Antiviral Research 95 (2012) 229–236

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Influenza virosome/DNA vaccine complex as a new formulation to induce intra-subtypic protection against influenza virus challenge

Masoumeh Tavassoti Kheiri [a], Abbas Jamali [a,*], Mohammad Shenagari [b], Hamidreza Hashemi [c], Farzaneh Sabahi [d], Fatemeh Atyabi [e], Reza Saghiri [f]

[a] Influenza Research Laboratory, Department of Virology, Pasteur Institute of Iran, Tehran, Iran
[b] Department of Microbiology, Faculty of Medicine, Guilan University of Medical Sciences, Rasht, Iran
[c] Department of Medical Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[d] Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[e] Medical Nanotechnology Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[f] Department of Biochemistry, Pasteur Institute of Iran, Tehran, Iran

ARTICLE INFO

Article history:
Received 4 April 2012
Revised 4 July 2012
Accepted 7 July 2012
Available online 15 July 2012

Keywords:
Virosomal influenza vaccine
Virosome/DNA complex
Nucleoprotein
Antigenic drift

ABSTRACT

Influenza virosome is one of the commercially available vaccines that have been used for a number of years. Like other influenza vaccines, the efficacy of the virosomal vaccine is significantly compromised when circulating viruses do not have a good match with vaccine strains due to antigenic drift or less frequent emergence of a pandemic virus. A major advantage of virosome over other influenza vaccine platforms is its intrinsic adjuvant activity and potential carrier capability which have been exploited in this study to broaden vaccine protectivity by incorporating a conserved component of influenza virus in seasonal vaccine formulation. Influenza nucleoprotein (NP)-encoding plasmid was adsorbed onto surface of influenza virosomes as a virosome/DNA vaccine complex. Mice were immunized with a single dose of the influenza virosome attached with the NP plasmid or NP plasmid alone where both influenza virosomes and NP gene were derived from influenza A virus H1N1 New/Caledonia strain. Analysis of the cellular immune responses showed that 5 μg (10-fold reduced dose) of the NP plasmid attached to the virosomes induced T cell responses equivalent to those elicited by 50 μg of NP plasmid alone as assessed by IFN-γ and granzyme B ELISPOT. Furthermore, the influenza virosome/NP plasmid complex protected mice against intra-subtypic challenge with the mouse adapted H1N1 PR8 virus, while mice immunized with the virosome alone did not survive. Results of hemagglutination inhibition test showed that the observed intra-subtypic cross-protection could not be attributed to neutralizing antibodies. These findings suggest that influenza virosomes could be equipped with an NP-encoding plasmid in a dose-sparing fashion to elicit anti-influenza cytotoxic immune responses and broaden the vaccine coverage against antigenic drift.

1. Introduction

Influenza virus causes serious morbidity and mortality in human populations (Nicholson et al., 2003; Palese, 2004; Stohr et al., 2006). During seasonal epidemics, 5–15% of the worldwide population is typically infected, resulting in 3–5 million cases of severe illness and up to 500,000 deaths per year (Stohr et al., 2006). Vaccination is the most effective means for the prevention of influenza virus infection (Rimmelzwaan and Osterhaus, 2001). Current licensed influenza vaccine strategies are based upon induction of neutralizing antibodies mainly against haemagglutinin and include whole-inactivated virus, split virus, subunit and virosomal vaccines (Geeraedts and Huckriede, 2011; Herzog et al., 2009). Virosomal influenza vaccines have been on the European market for over 11 years, and more than 41 million doses have been sold (Herzog et al., 2009). Virosome is a reconstituted envelope of the influenza virus devoid of viral genetic material that preserve the receptor-binding and membrane fusion activity of the viral haemagglutinin (Stegmann et al., 1987). Virosomal vaccines have been shown to induce antibody titers comparable to the whole inactivated or subunit vaccines. Moreover, they appear to induce a long-term sero-protection rate in the elderly (Zamparo and Little, 2011).

Efficacy of the virosomal influenza vaccine like other available influenza vaccine formulations is significantly compromised when circulating viruses do not have a good match with vaccine strains

* Corresponding author. Address: Influenza Research Laboratory, Department of Virology, Pasteur Institute of Iran, P.O. Box 1316943551, Tehran, Iran.
E-mail address: jamali@pasteur.ac.ir (A. Jamali).

0166-3542/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2012.07.003

due to antigenic drift or inaccurate epidemiological predictions. Antigenic drift is the gradual evolution of viral strains due to frequent mutations (Both et al., 1983). Most of these mutations are 'neutral' as they do not affect the conformation of the proteins; however, some mutations cause changes to the viral proteins such that the binding of host neutralizing antibodies is compromised. Influenza virus strains that are not matched with the seasonal vaccine circulate on a regular basis and can have a significant impact on vaccine effectiveness (Carrat and Flahault, 2007). Indeed it has been suggested that approximately once every decade the mismatch between virus and vaccine is enough to reduce vaccine effectiveness by 70% (Carrat and Flahault, 2007). Consequently, infecting viruses can no longer be inhibited effectively by host antibodies raised against the previously circulating strains, allowing the virus to spread more rapidly among the population by escape selection from the adaptive immune response (Webby and Webster, 2001). To circumvent this problem, many studies have been conducted against more conserved influenza virus proteins. These vaccines have been shown to protect against multiple influenza A virus subtypes in animal models and are promising complements or alternatives for the currently used HA-based, strain-specific vaccines (Hillaire et al., 2011; Stanekova and Vareckova, 2011). DNA plasmids (pDNA) expressing conserved influenza proteins especially nucleoprotein (NP) elicit T-cell responses and provide cross-protection against lethal challenge in mice. However, the initial clinical results using naked pDNA immunization have not been promising in large animals and humans (Ulmer et al., 1993, 1998). A major drawback in DNA vaccines development is the need for relatively high doses of pDNA to elicit minimal responses (Liu, 2011). Hence, there has been an ongoing challenge to find new strategies to improve potency of pDNA immunogens.

Interestingly, influenza virosomes appear to be a flexible platform that is particularly suited to resolve these drawbacks of DNA vaccines (Daemen et al., 2005). Adjuvant property of influenza virosomes is related to strong capacity of virosomes to induce maturation of DCs and trigger the secretion of various cytokines such as TNF-α, IFN-γ and IL-12 (Gluck et al., 2004; Moser et al., 2003). In this study, we introduce an alternative vaccine formulation composed of influenza virosome/NP pDNA complex to broaden immunogenicity and protectivity of currently used seasonal flu vaccines.

2. Material and methods

2.1. Construction of NP-expressing plasmid

PcDNA3 plasmid encoding influenza virus NP gene was constructed and characterized as described before (Jamali et al., 2010). Briefly, NP gene was amplified by RT–PCR from influenza A/New Caledonia/20/99 H1N1 virus (hereafter called NC virus) and cloned into pcDNA3 plasmid. Expression of NP was analyzed by immunoflourescence staining. pcDNA3–NP was amplified in *Escherichia coli* DH5-α and purified using Endo-Free Mega kit (Qiagen, USA).

2.2. Formulation of influenza virosome/NP pDNA complex

Influenza virosomes with pDNA attached to the surface were prepared from NC virus as described by Schoen et al. (1999) and modified by de Jonge et al. (2007). Briefly, the influenza viral membrane was solubilized with detergent 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DCPC) followed by reconstitution after removal of DCPC by dialysis. Cationic lipid 1,2-dioleoyl-3-trimethyl-ammoniumpropane (DOTAP, Avanti Polar Lipids, Inc., USA) was added 34% relative to total lipid and 43% relative to viral lipid prior to detergent removal. For the binding of NP pDNA to the surface of the virosome, 5 μg of pDNA was incubated with 20 nmol of virosomal phospholipids at room temperature for 30 min. This molar ratio of NP pDNA:virosome corresponds to the saturation capacity of the virosome as described previously (Schoen et al., 1999).

2.3. NP pDNA transfection by influenza virosomes

Carrier potential of influenza virosomes was evaluated *in vitro* by transient transfection of NP pDNA into BHK-21 cells and subsequent immunofluorescence staining using specific antibodies. BHK-21 cells were transfected with 1 μg of the pcDNA3–NP or pcDNA3 empty vector loaded on 4 nmol of influenza virosomes. As positive control, 1 μg of NP pDNA was transfected by Lipofectamine 2000® (Invitrogen) according to manufacturer's instruction. Two days post-transfection, the cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS). Next, the cells were treated with Triton X-100 and then anti-NP monoclonal antibody (Ab) (Serotec, USA), followed by incubation with anti-mouse IgG–rhodamine conjugate (Invitrogen, USA).

2.4. Immunization schedule

Six to eight-week old female BALB/c mice were obtained from the animal facilities of Pasteur Institute of Iran (Karaj, Iran). Mice were housed for 1 week before the experiment, given free access to food and water, and maintained on a 12-h light/12-h dark cycle. All experiments were performed according to the Animal Care and Use Protocol of the Pasteur institute of Iran. Mice were divided into five groups and immunized once intradermally. Group I and II were inoculated with 5 and 50 μg of pDNA–NP, hereafter referred to as pNP-5 and pNP-50 respectively. Group III received pDNA/virosome complex in which 5 μg of pDNA–NP was attached to the surface of influenza virosomes (containing 15 μg HA) and group IV injected with virosomes without NP–pDNA, hereafter referred to as V-NP and V-only respectively. As negative control, mice were immunized with PBS.

2.5. Virus challenge

For challenge experiments, three weeks after immunization, the mice were slightly anesthetized with a mixture of Ketamine/Xylazine (1.98 and 0.198 mg per mouse, respectively) and challenged intranasally with 4 LD_{50} of influenza viruses in 50 μl PBS. The mouse-adapted strains used in this study included PR8 [A/Puerto Rico/8/34 (H1N1)] and NC. PR8 virus was a kind gift from Prof. Anke Hueckride, University of Groningen, Netherlands. NC virus was adapted to mice by sequential lung-to-lung passages of the virus in six to eight week-old BALB/c mice. Briefly, female 6-week-old BALB/c mice were inoculated intranasally under light anesthesia with 50 μl of NC virus. Lungs were harvested after 3–4 days; homogenized and 50 μl of the clarified homogenate was used as the inoculum for the next passage. After a total of 10 passages, filtered mouse lung homogenates served as the viral challenge stocks. Adaptation process was verified by rapid and widespread viral replication in the lungs and death of the unimmunized mice in 6–8 days. Mortality rate and weight changes were recorded regularly for 2 weeks after challenge.

2.6. Lung virus titration

The virus titers were measured in mice lungs 5 days after the challenge. Lungs were harvested from five mice out of each group and prepared as clarified homogenates. MDCK cells were inoculated with serial $\log_{10}$ dilution of the lung homogenates and the

50% tissue culture infectious dose (TCID50) was calculated by the Reed–Muench method after 72 h.

2.7. Hemagglutination inhibition (HI) assay

To measure anti-HA Ab titers by HI assay, sera were heat-inactivated by incubation at 56 °C for 30 min. In order to reduce non-specific inhibition of hemagglutination, 225 μl of a 25% kaolin suspension was added to heat-inactivated sera. The mixture was vortexed and incubated for 20 min at room temperature. After centrifugation for 2 min at 6500*g*, 50 μl of the supernatant was harvested and serially diluted (twofold) in PBS in U-bottom 96-well plates (Greiner, Germany). Four HA units of NC or PR8 virus was added to each sample separately and incubated at room temperature (RT) for 1 h, followed by addition of 0.5% chicken red blood cells and incubation for 30 min at RT. The HI titer for each sample was reported as the reciprocal of the highest serum dilution that completely inhibited hemagglutination.

2.8. Splenocyte isolation

Three weeks after immunization, spleens of immunized mice were smashed into a cell homogenizer and red blood cells were osmotically lysed using Tris-ammonium chloride buffer (NH_4Cl 0.16 M, Tris 0.17 M). Cells were washed twice with complete RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Viable cells were counted by trypan blue (0.4% w/v) exclusion.

2.9. IFN-gamma ELISPOT assay

A commercial IFN-γ ELISPOT kit (R&D, USA) was used according to the manufacturer's instruction. Briefly, in 96-well PVDF-backed microplate coated with monoclonal antibody specific for mouse IFN-γ, 2×10^5 splenocytes were incubated with H–$2K^d$-restricted CTL epitope of influenza virus NP ($NP_{147-155}$, TYQRTRALV) at 10 μg/ml for 24 h at 37 °C and 5% CO_2. As positive control, PHA (Sigma, USA) was added to a final concentration of 5 μg/ml and an irrelevant peptide served as negative control. After cell removal by washing steps, biotinylated polyclonal antibody specific for mouse IFN-γ was added and the plates were incubated overnight at 4 °C. After adding Streptavidin-AP and washing steps, a chromogen mixture of 5-bromo-4-chloro-3 indolylphosphate *p*-toluidine salt (BCIP) and nitro blue tetrazolium chloride (NBT) was added to each well and incubated for 1 h at room temperature. The microplates were rinsed with deionized water and dried completely at room temperature for 90 min. Spot forming cells (SFCs) were enumerated under a dissection microscope (Leica Microscopy system, Heerbrugg, Switzerland).

2.10. Granzyme B ELISPOT assay

Granzyme-B ELISPOT kit (R&D) was used according to the manufacturer's instruction. Briefly, to prepare effector cells, 2×10^6 cells/ml splenocytes were incubated with 10 μg/ml of $NP_{147-155}$ peptide for 24 h at 37 °C and 5% CO_2. The p815 target cells (mouse mastocytoma cell line) were pulsed with 10 μg/ml of $NP_{147-155}$ peptide for 4 h at 37 °C and 5% CO_2. Next, the target cells were co-cultured with 100 μl of effector cells for 5 h at the effector/target (E:T) ratios of 10:1 and 1:1. Recombinant mouse granzyme B provided by the manufacturer served as a positive control. A biotinylated polyclonal antibody specific for mouse granzyme B was used as detection antibody. After cell removal by washing steps, spot forming cells (SFCs) were enumerated under a dissection microscope (Leica Microscopy system, Heerbrugg, Switzerland).

2.11. Statistical analysis

All experiments were performed in triplicate and/or repeated three times. Antibody titer, granzyme B/IFN-γ release (ELISPOT assay) and MTT assay results were analyzed by one-way analysis of variance followed by Tukey's test. Survival rates were analyzed by Kaplan–Meier and log rank tests. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Expression of NP gene transfected by influenza virosomes

BHK-21 cells were transfected with the pcDNA3–NP plasmid using influenza virosomes and expression of NP was confirmed by immunofluorescence (Fig. 1).

3.2. Influenza virosomes/NP pDNA complex induces intra-subtypic cross-protection

The virosome–NP pDNA complex was tested for the ability to provide protection against lethal challenge with two H1N1 subtype viruses (Fig. 2). Mice were monitored daily for the signs of the clinical illness (weight loss) and survival. All mice in the V-only and V-NP groups showed 100% survival when challenged with the NC

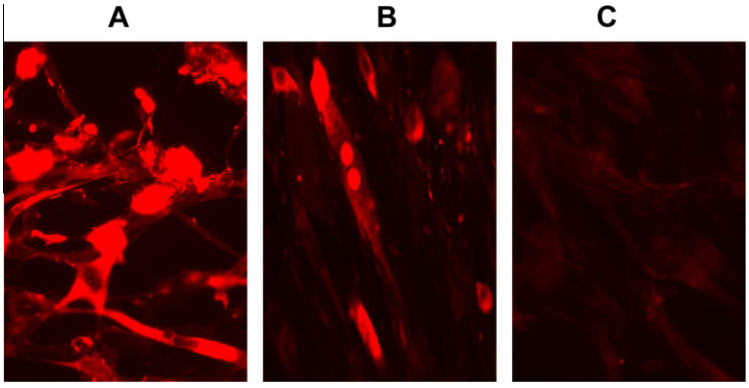

Fig. 1. Immunofluorescence of BHK-21 cells transfection by influenza virosomes containing pcDNA3–NP. BHK-21 cells were transfected with 1 μg of pcDNA3–NP loaded on the surface of influenza virosomes. Two days post-transfection, the cells were fixed with 4% formaldehyde in PBS, treated with Triton X-100 and then anti-NP monoclonal Ab, followed by incubation with anti-mouse IgG–rhodamine conjugate. (A) BHK-21 cells transfected by pNP–influenza virosomes. (B) BHK-21 cells transfected by pNP–Lipofectamine 2000®. (C) BHK-21 cells transfected by empty vector (pcDNA3) loaded on influenza virosomes.

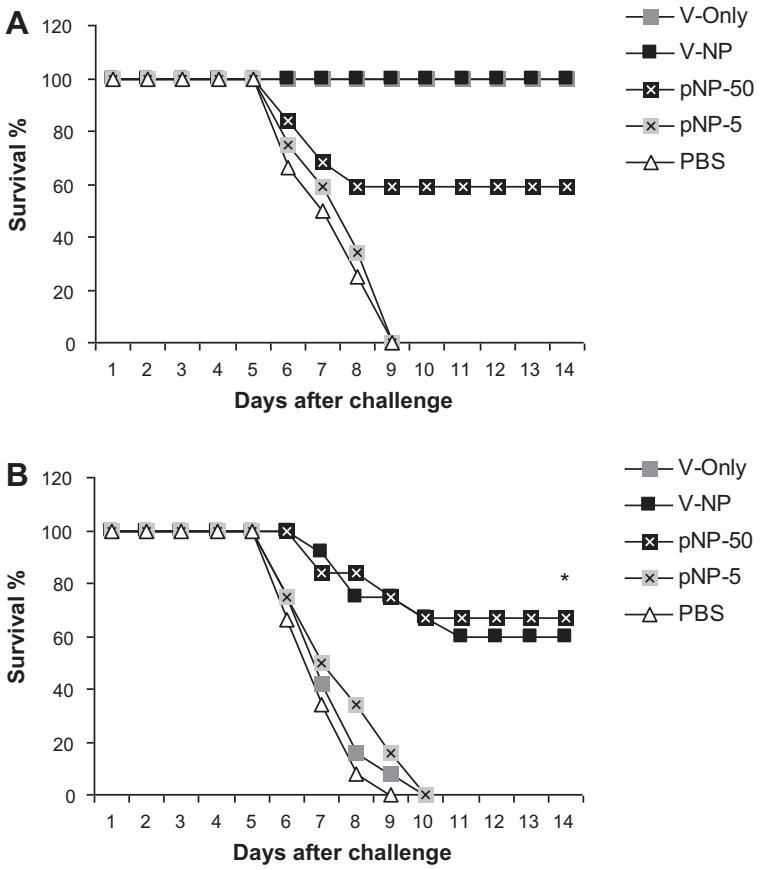

Fig. 2. Resistance of immunized mice against infection by A/New Caledonia/20/99 and A/Puerto Rico/8/34 influenza viruses. Groups of mice (n = 12 per group) were injected intradermally with 5 μg of NP DNA-influenza virosome (V-NP), 50 or 5 μg of NP DNA, influenza virosomes without NP pDNA (V-only), or PBS. Three weeks after immunization, mice were challenged with A/New Caledonia/20/99 virus as a homologous strain (A) or with A/Puerto Rico/8/34 virus (B) as a drift strain. *P < 0.001, V-NP vs. V-only after drift virus challenge (B).

virus (Fig. 2A). Next, to evaluate the protective efficacy against strains of influenza virus within the same subtype, the mice immunized with NC-based virosomes were challenged with PR8 virus (Fig. 2B). The V-only group succumbed to death after intra-subtypic challenge. Also, NP-5 group failed to develop protective immunity while V-NP group showed significant protection (60%) compared to both V-only and pNP-5 groups (0%) against lethal challenge with drift virus (PR8 virus) (P < 0.001).These data indicated that either a higher NP–pDNA dose or a low dose formulated with virosome is required for threshold protective responses. Regarding NP pDNA vaccine, only NP-50 group showed significant survival compared to PBS group after challenge with both homologous and drift virus (P < 0.001). Negative controls did not show any survival after challenge.

3.3. Influenza virosome/NP pDNA complex prevents weight loss of mice after intra-subtypic challenge

All immunized mice were followed daily for weight loss after challenge with two H1N1 subtype viruses. As shown in Fig. 3A, both virosome-immunized groups (V-only and V-NP) experienced only a small and transient weight loss after homologous virus challenge compared to pNP-50, pNP-5 and PBS groups (p < 0.001). The smallest weight loss was observed in V-NP group (4%) after NC virus challenge and this recovered to the initial level within 10 days (V-NP versus V-only: p < 0.05) (Fig. 3A). The pNP-50 group lost about 20% of their body weight initially and this only recovered to 90% at the end of the 14-day follow-up. Both pNP-5 and PBS groups exhibited severe weight loss (30%) and died within 6–10 days post challenge. Regarding intra-subtypic challenge (PR8 virus), the V-NP or pNP-50 groups exhibited a similar weight loss pattern (maximum 20%) and they began to recover from disease at 10 days post challenge (Fig. 3B). In contract, V-only group lost a significant amount of body weight (30%) and all of them died within 7–10 days post-challenge. Similar to the homologous challenge, both pNP-5 and PBS groups exhibited severe weight loss (30%) and died within 6–10 days post challenge (Fig. 3B).

3.4. Influenza virosome/NP pDNA complex enhances clearance of both of the challenging viruses from lungs

To evaluate the effect of NP pDNA incorporation in virosome formulation on the virus clearance rate in the lungs, the immunized mice were challenged with 4 LD_{50} of influenza NC and PR8 viruses. As shown in Fig. 4A, influenza virus titer after homologous challenge (NC virus) was significantly lower in V-NP, V-only and pNP-50 immunized mice than pNP-5 and PBS groups (P < 0.05). Moreover, maximal clearance of the virus was observed in V-NP group. In contrast, after intra-subtypic challenge (PR8 virus), V-NP but not V-only group showed a significant reduction in lung virus titers compared to PBS group (P = 0.015) (Fig. 4B). Regarding NP pDNA vaccine, only mice receiving 50 μg of NP pDNA (NP-50 group) successfully suppressed virus amplification in lungs compared to PBS group (P = 0.017). There was no significant difference between NP-50 and V-NP groups.

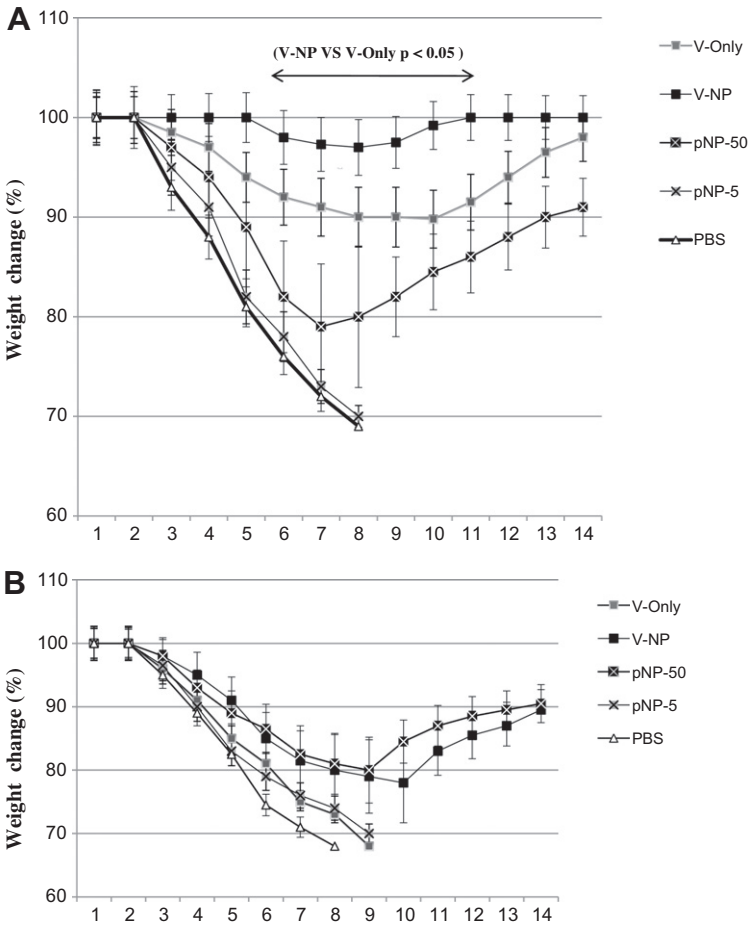

Fig. 3. Protective efficacy of influenza virosome/pNP DNA vaccine complex against weight loss after influenza virus challenge. Groups of mice (n = 12 per group) were injected intradermally with 5 μg of NP DNA-influenza virosome (V-NP), 50 or 5 μg of NP pDNA, influenza virosomes without NP (V-only), or PBS three weeks after immunization, mice were challenged with A/New Caledonia/20/99 as a homologous virus (A) or with A/Puerto Rico/8/34 (B) as a drift virus. Weight changes were measured for 14 days after the challenge.

3.5. Anti-HA antibodies induced by influenza virosome derived from NC virus fail to cross-neutralize PR8 virus

HI titers were determined three weeks after immunization. Sera from the mice were twofold diluted and incubated with 4 HA unit of NC or PR8 virus. As indicated in Table 1, there was no significant difference between the means of HI titers of the V-only and V-NP groups. The mice receiving NP pDNA and negative controls had HI titers <4 (HI titer of four was the minimal measured titer). Furthermore, we investigated whether sera from the mice immunized with the virosomes reconstituted from NC virus neutralize PR8 virus in HI assay. Sera from the mice were incubated with 4 HA unit of PR8 virus. No HI titer higher than four was demonstrated, indicating lack of cross-reactive antibodies in the sera from the mice immunized with NC-derived virosomes (Table 1).

3.6. Cellular immune responses

To assess cell-mediated immunity to NP, IFN-γ and Granzyme B production was measured using ELISPOT as described in Section 2. As shown in Fig. 5, no significant difference was observed between the pNP-50 and V-NP groups. Moreover, both V-NP and pNP-50 groups showed a significantly higher frequency of IFN-γ and granzyme B-secreting T cells compared to the pNP-5 ($P < 0.001$). There was no significant difference between pNP-5 and negative controls.

4. Discussion

The data presented here describe a new influenza vaccine formulation composed of a modified virosomal platform in which the incorporation of a low dose of NP-encoding plasmid broadened immunogenicity of a conventional virosome-based seasonal flu vaccine by inducing intra-subtypic protection against drifted influenza viruses.

Current inactivated influenza vaccines provide protective immunity by induction of the strain-specific neutralizing antibodies (Geeraedts and Huckriede, 2011). However, seasonal influenza vaccine mismatch as a consequence of antigenic drift has significant epidemiological and economic impacts on influenza vaccine effectiveness as reviewed by Carrat and Flahault (2007). Our results showed that virosomal vaccine, as expected, completely protected against the homologous virus lethal challenge without any clinical signs of disease while could not protect against the drift strain. Analysis of serum antibodies in NC virosome-inoculated group showed neutralizing activity against the original virus without cross-neutralizing antibodies against the drift (PR8) virus. In agreement with this study, many investigations have shown that the inactivated influenza vaccine fails to protect against antigenic drift variants within a subtype of the influenza virus (Ada and Jones, 1986; Smirnov et al., 2004). Although cholera toxin has been co-administrated as a mucosal adjuvant with inactivated influenza virus to elicit cross-protective neutralizing antibodies (Quan et al.,

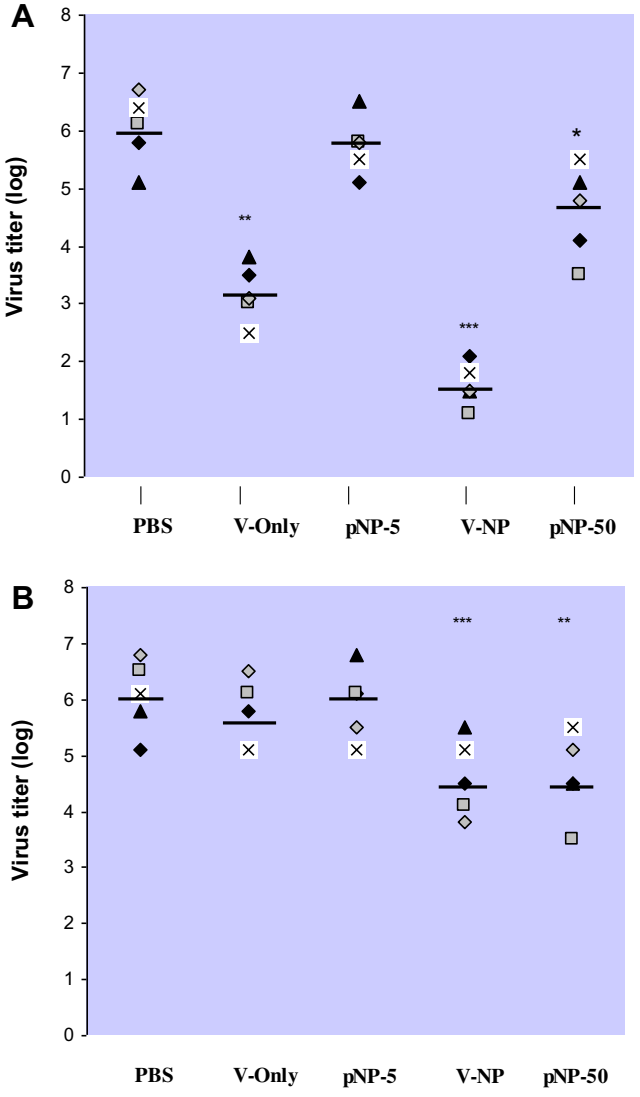

Fig. 4. Viral replication in the lungs following influenza virus challenge. Groups of mice ($n = 5$ per group) were injected intradermally with 5 μg of NP pDNA–influenza virosome (V-NP), 50 or 5 μg of NP pDNA, influenza virosomes without NP pDNA (V-only), or PBS. Three weeks after immunization, the mice were challenged with A/New Caledonia/20/99 as a homologous virus (A) or with A/Puerto Rico/8/34 (B) as a drift virus. Viral titers in the lungs were measured 5 days after intranasal infection. A: $^{***}P < 0.05$ V-NP vs. V-only; $^{**}P < 0.05$ V-only vs. pNP-5; $^{*}P < 0.05$ pNP-50 vs. pNP-5. (B): $^{***}P < 0.05$, V-NP vs. V-only; $^{**}P < 0.05$ pNP-50 vs. V-only.

Table 1
Anti-HA antibodies induced by influenza virosome against homologous and intra-subtypic influenza strains.

Groups	Anti-HA antibodies against homologous strain	Anti-HA antibodies against drift strain
V-only	89.6 ± 35	<4
V-NP	76.8 ± 28	<4
pNP-5	<4	<4
pNP-50	<4	<4
PBS	<4	<4

Groups of mice ($n = 5$ per group) were injected intradermally with 5 μg of NP DNA–influenza virosome complex (V-NP), 50 or 5 μg of NP DNA, influenza virosomes without NP pDNA (V-only), or PBS. HI titers were determined three weeks after immunization. Sera from the mice were twofold diluted and incubated with 4 HA unit of A/New Caledonia/20/99 (homologous strain) or A/Puerto Rico/8/34 (drift strain).

2008), but it was withdrawn from the market after reports of the consequent palsy syndrome (Mutsch et al., 2004).

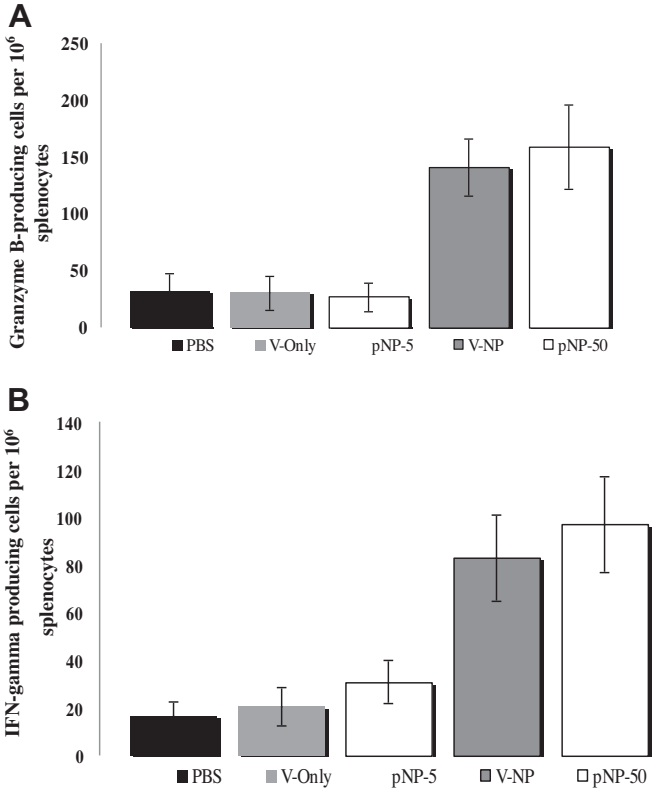

Fig. 5. Cellular immune responses induced by immunization with influenza virosomes/NP plasmid complex in mice. Groups of mice ($n = 5$ per group) were injected intradermally with 5 μg of NP pDNA–influenza virosome (V-NP), 50 or 5 μg of NP pDNA, influenza virosomes without NP pDNA (V-only), or PBS. Spleen cells were harvested 3 weeks after immunization and re-stimulated *in vitro* with the NP peptide (see Section 2.9). The frequency of granzyme B- (A) and IFN-γ-secreting T cells (B) were determined by ELISPOT assay.

In addition to being used as a commercial formulation for seasonal flu vaccine, intrinsic adjuvant activity of virosome has been demonstrated in several studies (Gluck et al., 2004; Moser et al., 2003). Priming of naïve $CD8^+$ T cells requires efficient processing of the antigen and presentation of the epitopes on MHC-I complexes of APCs. Moreover, to express co-stimulatory molecules, the APCs need to be activated. Virosomal HA binds sialic acid receptors on the surface of APCs with a high affinity (Matlin et al., 1981). APCs are hardly transfected with pDNA (Dokka et al., 2000). Efficient transfection of APCs has to be considered in designing successful delivery systems for genetic vaccines (Donnelly et al., 2005). As reported by Cusi et al., (2004) immunopotentiating reconstituted influenza virosomes (IRIV) transfected dendritic cells (DCs) with a significant efficiency as described.

The influenza vaccine platform described in this study consists of a balanced mixture of viral envelope phospholipids and the cationic lipid 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP). Recently, DOTAP liposome was demonstrated to induce p38 activation which resulted in Th_1 cytokine release such as IL-12 (Yan et al., 2007; Yu et al., 2003). Besides, DOTAP induced down-regulation of the IL-1 which could suppress the Th_2 response (Yan et al., 2007). Therefore, DOTAP triggers predominantly Th_1 cytokines which favor development of cell-mediated immune responses. Hong et al. showed that the addition of cationic lipid/non-coding DNA complexes (CLDC) as adjuvant to whole inactivated influenza A virus vaccine induces robust adaptive immune responses in mice (Hong et al., 2010). We have shown that DOTAP contributes to adjuvant activity of cationic influenza virosomes loaded with pDNA in a synergistic fashion (Jamali et al., submitted for publication).

Key role of cytotoxic T lymphocyte (CTL) responses in clearing influenza A virus infections in mice and humans have been shown in several studies (Hillaire et al., 2011; Tao et al., 2009; Ulmer et al., 1993). Amino acid sequence of NP is highly conserved among human and avian subtypes and NP DNA vaccination has been shown to induce cross-reactive cell-mediated immune responses in animal models (Donnelly et al., 1997; Tao et al., 2009; Ulmer et al., 1993, 1998). In the present study, addition of a 10-fold reduced dose (5 μg) of the NP pDNA to the modified virosomal platform induced CTL responses comparable to those induced by 50 μg of NP pDNA as demonstrated by granzyme B and IFN-γ release. In agreement, a pDNA expressing the parathyroid hormone-related peptide (PTH-rP), a protein secreted by prostate and lung carcinoma cells, was used at a reduced dose (5 μg) with influenza virosomes and elicited anti-tumor CTL responses in mice (Correale et al., 2001). To date, different dose-sparing strategies have been evaluated for NP pDNA-based vaccines. Degano et al. demonstrated that administration of gold particles coated with nanogram amounts of NP plasmid using gene gun elicited a strong and specific anti-NP CTL response (Degano et al., 1998). Furthermore, electroporation-assisted delivery systems have been tested to enhance the potency of DNA vaccines (Chiarella et al., 2010). However, these procedures require the special apparatus itself, which may be cost prohibitive for worldwide immunization programs in underdeveloped countries. Lipid carrier-mediated delivery formulations such as Vaxfectin® have shown promising effects in NP plasmid dose-sparing (Jimenez et al., 2007). As mentioned earlier, haemagglutinin (HA) antigens embedded in phospholipid bilayer of virosome make a great contribution to immunogenicity of this carrier by eliciting neutralizing antibodies against circulating influenza virus which is lacking in synthetic liposomes such as vaxfectin®.

In our experimental setup, to mimic the dosage of seasonal flu vaccine, protectivity of a single dose of this formulation was evaluated in mice. In accordance with previous studies, NP–pDNA injection partially protected mice against lethal challenge; however, slightly higher level of protection achieved in this study may be attributed to the intradermal route of administration as reviewed by Kim et al. (2011). Adsorption of NP–pDNA to the surface of cationic influenza virosomes in a dose-spared formulation remarkably protected mice against drift virus lethal challenge. As described above, there was no cross-reactive antibody in the sera from the mice immunized with NC virosomes against PR8 virus. Furthermore, challenge study proved *in vitro* neutralization results obtained by HI. All the mice immunized with NC virosomes alone succumbed to death after lethal challenge with PR8 virus. Hence, the observed cross-protection was not because of induction of cross-neutralizing antibodies. In contrast, amino acid sequence alignment of NP protein in PR8 and NC virus showed a high level of sequence conservation (95% NP compared to 88% HA) and there was no mutation in the dominant CTL epitope of NP protein ($NP_{147-155}$) (data not shown). It is noteworthy that a retrospective study by Nordin et al. showed that vaccine protectivity against a mismatched strain dramatically reduced to 35% compared to 61% in matched circulating strain (Nordin et al., 2001). Interestingly, with our formulation a 100% protection against homologous strain and 60% intra-subtypic protection against drift virus were achieved. Furthermore, virosome/NP–pDNA complex showed a superior prevention from clinical illness (weight loss) compared to the virosome alone.

In summary, a reduced dose of NP pDNA combined with influenza virosomes not only fully protected against homologous strain but also induced significant protection against intra-subtypic challenge with drift virus. Since influenza epidemic and pandemic strains cannot be predicted with certainty, incorporation of NP–pDNA into virosomal seasonal vaccine formulation would be a technically and economically feasible strategy to broaden influenza vaccine coverage. We would suggest further improvement of our proposed formulation by co-expression of other conserved influenza virus proteins specially M1 and M2. Also, acute and long-term CTL responses elicited by V-NP vaccination remain to be characterized in further studies. Finally, evaluation of the potential protection against other H1N1 strains and hetero-subtypic protection against pre-pandemic strains such as H5N1 definitely will shed more light on the immunogenicity of the virosome–pDNA formulation.

Acknowledgments

This work was supported in part by a grant from Iran National Science Foundation (INSF); as well as a fund from Pasteur Institute of Iran, Tehran, Iran. We also thank Prof. Anke Huckriede and Dr. Aalzen de Haan for training Dr. Abbas Jamali in the field of virosome technology at University Medical Center Groningen, The Netherlands.

References

Ada, G.L., Jones, P.D., 1986. The immune response to influenza infection. Curr. Top. Microbiol. Immunol. 128, 1–54.
Both, G.W., Sleigh, M.J., Cox, N.J., Kendal, A.P., 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J. Virol. 48, 52–60.
Carrat, F., Flahault, A., 2007. Influenza vaccine: the challenge of antigenic drift. Vaccine 25, 6852–6862.
Chiarella, P., Fazio, V.M., Signori, E., 2010. Application of electroporation in DNA vaccination protocols. Curr. Gene Ther. 10, 281–286.
Correale, P., Cusi, M.G., Sabatino, M., Micheli, L., Pozzessere, D., Nencini, C., Valensin, P.E., Petrioli, R., Giorgi, G., Zurbriggen, R., Gluck, R., Francini, G., 2001. Tumour-associated antigen (TAA)-specific cytotoxic T cell (CTL) response in vitro and in a mouse model, induced by TAA-plasmids delivered by influenza virosomes. Eur. J. Cancer 37, 2097–2103.
Cusi, M.G., Terrosi, C., Savellini, G.G., Di Genova, G., Zurbriggen, R., Correale, P., 2004. Efficient delivery of DNA to dendritic cells mediated by influenza virosomes. Vaccine 22, 735–739.
Daemen, T., de Mare, A., Bungener, L., de Jonge, J., Huckriede, A., Wilschut, J., 2005. Virosomes for antigen and DNA delivery. Adv. Drug Deliv. Rev. 57, 451–463.
de Jonge, J., Leenhouts, J.M., Holtrop, M., Schoen, P., Scherrer, P., Cullis, P.R., Wilschut, J., Huckriede, A., 2007. Cellular gene transfer mediated by influenza virosomes with encapsulated plasmid DNA. Biochem. J. 405, 41–49.
Degano, P., Sarphie, D.F., Bangham, C.R., 1998. Intradermal DNA immunization of mice against influenza A virus using the novel PowderJect system. Vaccine 16, 394–398.
Dokka, S., Toledo, D., Shi, X., Ye, J., Rojanasakul, Y., 2000. High-efficiency gene transfection of macrophages by lipoplexes. Int. J. Pharm. 206, 97–104.
Donnelly, J.J., Friedman, A., Ulmer, J.B., Liu, M.A., 1997. Further protection against antigenic drift of influenza virus in a ferret model by DNA vaccination. Vaccine 15, 865–868.
Donnelly, J.J., Wahren, B., Liu, M.A., 2005. DNA vaccines: progress and challenges. J. Immunol. 175, 633–639.
Geeraedts, F., Huckriede, A., 2011. Influenza vaccines: what do we want and how can we get it? Adv. Exp. Med. Biol. 780, 161–174.
Gluck, R., Moser, C., Metcalfe, I.C., 2004. Influenza virosomes as an efficient system for adjuvanted vaccine delivery. Expert Opin. Biol. Ther. 4, 1139–1145.
Herzog, C., Hartmann, K., Kunzi, V., Kursteiner, O., Mischler, R., Lazar, H., Gluck, R., 2009. Eleven years of Inflexal V-a virosomal adjuvanted influenza vaccine. Vaccine 27, 4381–4387.
Hillaire, M.L., Osterhaus, A.D., Rimmelzwaan, G.F., 2011. Induction of virus-specific cytotoxic T lymphocytes as a basis for the development of broadly protective influenza vaccines. J. Biomed. Biotechnol., 939860.
Hong, D.K., Chang, S., Botham, C.M., Giffon, T.D., Fairman, J., Lewis, D.B., 2010. Cationic lipid/DNA complex-adjuvanted influenza A virus vaccination induces robust cross-protective immunity. J. Virol. 84, 12691–12702.
Jamali, A., Sabahi, F., Bamdad, T., Hashemi, H., Mahboudi, F., Kheiri, M.T., 2010. A DNA vaccine-encoded nucleoprotein of influenza virus fails to induce cellular immune responses in a diabetic mouse model. Clin. Vaccine Immunol. 17, 683–687.
Jimenez, G.S., Planchon, R., Wei, Q., Rusalov, D., Geall, A., Enas, J., Lalor, P., Leamy, V., Vahle, R., Luke, C.J., Rolland, A., Kaslow, D.C., Smith, L.R., 2007. Vaxfectin-formulated influenza DNA vaccines encoding NP and M2 viral proteins protect mice against lethal viral challenge. Hum. Vaccin. 3, 157–164.
Kim, Y.C., Jarrahian, C., Zehrung, D., Mitragotri, S., Prausnitz, M.R., 2011. Delivery systems for intradermal vaccination. Curr. Top. Microbiol. Immunol. 351, 77–112.
Liu, M.A., 2011. DNA vaccines: an historical perspective and view to the future. Immunol. Rev. 239, 62–84.

Matlin, K.S., Reggio, H., Helenius, A., Simons, K., 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. J. Cell Biol. 91, 601–613.
Moser, C., Metcalfe, I.C., Viret, J.F., 2003. Virosomal adjuvanted antigen delivery systems. Expert Rev. Vaccines 2, 189–196.
Mutsch, M., Zhou, W., Rhodes, P., Bopp, M., Chen, R.T., Linder, T., Spyr, C., Steffen, R., 2004. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N. Engl. J. Med. 350, 896–903.
Nicholson, K.G., Wood, J.M., Zambon, M., 2003. Influenza. Lancet 362, 1733–1745.
Nordin, J., Mullooly, J., Poblete, S., Strikas, R., Petrucci, R., Wei, F., Rush, B., Safirstein, B., Wheeler, D., Nichol, K.L., 2001. Influenza vaccine effectiveness in preventing hospitalizations and deaths in persons 65 years or older in Minnesota, New York, and Oregon: data from 3 health plans. J. Infect. Dis. 184, 665–670.
Palese, P., 2004. Influenza: old and new threats. Nat. Med. 10, S82–87.
Quan, F.S., Compans, R.W., Nguyen, H.H., Kang, S.M., 2008. Induction of heterosubtypic immunity to influenza virus by intranasal immunization. J. Virol. 82, 1350–1359.
Rimmelzwaan, G.F., Osterhaus, A.D., 2001. Influenza vaccines: new developments. Curr. Opin. Pharmacol. 1, 491–496.
Schoen, P., Chonn, A., Cullis, P.R., Wilschut, J., Scherrer, P., 1999. Gene transfer mediated by fusion protein hemagglutinin reconstituted in cationic lipid vesicles. Gene Ther. 6, 823–832.
Smirnov, Y.A., Gitelman, A.K., Govorkova, E.A., Lipatov, A.S., Kaverin, N.V., 2004. Influenza H5 virus escape mutants: immune protection and antibody production in mice. Virus Res. 99, 205–208.
Stanekova, Z., Vareckova, E., 2011. Conserved epitopes of influenza A virus inducing protective immunity and their prospects for universal vaccine development. Virol J. 7, 351.
Stegmann, T., Morselt, H.W., Booy, F.P., van Breemen, J.F., Scherphof, G., Wilschut, J., 1987. Functional reconstitution of influenza virus envelopes. EMBO J. 6, 2651–2659.
Stohr, K., Kieny, M.P., Wood, D., 2006. Influenza pandemic vaccines: how to ensure a low-cost, low-dose option. Nat. Rev. Microbiol. 4, 565–566.
Tao, P., Luo, M., Pan, R., Ling, D., Zhou, S., Tien, P., Pan, Z., 2009. Enhanced protective immunity against H5N1 influenza virus challenge by vaccination with DNA expressing a chimeric hemagglutinin in combination with an MHC class I-restricted epitope of nucleoprotein in mice. Antiviral Res. 81, 253–260.
Ulmer, J.B., Donnelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowski, S.H., Deck, R.R., DeWitt, C.M., Friedman, A., et al., 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259, 1745–1749.
Ulmer, J.B., Fu, T.M., Deck, R.R., Friedman, A., Guan, L., DeWitt, C., Liu, X., Wang, S., Liu, M.A., Donnelly, J.J., Caulfield, M.J., 1998. Protective CD4+ and CD8+ T cells against influenza virus induced by vaccination with nucleoprotein DNA. J. Virol. 72, 5648–5653.
Webby, R.J., Webster, R.G., 2001. Emergence of influenza A viruses. Philos. Trans. R. Soc. Lond. B Biol. Sci. 356, 1817–1828.
Yan, W., Chen, W., Huang, L., 2007. Mechanism of adjuvant activity of cationic liposome: phosphorylation of a MAP kinase, ERK and induction of chemokines. Mol. Immunol. 44, 3672–3681.
Yu, J.J., Tripp, C.S., Russell, J.H., 2003. Regulation and phenotype of an innate Th1 cell: role of cytokines and the p38 kinase pathway. J. Immunol. 171, 6112–6118.
Zamparo, E., Little, D., 2011. Immunogenicity and effectiveness of virosomal adjuvanted vaccines against influenza: a brief review of their utility in the elderly population. J. Prev. Med. Hyg. 52, 116–119.