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Influenza M1 VLPs containing neuraminidase induce heterosubtypic cross-protection

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Abstract

Influenza virus like particles (VLPs) containing hemagglutinin were previously demonstrated to induce protection against the homologous strains. However, little information is available on the protective role of neuraminidase (NA), the second major glycoprotein. In this study, we developed VLPs (NA VLPs) containing NA and M1 derived from A/PR/8/34 (H1N1) influenza virus, and investigated their ability to induce protective immunity. Intranasal immunization with NA VLPs induced serum antibody responses to H1N1 and H3N2 influenza A viruses as well as significant neuraminidase inhibition activity. Importantly, mice immunized with NA VLPs were 100% protected against lethal infection by the homologous A/PR/8/34 (H1N1) as well as heterosubtypic A/Philippines/82 (H3N2) virus, although body weight loss was observed after lethal challenge with heterosubtypic H3N2 virus. The present study therefore provides evidence that influenza VLPs containing M1 and NA are capable of inducing immunity to homologous as well as antigenically distinct influenza A virus strains.

Keywords

Influenza; Virus-like particles; Neuraminidase; Protection

Introduction

The principal target of currently licensed influenza vaccines is hemagglutinin (HA), the most abundant viral surface antigen. Antibody to the hemagglutinin (HA) of influenza viruses has virus neutralizing activity and shows a correlation with protective immunity to influenza.
The current trivalent influenza vaccines are standardized on the basis of the content of the HA components. Sixteen HA subtypes have been identified among influenza A viruses (Fouchier et al., 2005). The protective efficacy of the vaccine is highly dependent on the degree of homology between the HA of the vaccine strain and the predicted epidemic strain (Couch, 2008). However, current vaccines based on immunity to HA do not provide good protection against antigenically heterologous viruses (Wilson and Cox, 1990). The degree of HA sequence diversity between subtypes is large and the antigenic properties of the HA molecules of the virus vary because of mutation to escape from the pressure of preexisting immunity (Fouchier et al., 2005; Wilson and Cox, 1990).

Neuraminidase (NA), the second major surface protein and determinant of 9 serotypes, is not standardized in current vaccines (Fouchier et al., 2005). It was proposed that the NA molecules are relatively slower in antigenic evolution as compared with the HA (Kilbourne et al., 1990). Antibodies against NA do not block infection, but they were shown to inhibit the enzymatic activity of NA, to cause virus aggregation, and limit the spread of virus (Chen et al., 2000; Compans et al., 1969; Johansson et al., 1989; Johansson and Kilbourne, 1993). Also, NA antibodies could provide immunity to influenza in humans (Couch et al., 1974; Murphy et al., 1972). In addition, the NA protein has become an important target for antiviral drugs (oseltamivir, zanamivir). Thus, it would be desirable to ensure that influenza vaccines induce anti-NA antibodies as well.

Previous studies used different approaches to study the role of NA in vaccination against influenza, demonstrating reduction in morbidity and mortality. These include purified NA proteins (Brett and Johansson, 2005; Deroo et al., 1996; Johansson, 1999; Johansson et al., 1998; Martinet et al., 1997), DNA plasmid (Chen et al., 2005; Chen et al., 2000; Li et al., 2006; Qiu et al., 2006; Sandbulte et al., 2007), and a variety of live virus-vectored vaccines expressing NA (Gao et al., 2006; Pavlova et al., 2009; Qiao et al., 2003; Sylte et al., 2007; Webster et al., 1988). However, preparation of soluble recombinant protein vaccines is laborious and may increase the vaccine cost, and DNA vaccines are not very effective in inducing antibody responses and thus require multiple immunizations. Recombinant live vectored vaccines may have concerns about anti-vector immunity and safety for use in humans.

Influenza virus-like particles (VLPs) have been suggested as a promising vaccine candidate against influenza (reviewed in (Kang et al., 2009a)). Vaccination with VLPs containing HA was demonstrated to induce protection to the homologous and closely related influenza viruses of seasonal and potential pandemic strains (Quan et al., 2007; Quan et al., 2010c; Song et al., 2010). Protective immunity of influenza VLPs containing both HA and NA was also reported (; Bright et al., 2007, 2008; Pushko et al., 2007). However, NA-mediated protective immune responses by vaccination with VLPs have not been well studied. In this study, we generated influenza VLPs containing M1 and NA derived from A/PR/8/34 virus (NA VLPs), and investigated their immunogenicity to homologous and heterosubtypic influenza viruses. Potential mechanisms of protection mediated by NA VLP vaccination have been explored and are discussed.
Results

Characterization of influenza VLPs containing neuraminidase (NA VLPs)

To determine the immunogenicity and protective efficacy of neuraminidase (NA), we produced influenza NA VLPs in insect cells by co-infecting with recombinant baculoviruses (rBV) expressing the M1 and NA proteins derived from PR8 (A/PR8/1934) virus, and characterized the VLPs. The incorporation of NA and M1 into VLPs was confirmed by western blot (Fig. 1A). The functional activity of NA incorporated into VLPs was found to be dependent on concentration of VLPs as determined by a neuraminidase enzyme assay (Fig. 1B). Influenza HA VLPs without NA did not show neuraminidase activity (Fig. 1B). The size and morphology of NA VLPs resembled influenza virus particles (Fig. 1C) although rod-like HA spikes were not detected. Taken together, these results show that NA VLPs produced in insect cells contain NA functional activity and are structurally similar to influenza virions.

Vaccination with NA VLPs elicits antibody response specific to NA

To evaluate the immunogenicity of NA VLPs, mice (n = 12) were immunized intranasally with 5 μg (total proteins) of NA VLPs using a prime-boost regimen. At 2 weeks after priming, we determined levels of total IgG serum antibodies binding to inactivated A/PR8 virus or CV-1 cells infected with recombinant vaccinia virus expressing NA (Fig. 2). Low levels of antibodies binding to NA expressed on the surface of cells or inactivated virus were observed after priming. After boost immunization, significantly higher levels of NA specific antibodies were induced (Fig. 2A). Similarly high levels of antibodies binding to inactivated A/PR8 virus were detected in boost immune sera (Fig. 2B). IgG responses after boost were significantly higher than those after priming (P<0.01). When isotypes of antibodies to inactivated A/PR8 virus were determined, IgG1 was detected at a significantly higher level than IgG2a (Fig. 2C). To determine the cross reactivity of NA VLP immune sera, the heterosubtypic H3N2 (A/Philippines/82) viral antigen was used as an ELISA coating antigen (Fig. 2D). Substantial levels of total IgG and IgG1 isotype antibodies but not IgG2a antibody specific to inactivated A/Philippines/82 virus were observed (Fig. 2D). Overall, these results indicate that NA VLP immunization induces antibodies reactive to the homologous as well as heterosubtypic NA antigens.

Vaccination with NA VLPs induces complete protection against the homologous virus without weight loss

Next, we determined whether immune sera from N1 VLP vaccination would inhibit NA activity (Fig. 3A). Six-fold higher levels of NA inhibition activity were found in immune sera compared to naïve sera (P<0.001). No significant hemagglutinin inhibition activity (HAI) or neutralizing activity was found in the immune sera (data not shown). Therefore, immunization with NA VLPs induces serum antibody responses that can inhibit neuraminidase activity.

To determine protective efficacies, mice were challenged with a high lethal dose of A/PR8/1934 virus (20 × LD50) at 4 weeks after boost immunization with NA VLPs. As shown in Fig. 3B and C, all naïve mice died after infection. In contrast, mice vaccinated with NA

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VLPs were completely protected with 100% survival without weight loss. These results show that VLPs containing NA can confer completely protective immunity against lethal challenge in a mouse model.

**NA VLP vaccination provides effective control of challenge virus replication**

The efficiency of virus clearance in lungs provides a sensitive indicator for assessing protective efficacy. At day 4 post challenge, mice were sacrificed and viral titers in lung extracts were determined. Compared to naïve mouse control groups, mice immunized with NA VLPs showed significantly lower lung viral titers by 10,000 fold ($P<0.001$) (Fig. 4A). The production of the inflammatory cytokine interferon gamma (IFN-γ) was also determined in lungs collected at day 4 after challenge infection. Naïve mice infected with A/PR8/34 virus showed high levels of IFN-γ in lung extracts. The vaccinated mice showed significantly lower levels of IFN-γ similar to those in uninfected naïve mice (Fig. 4B). Therefore, these results show that vaccination with NA VLPs induces protective immunity without disease symptoms and confers effective control of viral replication, resulting in reduced proinflammatory cytokine production.

**NA VLP vaccination induces effective antibody secreting cell responses**

To determine antibody secreting cell responses (ASC), spleen or bone marrow cells were harvested at day 4 post challenge infection and cultured in vitro for 2 and 6 days. The number of B cells in a memory state is relatively low with VLP vaccination and antigenic challenge allows an easy detection of ASC responses indicating anamnestic responses (Song et al., 2010). Significantly higher levels of antibodies specific to A/PR8/34 viral antigens were secreted into supernatants after 6 days culture of splenocytes than those obtained after 2 days culture ($P<0.001$, Fig. 5). Importantly, cells from bone marrow produced substantial amounts of antibodies specific to A/PR8 virus at day 2 post culture, which is significantly higher than those in spleen cell cultures at day 2 (Fig. 5). Levels of antibodies in bone marrow cultures continued to increase during the 6 day of culture ($P<0.05$, Fig. 5). These results indicate that vaccination with NA VLPs can induce the generation of antibody secreting plasma cells in bone marrow. In addition, NA VLPs vaccination can induce memory B cells in spleens, which can rapidly differentiate into antibody secreting plasma cells upon exposure to influenza viral infection.

**NA VLP vaccination induces heterosubtypic cross protection**

To determine whether vaccination of mice with VLPs containing N1 NA (A/PR8, H1N1) can induce heterosubtypic cross protection, immunized mice were challenged with a lethal dose of A/Phil (H3N2) virus. At day 4 post challenge with A/Phil virus, lung virus titers were observed at approximately 20-fold lower in NA VLP immunized mice compared to naïve mice (Fig. 6A). All naïve mice infected with A/Phil virus died by day 7. Mice vaccinated with N1 NA VLPs were 100% protected against lethal challenge with A/Phil virus although substantial body weight loss was observed (Fig. 6B and C). Immunized mice showed a recovery in the body weight from day 9. In terms of virus clearance and body weight changes, the protective efficacy after heterosubtypic virus challenge was lower compared to the protection against the homologous virus. Nonetheless, the results provide evidence that vaccination with VLPs containing NA may be able to confer cross-protection.
Protective role of NA VLP immune sera

The capability of NA VLP immune sera to provide protection was tested in naïve mice. Although NA specific antibodies do not have direct virus neutralizing activity, we wanted to determine other possible immune mechanisms in providing protection. Mixtures of a lethal dose of A/PR8 and NA VLP immune or naïve sera were used to intranasally infect naïve mice. This method was previously well used to assess the protective role of polyclonal immune sera (Quan et al., 2010c; Song et al., 2011b). Naïve mice that were infected with a mixture of virus and naïve sera showed severe body weight loss reaching to 75% of original weight and showed a 20% survival rate (Fig. 7A and B). In contrast, NA VLP immune sera conferred complete protection to naïve mice that were infected with a lethal dose of A/PR8 virus. Therefore, these results suggest that anti-NA immune sera play an important role in providing protection.

Since NA antibodies do not have virus neutralizing activity, we studied the potential roles of lung airway macrophage cells in conferring anti-NA antibody-mediated protection (Fig. 7C and D). Previous studies demonstrated the selective depletion of lung dendritic and macrophage cells by intranasal administration with clodronate-liposomes (Bosio and Dow, 2005; McGill et al., 2008). Similarly, we showed selective depletion of lung dendritic and macrophage cells by intranasal administration of clodronate-liposomes (Song et al., 2011a, 2011b). When naïve mice were pretreated with clodronate-liposome, these mice showed severe body weight loss and 50% survival rate after infection with a lethal dose of A/PR8 virus mixed with N1 VLP immune sera (Fig. 7C and D). Whereas, sera from mice that had recovered from sub-lethal infection with A/PR8 virus provided good protection even in the group pretreated with clodronate-liposomes (Fig. 7C and D), indicating that protective antibody responses to HA might be different from those to NA in the mechanism of protection. These results indicate that dendritic and macrophage cells might be important for conferring NA immune serum-mediated protection.

Discussion

VLPs provide a promising platform of vaccine candidates presenting an antigen in an immunogenic form. Although both HA and NA are glycoproteins expressed on the surfaces of influenza virions, the role of NA immune responses in conferring vaccine-induced protective immunity has not been as well investigated compared to that of HA. Among the factors in limiting the study of NA-specific antibodies in protection is the skewed immune responses to HA after influenza vaccination. The immunodominant effects of HA as an antigen were found to occur when HA and NA are present in the same viral particle (Johansson and Kilbourne, 1993; Johansson et al., 1987). Influenza VLPs provide an excellent vehicle to present NA as the major vaccine component in a membrane-anchored conformation. In this study, we investigated the generation of VLPs containing N1 NA, their immunogenicity, and protective immunity against homologous and heterosubtypic influenza A viruses. Influenza M1 VLPs containing NA were highly immunogenic inducing NA-specific functional antibodies, and conferred protection against the homologous H1N1 A/PR8 virus and the heterosubtypic H3N2 A/Philippines/82 virus. The contribution of influenza M1 to protection is known to be minimal or not significant (Chen et al., 1999;
Chen et al., 1998; Wiesener et al., 2011) despite the induction of M1 specific antibodies (Chen et al., 1998). In a previous study, mice immunized with 20 μg of total VLPs containing influenza M1 as a core protein did not induce detectable levels of antibodies specific to M1 (Song et al., 2011b). Thus, immune responses to NA in 5 μg NA VLPs are likely to be the major contributor to protection observed in this study.

Immunization with NA VLPs induced NA-specific antibody responses that can inhibit neuraminidase activity. The pattern of antibody isotypes provides insight into the potential immune cells that are involved in inducing antibody responses to an antigen (Mongini et al., 1982; Pulendran, 2004). Analysis of antibody isotypes in immune sera showed that IgG1 antibody was predominantly induced by immunization of mice (BALB/c) with NA VLPs. Soluble recombinant HA protein antigens are also known to induce IgG1 dominant antibody responses compared to whole inactivated virus or HA VLP antigens, which are capable of inducing IgG2a antibodies as a dominant isotype (Bright et al., 2007, 2008; Song et al.). Therefore, it is speculated that vaccine immunogens that are able to activate co-stimulatory and/or innate immune sensing molecules on antigen presenting cells may be effective in inducing IgG2a dominant isotype antibody responses. In support of this hypothesis, we found in a preliminary study that mice with deficiency in MyD88, a key signaling molecule for innate to adaptive immunity, displayed a significant defect in inducing IgG2a (or IgG2c) antibody responses after influenza HA VLP immunization (data not shown). In addition, our recent studies demonstrated that whole inactivated virus or HA VLPs with a loss of hemagglutination activity induced IgG1 dominant isotype antibody responses (Kim et al., 2010; Quan et al., 2010b; Quan et al., 2009a). Probably, NA VLPs without HA, which also lack hemagglutination activity, might be less effective in inducing IgG2a dominant immune responses compared to HA VLPs with hemagglutination activity.

The immune mechanisms by which NA-specific antibodies confer protection appear to be different from those by HA specific antibodies induced by conventional vaccination. Antibodies specific to HA are able to neutralize and block the infection of influenza virus, possibly inducing sterilizing immunity. In contrast, NA antibodies block the release of infectious virus from the surface of infected cells, causing virus aggregation, limiting viral spread, and thus allowing the host to develop adaptive immunity (Compans et al., 1969; Schulman et al., 1968). Anti-NA sera failed to prevent infection of cells in vitro, but passive immunization reduced morbidity and mortality in mice after infection (Kasel et al., 1973; Schulman et al., 1968; Webster et al., 1968). We found that immune responses to NA VLP vaccination effectively controlled lung viral replication resulting in several thousand-fold lower titers and complete protection without morbidity, despite not being able to prevent the infection. This study also suggests that protection by NA VLP immune sera seems to be dependent on the intactness of lung dendritic and macrophage cells. This is suggested by the finding of reduced protection when mice were treated with clodronate; one interpretation is that NA-specific antibodies limit infection in a macrophage-dependent manner. Another interpretation is that multiple immune mediators contribute toward protection; macrophages provide some control of infection; when this cell population is absent, overall protection is significantly less, since residual protection reflects the contribution of NA-specific antibodies alone and no longer the sum of innate and NA-specific responses. In contrast,
immune sera from infected mice were protective independent of lung dendritic and macrophage cells, indicating the role of virus-neutralizing activity in mediating protection.

Our previous studies demonstrated that immunization with HA VLP vaccines (HA+M1) induced strong protective immunity and high virus neutralizing titers (Quan et al., 2010a; Quan et al., 2008b; Quan et al., 2009b). Low doses in a range of 0.2 μg to 0.5 μg of total proteins of HA VLPs were capable of providing 100% protection against homologous virus challenge without body weight loss (Quan et al., 2009b). In this study, we used 5 μg of total proteins of NA VLPs. This indicates that HA is likely to be a more effective immunogen than NA in providing protection against homologous virus.

Regarding heterosubtypic protection, we reported that influenza VLPs containing HA which originated from either A/PR8 (H1N1) or A/Aichi/68 (H3N2) virus did not confer protection against an antigenically different strain (A/Philippines/82) (Quan et al., 2008b). NA DNA vaccines were poor in inducing heterosubtypic immunity, not providing protection against heterosubtypic H1N1 or H7N7 viruses although they provided survival protection against homologous or heterologous virus within the same subtype (Chen et al., 2000; Webster et al., 1988). The cross protection by NA vaccines was also weak or partial if the strains were distantly related, even within the same subtype (Sandbulte et al., 2007; Sylte et al., 2007). In the present study, vaccination with N1 NA VLPs provided 100% survival protection after lethal challenge with the H3N2 virus. Since NA VLP vaccine was administered intranasally, mucosal responses such as the induction of IgA antibody might have contributed to heterosubtypic protection, which remains to be determined. NA VLPs would also be a promising supplementary vaccine which is capable of enhancing cross protection. However, the heterosubtypic cross protection against the H3N2 virus (A/Philippines/82) was low since there was visible body weight loss and relatively high lung viral titers compared to the homologous protection. The NA VLP immune sera showed higher binding to the homologous virus compared to the heterologous virus, correlating with the degree of protection. Previous studies with HA-containing VLPs used very high challenge doses, making it difficult to compare the efficacy of HA and NA VLPs. Considering natural infections are sublethal in most humans, the relatively low challenge doses used for these NA VLP studies are relevant. The extent to which NA and HA VLP vaccines protect against heterologous infection will be compared in future studies.

Overall, the immunity to NA after vaccination is likely to induce protection against homologous or antigenically closely related strains but its protective efficacy against antigenically distinct strains may be low. The levels of NA antibody would be an important factor in protection. However, there is a concern that human influenza vaccines do not contain enough NA protein to induce a strong protective antibody response to NA. A low level of antibodies against NA is induced by immunization with either live-attenuated influenza vaccine or conventional inactivated monovalent vaccine, and the humoral immune response is skewed toward HA (Brett and Johansson, 2005; Johansson et al., 1989). To address this concern, previous studies demonstrated enhanced efficacy of protection against homologous and heterologous viruses by co-immunizing with HA and NA vaccines, using recombinant vaccines co-expressing HA and NA, or use of a high dosage of influenza vaccine (Brett and Johansson, 2005; Cate et al., 2010; Chen et al., 1999; Johansson and
Brett, 2007; Johansson et al., 1998; Pavlova et al., 2009; Qiao et al., 2003). In this regard, NA VLPs might be a promising candidate vaccine for use in combination with current vaccination to extend the level of cross protection.

**Materials and methods**

**Virus and cells**

Influenza A viruses, A/PR/8/1934 (H1N1, abbreviated as A/PR8) and A/Philippines/2/82 (A/Phil) were grown in 10-day old embryonated hen’s eggs and purified from allantoic fluid by using a discontinuous sucrose gradient (15%, 30% and 60%) layers. The purified virus was inactivated by mixing the virus with formalin at a final concentration of 1:4000 (v/v) as described (Novak et al., 1993; Quan et al., 2008a; Sha and Compans, 2000). Inactivation of the virus was confirmed by a plaque assay on confluent monolayer Madin-Darby canine kidney (MDCK) cells and inoculation of the virus into 10-day old embryonated hen’s eggs. For use in challenge experiments, mouse adapted A/PR8 and A/Phil viruses were prepared as lung homogenates of infected mice. Spodoptera frugiperda Sf9 cells were maintained in suspension in serum free SF900II medium (GIBCO-BRL) at 27 °C in spinner flasks. MDCK and CV-1 cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM).

**Preparation of influenza NA VLPs and HA VLPs**

To clone a NA gene, influenza A/PR8 virus was used to infect MDCK cells and total viral RNA was extracted using RNeasy Mini kit (Qiagen). Reverse transcription (RT) and PCR were performed on extracted viral RNA using One-Step RT-PCR system (Invitrogen) with NA gene specific oligonucleotide primers. The following primer pairs were used for the synthesis of NA: 5′-AAAGAATTCAAAATGAATCCAAATCA GAAAATAATA-3′ and 5′- TTACTCGAGCTACTTGTCAATGCTGAATGGCAACT-3′. Following RT-PCR, a PCR DNA fragment obtained by the NA gene specific primer pair was cloned into the pBlueScriptII vector (Invitrogen) and then pFastBac vector. The nucleotide sequences of the NA gene was confirmed by DNA sequencing.

The construct of a baculovirus (BV) expressing influenza A M1 was prepared as previously described (Quan et al., 2007). To generate a recombinant baculovirus (rBV) expressing NA, the transfer vector, pFastBac plasmid and recombinant Bacmid baculovirus DNAs (rAcNPV) containing PR8 NA isolated from transformed DH10Bac cells were used to transfect Sf9 insect cells as described (Kang et al., 2009b). To produce VLPs, Sf9 cells were co-infected with rBVs expressing NA and M1 at a multiplication of infection 2 and 1 respectively, and culture supernatants containing released VLPs were harvested after 2–3 day of culture post infection. After removing cell debris by low-speed centrifugation (2000 g for 20 min at 4 °C), influenza M1-derived NA VLPs (NA VLPs) in cleared culture supernatants were purified by 20%, 30% and 60% sucrose gradient ultracentrifugation. The NA VLPs were found to be located in the fraction between 30% and 60% sucrose. Characterization of influenza VLPs containing A/PR8 NA was performed by western blot using mouse polyclonal antibodies (described below) against recombinant vaccinia virus encoding A/PR8 NA. Anti-M1 antibody (Genesis Biotech Inc) was used to determine M1 in
the NA VLPs. Neuraminidase activity of purified influenza NA VLPs were estimated by NA-Star influenza neuraminidase inhibitor resistance detection kit (Applied Biosystems). Influenza M1 VLPs containing HA derived from A/PR8 (HA VLPs) were prepared as described (Quan et al., 2007).

**Electron microscopy**
To examine budding of VLPs, Sf9 cells infected with rBVs expressing M1 and NA were fixed with 0.25% glutaraldehyde and 1% osmium tetraoxide, dehydrated with ethanol, and then embedded in Epon resin. Thin sections were stained with lead citrate and uranyl acetate and observed by electron microscopy. For negative staining of VLPs, sucrose gradient-purified VLPs (1 to 5 μg) were applied to a carbon-coated Formvar grid for 30 s. Excess VLP suspension was removed by blotting with filter paper, and the grid was immediately stained with 1% sodium phosphotungstate for 30 s. Excess stain was removed by filter paper, and the samples were examined using a transmission electron microscope.

**Production of recombinant vaccinia virus encoding A/PR8 neuraminidase**
The A/PR8 neuraminidase gene fragment from pFastBAc vector described above was cloned in to pRB21 plasmid by the insertion of neuraminidase between the EcoR I and Hind III enzyme sites. Following infection and transfection of CV-1 cells with vaccinia virus and pRB21 plasmid vector respectively, recombinant vaccinia virus was identified and isolated as described (Kang et al., 2005). For analysis of the NA gene incorporation, the cell lysate was amplified by PCR with NA specific primers. The recombinant vaccinia viruses expressing NA were amplified, purified and titrated. Recombinant vaccinia viruses (10^5 pfu) expressing NA were injected into mice twice with a 4-week interval, and sera were used for detection of neuraminidase in VLPs by western blot.

**Neuraminidase activity**
Neuraminidase activity assay was tested by incubation of NA VLPs with a chemiluminescent derivative of neuraminic acid (NA-Star Substrate) and following luminometric detection of the reaction product using the NA-Star Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems). The samples containing NA VLPs were diluted in NA-Star Assay Buffer [26 mM MES (pH 6.0) with 4 mM CaCl2]. Inactivated whole influenza virus and HA VLPs were used as controls. Standard neuraminidase was purified from bacteria Vibrio Cholerae (Roche, 1 U/ml). The luminometric detection was performed on a Modulus Microplate Multimode Reader (Turner Biosystems) and chemiluminescent signal [Relative luminence unit (RLU)] was presented.

**Immunization and challenge**
Female inbred BALB/c mice (Charles River) aged 6 to 8 weeks were used. Groups of mice (12 mice per group) were intranasally immunized twice with 5 μg (total proteins) of NA VLPs (0.006 units of enzyme activity) at a 4-week interval. For virus challenge, isoflurane-anesthetized mice were intranasally infected with A/PR8 virus (20 × LD50), or A/Phil (5 × LD50) in 50 μl of phosphate-buffered saline (PBS) per mouse at week 4 after boost. For measurement of immune response parameters, six mice from each group were sacrificed.
prior to challenge or at day 4 post-challenge. Mice were observed daily to monitor changes in body weight and to record the mortality endpoint (25% loss in body weight). We followed an approved Emory IACUC protocol for this study.

**Neuraminidase inhibition assay**

Neuraminidase activity and inhibition assay was performed following the manufacturer’s procedure of the NA-Star influenza neuraminidase inhibitor resistance detection kit. Sera samples from NA VLPs immunized sera and naïve mouse were diluted in NA-Star Assay Buffer and incubated with $10^5$ pfu (plaque forming unit) of A/PR8 live virus for 30 min at 37 °C. After incubation with substrate, the chemiluminescent signal [Relative luminescence unit (RLU)] was measured by injecting NA-Star accelerator.

**Antibody responses**

Blood samples were collected by retro-orbital plexus puncture before immunization and at week 2 after the first and second immunization. Influenza virus specific antibodies IgG were determined in sera by enzyme-linked immunosorbent assay (ELISA) as described previously (Quan et al., 2007). As coating antigens to measure virus specific antibodies, we used two different coating antigens. One is to determine the reactivity of sera to NA expressed on CV-1 cells. To determine the cell surface-expressed neuraminidase, CV-1 cells were infected with a recombinant vaccinia virus encoding neuraminidase at MOI 0.5 and incubated for 18 h. Cells were then washed and fixed with 0.25% glutaraldehyde for 10 min and used for cell surface ELISA. Another is to determine the reactivity to egg-grown inactivated influenza A/PR8 (H1N1) or A/Philippines/82 (A/Phil, H3N2) virus. Viral antigens were coated onto the ELISA plates in coating buffer (0.1 M sodium carbonate, pH 9.5, and 4 μg inactivated A/PR8 or A/Phil viruses per ml) at 4 °C overnight. Serum IgG1 or IgG2a antibody responses were determined using inactivated A/PR8 or A/Phil virus as ELISA plate coating antigens (Quan et al., 2007, 2008b).

**Hemagglutinin inhibition (HAI) assay and neutralizing activity**

For determination of hemagglutination-inhibition (HAI) titers, serum samples were first treated with receptor destroying enzyme by incubation overnight at 37 °C, and then incubated 30 min at 56 °C. Sera were serially diluted, mixed with 4 HA units (HAU) of influenza A/PR8 virus, and incubated for 30 min at room temperature prior to adding 0.5% chicken red blood cells. The reciprocal of the highest serum dilution preventing hemagglutination was scored as the HAI titer as described (Quan et al., 2010c). Virus neutralization was performed using by a plaque assay on MDCK cells following a previously described procedure (Quan et al., 2007).

**Lung viral titers and cytokine assays**

Whole lung extracts prepared as homogenates using frosted glass slides were centrifuged at 1000 RPM for 10 min to collect supernatants. Lung tissues were placed on the glass slide, minced and rinsed in the DMEM media repeatedly. The lung extract containing viruses was then collected. Determination of viral titers in lung extracts was performed using MDCK cells as previously described (Quan et al., 2007). The detection limit of the virus plaque
assay is 50 pfu/ml. Cytokine ELISA was performed as described (Quan et al., 2007). Ready-Set-Go IFN-γ kits (eBioscience, San Diego, CA) were used for detecting cytokine levels in lung extracts following the manufacturer’s procedure.

**Virus-specific antibody secreting cell (ASC) responses from spleen and bone marrow**

Virus-specific antibody secreting cell responses were determined from bone marrow and spleen at day 4 post-challenge. Since there is a correlation between the number of antibody secreting cell spots and antibody levels secreted into culture supernatants, ELISA was used to determine ASC response as described (Kang et al., 2011; Song et al., 2010). Culture plates (96-well) were coated with inactivated A/PR8 viral antigen overnight. To measure antibody-producing cells in vitro, bone marrow and spleen cells were prepared and placed in coated culture plates. Cells from spleen or bone marrow were added to culture plates after washing. Six mice were used in each group. After 2 and 6 days, cells were removed from plates and secondary antibody IgG-HRP and substrate OPD was added and read at 450 nm (Song et al., 2010).

**Protective efficacy test of immune sera and effects of clodronate-liposomes**

To measure protective efficacy of immune sera in vivo, serum samples (4 x diluted) from immunized mice, infected mice after recovery, or naïve mice were pre-incubated with a lethal dose of influenza virus at room temperature for 30 min as described (Quan et al., 2008a; Song et al., 2011b). A mixture of a lethal infectious dose of A/PR8 (H1N1) influenza virus (3 LD50) and sera was administered to naïve mice (n=4 BALB/c), and body weight changes and survival rates were monitored daily. Liposome-encapsulated clodronate were prepared as previously described (Van Rooijen and Sanders, 1994). Four hrs prior to infection with virus-serum mixture, some groups of naïve mice (n = 4 BALB/c) were intranasally treated with clodronate-liposomes to deplete dendritic and macrophage cells as described (Bosio and Dow, 2005; McGill et al., 2008; Thepen et al., 1991). Clodronate was a kind gift of Roche Diagnostics GmbH, Mannheim, Germany.

**Statistics**

All parameters were recorded for individuals within all groups. Statistical comparisons of data were carried out using the t-test of the SigmaPlot program (Systat Software, Inc.). A P value less than 0.05 was considered to be significant.

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Fig. 1. Characterization of N1 NA VLPs
(A) Influenza NA VLPs containing NA and M1 proteins on Western blot. NA VLPs (10, 5, 1 μg) were separated by SDS-PAGE. NA protein was detected by blotting with mouse serum prepared as described in methods. M1 protein was determined by anti-M1 IgG antibody. (B) Neuraminidase activity (relative luminescence unit (RLU)). NA VLPs: M1 VLPs containing NA (derived from A/PR8 virus), PR8 whole virus: inactivated whole virus, HA VLPs: M1 VLPs containing HA (derived from A/PR8 virus). (C) Electron microscopy of influenza NA VLPs.
Fig. 2. IgG, IgG1 and IgG2a antibody responses
Groups of mice were immunized with NA VLP intranasally with an interval of 4 weeks and blood samples were collected at week 2 after prime (1st sera) and boost (2nd sera). Individual mouse serum was used and the average of antibody response from each group was determined. (A) IgG antibody reactive to N1 NA derived from A/PR8 virus. CV-1 cells were infected with recombinant vaccinia virus expressing A/PR8 NA and used for cell surface ELISA antigen. (B) IgG antibody reactive to the whole inactivated A/PR8 virus. (C) IgG1 and IgG2a isotype antibodies reactive to the whole inactivated A/PR8 (H1N1) virus. (D) IgG and isotypes IgG1 and IgG2a antibodies reactive to the whole inactivated A/Phil (A/Philippines/82, H3N2 subtype) virus.
Fig. 3. Protective immunity to the homologous A/PR8 virus
(A) Serum NA inhibition activity against A/PR8 virus. NA inhibition activity was
determined by using the NA-Star influenza neuraminidase inhibitor resistance detection kit.
Immune sera were from N1 VLP-immunized mice and naïve sera from naïve mice. (B)
Body weight changes. (C) Survival rates. (B–C) At week 4 after boost, mice were
challenged with a lethal dose (20 × LD50) of A/PR8 virus and were monitored daily to
record body weight changes and survival rates. Six mice were used in each group.
Lungs from individual mice in each group (6 mice) were collected at day 4 after A/PR8/34 virus challenge, and lung virus titers (A) and lung IFN-γ concentrations (B) were determined. NA VLP+PR8: NA VLP vaccinated mice with A/PR8 virus challenge. Naïve +PR8: Naïve mice with A/PR8 virus challenge.
Fig. 5. Antibody-secreting cell (ASC) responses
Spleen and bone marrow samples were collected from individual mice immunized with NA VLPs in each group (6 mice) at day 4 post challenge. Antibody levels produced during in vitro cultures for 2 days (D2) and 6 day (D6) as an indicator of ASC responses specific to the A/PR8 virus were expressed in ng/ml as determined by ELISA.
Fig. 6. Protective immunity to the heterosubtypic A/Phil (H3N2) virus
Protection against the heterosubtypic A/Phil (H3N2) virus was assessed by determination of lung virus titers, body weight changes and survival rates after A/Phil virus challenge (5 × LD50). (A) Lung viral titers at day 4 post challenge. (B) Body weight changes after A/Phil challenge. (C) Survival rates after A/Phil challenge. Six mice were used in each group.
Fig. 7. Protective efficacy of NA VLP immune sera and effects of clodronate-liposomes

Immune sera collected from NA VLP vaccinated mice at 2 weeks after boost vaccination were incubated with a lethal dose of A/PR8 virus at room temperature for 30 min. Groups of mice (n=4) were intranasally infected with a lethal dose mixed with NA VLP immune sera or naïve sera. (A) Body weight changes. (B) Survival rates. (C–D) To deplete dendritic cells and alveolar macrophages in lungs, groups of naïve mice (n=4) were intranasally instilled with clodronate-liposomes 4 h prior to lethal infection. A lethal dose of A/PR8 (H1N1) virus was incubated with sera collected from NA VLP vaccinated mice, infected mice (Infection), or naïve mice (Naïve). Each mixture of virus and sera was used to infect naïve mice with clodronate-liposome pretreatment. (C) Body weight changes against A/PR8 virus. (D) Survival rates against A/PR8 virus.