.M. (gray) and PBS-control (white) groups after H1N1-VLP vaccination

Discussion

Recently, non-living vaccines, particularly VLPs-based vaccines, have provided the most promising results. In line with this, a wide range of homologous and heterologous VLPs has been developed. In the present study, we tested the immunogenicity and protective efficacy of H1N1VLPs after two doses intranasally and intramuscular vaccination and investigated the mRNA levels of cytokines using relative quantitative real-time PCR.

The results of hemagglutination assay (HA) test showed that generated VLPs retained their functional activities. Indeed, comparison of HA titers of VLPs with homologs virus (A/PR8) illustrated that virus-induced HA was two-fold higher than that by VLP. This is consistent with the previous studies where the lower HA titer was due to the lower amounts of hemagglutinin protein in the context of VLPs (4,19,21-22). In agreement with previous studies, vaccinated mice with influenza VLPs induced high levels of antibody responses conferred highly effective protection against lethal influenza virus challenge, as viral replication in the lung was reduced to levels below the detection limit (16,19,21,23-27). The functional activities of the antibody responses were determined by an HAI assay, and as shown in (Figure 1), the HAI titers of sera from vaccinated mice were about 1:453 in I.N. and 1/160 in I.M. groups and were significantly higher than PBS-control group ($P \leq 0.05$ and $P \leq 0.0005$). Of note, the mice which received two doses of influenza VLPs had high level of serum HAI titers and were completely protected against influenza virus challenge with no significant weight loss and mortality (Figure 3B and Table 2). This is similar to the results from several early studies that investigated the immunogenicity of different influenza VLPs approaches in mice (4,23-28). We also observed that IgG antibody responses induced by influenza VLPs were significantly higher in both vaccinated groups (4-log, $P \leq 0.01$ and $P \leq 0.0001$) than PBS-control. However, the pattern of mucosal IgA antibody significantly ($P \leq 0.0001$) rose in I.N. vaccinated group (Figure 2). We compared the traditional parenteral route (I.M) and a mucosal route of vaccination. Characterization of the post-vaccination antibody responses identified differences between the two routes of vaccination. ELISAs and HAI revealed that mucosal (I.N.) vaccination was clearly superior to parenteral vaccination for the induction of protective neutralizing antibodies. In 2001, Terrence M. Tumpey *et al.*, documented the immunogenicity and protective efficacy of inactivated influenza vaccine administered intranasally to mice (22). Moreover, mucosal (I.N. routes) administered inactivated influenza vaccine has been indicated to elicit both serum and mucosal antibodies and conferred high level of antibodies and protection against virus challenge (22). Similar finding indicated mice protection following immunization with VLPs through I.N. and I.M. routes (29). Our findings were in agreement with previous studies suggesting VLPs can induce IgG responses through both the I.N. and I.M. routes, although I.N. route displayed more efficient responses which provides further evidence that VLPs are immunogenic. We also demonstrated that I.N. route induced higher IgA response than I.M. route in the mucosal tract. IgG and IgA antibodies are very important to fight influenza virus after lung infection. However, IgA antibody may contribute to heterotypic immunity by its ability to pass through the lung epithelium and, during this transcytosis, interfere with the production of viral proteins in an infected cell (30-33). Previous studies in mice showed mucosal but not parenteral vaccination induced subtype to cross reactive lung anti-HA antibodies against heterologous viruses (22,30-33). Mucosal vaccination may be more important than parenteral immunization in delivering viral antigens to the related anatomical site, specifically to dendritic cells in the respiratory tissue; which plays a critical role in communicating with T and B cells to develop immunological memory against influenza viruses (33-34).

Evaluation of cytokine responses after vaccination may provide a valuable tool and will be an appropriate measurement of the efficacy and safety of the vaccine. Cytokines are important molecules that facilitate the communication between immune cells and the surrounding tissue. Additionaly, they play a critical role in shaping the intensity and direction of the immune response (35). As illustrated in (Figure 4), we evaluated immune biomarker profiles after booster immunization. The gene expression of all four cytokines (IL-6, IL-4, IL-10 and IFN-g) was elevated after vaccination. The cytokine profile exhibited an increase in IFN-g, IL-10, IL-4 and IL-6 cytokines, respectively. It is likely that activated T-Helper cells activate B cells and the interaction between the T-Helper and B cells allows B-cells to differentiate into plasma cells and memory cells (30-35).

In conclusion, our findings indicated that a non-infectious genome-less VLP approach mimics parenteral virus with multiple viral antigens and epitopes that

stimulate a diverse set of immune responses such as innate immunity, specific serum IgG antibody, cell-mediated immunity and local antibodies with less reactogenicity associated with a live-attenuated or whole inactivated virus.

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