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Monophosphoryl Lipid A-Adjuvanted Virosomes with Ni-Chelating Lipids for Attachment of Conserved Viral Proteins as Cross-Protective Influenza Vaccine

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Induction of $CD8^+$ cytotoxic T cells (CTLs) to conserved internal influenza antigens, such as nucleoprotein (NP), is a promising strategy for the development of cross-protective influenza vaccines. However, influenza NP protein alone cannot induce CTL immunity due to its low capacity to activate antigen-presenting cells (APCs) and get access to the MHC class I antigen processing pathway. To facilitate the generation of NP-specific CTL immunity the authors develop a novel influenza vaccine consisting of virosomes with the Toll-like receptor 4 (TLR4) ligand monophosphoryl lipid A (MPLA) and the metal-ion-chelating lipid DOGS-NTA-Ni incorporated in the membrane. In vitro, virosomes with incorporated MPLA induce stronger activation of APCs than unadjuvanted virosomes. Virosomes modified with DOGS-NTA-Ni show high conjugation efficacy for his-tagged proteins and facilitate efficient uptake of conjugated proteins by APCs. Immunization of mice with MPLA-adjuvanted virosomes with attached NP results in priming of NP-specific CTLs while MPLA-adjuvanted virosomes with admixed NP are inefficient in priming CTLs. Both vaccines induce equally high titers of NP-specific antibodies. When challenged with heterosubtypic influenza virus, mice immunized with virosomes with attached or admixed NP are protected from severe weight loss. Yet, unexpectedly, they show more weight loss and more severe disease symptoms than mice immunized with MPLA-virosomes without NP. Taken together, these results indicate that virosomes with conjugated antigen and adjuvant incorporated in the membrane are effective in priming of CTLs and eliciting antigen-specific antibody responses in vivo. However, for protection from influenza infection NP-specific immunity appears not to be advantageous.

1. Introduction

Influenza A virus infections cause considerable mortality and morbidity to the human population during seasonal epidemics and occasional pandemics. Depending on the sequence of the hemagglutinin (HA) and neuraminidase (NA) carried on the surface of the virus particle, influenza A virus can be divided into various subtypes. During seasonal epidemics, caused by H1N1 or H3N2 viruses, 5–10% of the worldwide population is infected, resulting in 3–5 million people suffering from severe illness and up to 500 000 deaths per year.[1,2] During the last influenza pandemic caused by H1N1pdm virus in 2009, it was estimated that the overall cumulative incidence of infection was 24% and as many as 284 500 people succumbed to H1N1pdm-related illness.[3,4] Additionally, avian influenza viruses, such as H7N9 and H5N1, are also reported to cause high mortality in humans yet do not transmit efficiently from human to human.[5,6]

Vaccination is the primary strategy to prevent influenza virus infection. Currently, licensed influenza vaccines include whole inactivated influenza virus, split virus, subunit, and virosomal vaccines.[7] These vaccines mainly induce influenza strain-specific antibodies which can bind to the virus surface proteins, HA and NA. However, due to the high variability of HA and NA the vaccines need

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to be reformulated each year to match the circulating influenza virus strains but still they are unable to protect against newly emerging strains. An influenza vaccine which can induce cross-protective immune responses to diverse influenza virus strains is, therefore, urgently needed.

$CD8^+$ cytotoxic T cells (CTLs) to conserved influenza virus antigens, such as nucleoprotein (NP), are required for optimal cross-protective immune responses.[8,9,10] Influenza virus-specific CTLs can clear virus-infected cells and subsequently stop virus replication and virus spread in the body. Therefore, inducing CTLs to conserved influenza virus protein is an attractive approach for eliciting cross-protective immunity against various influenza virus strains.

Antigen-presenting cells (APCs) play a key role in priming CTL responses.[11] Priming of CTLs by non-replicating vaccines and by pathogens which do not infect APCs directly, relies on a mechanism called cross-presentation.[12] Cross-presentation requires uptake by APCs of sufficient amounts of antigen which can enter the MHC class I processing and presentation pathway as well as proper activation of the APCs to trigger physiological processes involved in cross-presentation.[11,12] A conserved protein, such as NP, alone is thus ineffective in inducing $CD8^+$ T cell immunity due to its low capacity to reach the MHC class I processing pathway and its inability to activate APCs.[13,14]

Uptake of antigen by APCs can be facilitated by use of a delivery system. Influenza virosomes are reconstituted viral membrane envelopes which consist only of the membrane lipids and the surface proteins of influenza virus. Virosomes retain the cell binding and membrane fusion abilities of the live virus. Due to these properties, virosomes can deliver encapsulated cargo to the cytosol of APCs and subsequently induce CTL responses.[15] However, only limited amounts of protein can be encapsulated into virosomes when adding the protein during virosome reconstitution.[16] Moreover, due to a lack of adjuvant virosomes are inefficient in activating APCs and thus in triggering cross-presentation, which limits the induction of CTL immunity.[17] Increasing the amount of antigen associated with the virosomes and adding an adjuvant to enable APC activation and thus improve cross-presentation may significantly enhance the induction of CTLs.

In this study, we used two strategies to modify influenza virosomes to overcome the above-mentioned drawbacks. Firstly, a nickel-chelating lipid was incorporated into the lipid bilayer of influenza virosomes. The presence of nickel-carrying lipids facilitates binding of large amounts of histidine (his)-tagged protein to the surface of lipid-based carriers.[18] Secondly, monophosphoryl lipid A (MPLA), a Toll-like receptor 4 (TLR4) ligand, was incorporated into the virosomal membrane.[19] TLR 4 engagement on APCs not only results in activation of the cells but can also promote cross-presentation.[20,21] These novel "all-in-one" influenza virosome particles, with his-tagged NP attached to the membrane and incorporated MPLA, were tested in vitro and in vivo.

2. Experimental Section

2.1. Material

Monophosphoryl lipid A (MPLA), 1,2-dihexanoylan-glyero-3-phosphocholine (DCPC), the nickel salt of 1,2-dioleoyl-sn-glycero-3-{[N-(5-amino-1-carboxypentyl) iminodiacetic acid] succinyl} (DOGS-NTA-Ni) and the ammonium salt of 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid) succinyl] (DOGS-NTA) were purchased from Avanti Polar Lipids (Alabaster, AL).

2.2. Expression and Purification of His-Tagged Recombinant Proteins

Recombinant pET32a-NP and pET32a-EGFP plasmids were generated by inserting the NP gene derived from A/Hongkong/2/1968 (H3N2, called HK68 in the following) or the gene encoding enhanced green fluorescent protein (EGFP) into vector pET32a (Merck Millipore, Germany). The recombinant plasmids were transfected into competent *Escherichia coli* AD494 (Merck Millipore) and the cells were cultured in LB broth supplemented with ampicillin and kanamycin. When an OD_{600} of 0.8 was reached, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce the expression of the his-tagged proteins. His-tagged proteins were purified by Ni-chelate affinity resin (Merck Millipore). Briefly, BugBuster protein extraction reagent (Merck Millipore) with 6 M urea was used for lysis of transfected *E. coli*. Ni-NTA resin was equilibrated with binding buffer (0.1 M HEPES, 0.5 M NaCl) and then incubated with *E. coli* lysate at 4 °C overnight with rotation. The resin column was then washed twice with washing buffer (0.1 M HEPES, 0.5 M NaCl, 20 mM imidazole). Then, 1.8 ml of elution buffer (0.1 M HEPES, 0.5 M NaCl, 400 mM imidazole) with 6 M urea were added and incubated with the column at room temperature for 10 min. After that, the eluted samples were collected and the presence of his-tagged protein was analyzed by SDS–PAGE on 10% gel stained with Coomassie blue. The purity of the proteins was more than 90% (Figure S1, Supporting Information). An endotoxin removal resin column (Thermo Fisher, Germany) was used to remove residual endotoxin from these proteins. Determination of residual endotoxin revealed that 99% of endotoxin was removed by this procedure. After that, proteins were dialyzed against HEPES-buffered saline (HBS; 5 mM HEPES, 3 mM EDTA, 0.15 M NaCl, pH 7.4) to remove imidazole and urea. The concentrations of the proteins were determined by micro-Lowry.[22] The purified proteins were stored at 4 °C until use.

2.3. Influenza Virus and Cell Culture

A/New Caledonia/20/1999 (H1N1, NC99 in the following) was kindly provided by Solvay Biologicals (Weesp, The Netherlands). A/HongKong/2/1968 (H3N2, HK68 in the following) virus for mouse challenge was kindly provided by Guus F. Rimmelzwaan (Erasmus MC, Rotterdam, The Netherlands). NC99 was inactivated by overnight incubation with 0.1% β-propiolactone (Thermo Fisher Scientific, Geel, Belgium) in citrate buffer (125 mM sodiumcitrate, 150 mM sodium chloride, pH 8.2) at 4 °C with rotation.

RAW-Blue™ cells were purchased from InvivoGen (USA) and maintained according to the manufacturer's protocol.

2.4. Preparation of Modified Influenza Virosomes

Influenza virosomes were prepared from inactivated NC99 influenza virus according to the protocol published before with some modification.[23,24] Briefly, DOGS-NTA-Ni or DOGS-NTA, dissolved in chloroform, were mixed with MPLA, dissolved in ethanol, at a ratio of 100 nmol DOGS:100 μg MPLA. The solvents were dried under a stream of nitrogen and the lipid film was further dried under vacuum in a SpeedVac centrifuge for 3 h. Meanwhile, inactivated influenza virus was disrupted with 0.1 mM DCPC in HBS and the nucleocapsid was removed by ultracentrifugation. The supernatant containing the viral surface proteins and membrane lipids (1 μmol phospholipids) was collected and was incubated with the dried film of lipid (containing MPLA and DOGS lipids) at room temperature for half an hour on ice. Tubes were inverted every 5 min to dissolve the lipid mixtures. Modified virosomes were reconstituted by removal of DCPC by dialysis against HBS buffer overnight with Slide-A-Lyzer Mini Dialysis Devices (10K MWCO) (Thermo Fisher, Germany). The buffer was changed on the second day for another 4 h of dialysis. Non-incorporated material was removed from the virosomes on a discontinuous sucrose gradient (10–30–50%, w/v). Subsequently, the sucrose in the virosomes was removed by dialysis against HBS. The concentration of virosomal proteins was determined by micro-Lowry. These modified virosomes were kept at 4 °C until further use.

The composition of and the nomenclature for the different virosome preparations used in the following are described in Table S1, Supporting Information.

2.5. Characterization of MPLA Incorporation

To evaluate the incorporation of MPLA into the virosomes, the mouse macrophage reporter cell line RAW-Blue™ was used. Lipid-modified virosomes were incubated with RAW-Blue™ cells (10^5/well) in a 96-well plate overnight at 37 °C in a 5% CO_2 incubator. QUANTI-Blue (Invivogen, USA) was added to 40 μl of the supernatant. After 10 min incubation at room temperature, the absorbance at 650 nm was measured in an ELISA reader. *E. coli* lipopolysaccharide (LPS; Sigma–Aldrich Chemie B.V Zwijndrecht, the Netherlands) and CpG oligodeoxynucleotides 1826 (CpG ODN; Eurogentec, Maastricht, the Netherlands) were used as positive controls.

2.6. Conjugation of His-Tagged Protein to Modified Virosomes

Plain virosomes without (V_{Ni-}) and with DOGS-NTA-Ni (V_{Ni+}) and virosomes with MPLA without (M/V_{Ni-}) and with DOGS-NTA-Ni (M/V_{Ni+}) (100 μg virosomal protein) were incubated with his-tagged EGFP (100–800 μg) at room temperature for 30 min with rotation. The conjugation of his-tagged EGFP with virosomes was analyzed by equilibrium density gradient centrifugation on a 10–30–50% sucrose gradient. The gradient was centrifuged in a TLS55 rotor at 35 000 rpm for 90 min. Subsequently, fractions of the gradient were analyzed for protein and fluorescence by ELISA reader (BioTech, USA). The fractions containing virosomes with attached his-tagged EGFP were collected and the sucrose was removed as before. The purified virosomes with attached his-tagged EGFP (V_{Ni+}-EGFP, M/V_{Ni+}-EGFP in the following) and the mixture of virosomes with unattached his-tagged EGFP (V_{Ni-}+EGFP, M/V_{Ni-}+EGFP in the following) were quantified by micro-Lowry and kept at 4 °C until use.

The binding of his-tagged NP to modified virosomes (100 μg virosomal protein + 100–400 μg NP) was performed as above. The purified virosomes with attached his-tagged NP (V_{Ni+}-NP, M/V_{Ni+}-NP in the following) and the mixture of virosomes with unattached his-tagged NP (V_{Ni-}+NP, V_{Ni-}/M+NP in the following) were quantified by micro-Lowry and kept at 4 °C until use.

2.7. Uptake of Free and Virosome-Conjugated EGFP by APCs

RAW-Blue™ cells were used to determine the uptake of his-tagged proteins as follows. V_{Ni+}-EGFP was prepared by incubating his-tagged EGFP (0 μg, 1.25 μg, 2.5 μg, or 5 μg) with V_{Ni+} (1 μg) as above. Free his-tagged EGFP (0 μg, 1.25 μg, 2.5 μg, or 5 μg) or V_{Ni+}-EGFP were then incubated with RAW-Blue™ cells (5×10^5/tube) at 37 °C for 1 h. Cells were washed with FACS buffer (PBS with 2% FBS) 3 times and analyzed on a FACS Calibur™ BD II flow cytometer. Data were analyzed by Kaluza® Flow Cytometry Analysis Software.

2.8. Vaccination

Animal experiments were performed according to the guidelines provided by the Dutch Animal Protection Act and the protocols were approved by the Animal Ethics Committee (DEC) of the University Medical Center Groningen (UMCG).

Six to eight weeks old female C57BL/6JOlaHsd mice (Envigo, The Netherlands) were separated into 5 groups of 12 mice. Mice were intramuscularly immunized with 25 μl vaccine in each hind leg on day 0 and day 21 under isoflurane anesthesia. Vaccines included virosomes with MPLA (M/V; 2.5 μg virosomal protein), M/V_{Ni+}-NP (2.5 μg virosomal protein with 10 μg his-NP), M/V_{Ni-}+NP (2.5 μg virosomal protein with 10 μg his-NP) or free NP protein alone (10 μg his-NP). PBS served as negative control. On day 28, mice were challenged by intranasal administration of 10^3 $TCID_{50}$ of HK68 virus in 40 μl of HBS under isoflurane anesthesia. On day 31, 6 mice in each group were sacrificed and serum samples, lung tissues, and spleen tissues were collected for further experiments. The remaining mice were monitored daily for 14 days to assess disease symptoms and potential recovery.

2.9. Elispot Assays

Influenza NP-specific IFN-γ producing cells in spleen were determined using an Elispot kit (eBioscience, The Netherlands) according to the manufacturer's protocol. Briefly, 96-well

Multiscreen PVDF filter plates (Millipore, Billerica, MA) were activated with 50 µl of 70% ethanol for 2 min and then washed three times with PBS. Anti-mouse IFN-γ capture antibodies were added to the plates and incubated at 4 °C overnight. On the next day, the plates were washed 3 times and blocked with DMEM medium with 10% FBS for 2 h at room temperature. Subsequently, splenocytes were added (5×10^5/well) with or without 5 µg ml^{-1} of NP peptide (ASNENMDAM; a H2-Db-restricted epitope of NP of H3N2 virus) and then incubated at 37 °C in IMDM complete medium overnight. After overnight incubation, plates were washed 5 times and IFN-γ producing cells were detected by using horseradish peroxidase (HRP) conjugated anti-mouse IFN-γ antibodies. Spots were detected by using an ACE staining kit (Sigma, Sigma–Aldrich, USA) and counted by AID Elispot reader (Autoimmune Diagnostika GmbH, Strassberg, Germany). The number of antigen-specific IFN-γ producing cells was calculated by subtracting the number of spots detected in the unstimulated samples from the number in stimulated samples.

2.10. Virus Titration in Lung Tissue

Lung tissue collected at day 3 post challenge was homogenized in 1 ml of PBS and centrifuged at 1200 rpm for 10 min. Supernatants were collected, aliquoted, and stored at −80 °C until use. Lung virus titers were determined by infection of MDCK cells in 96-well plates with serial dilutions of the lung supernatants as described before.[25]

2.11. ELISA and Microneutralization Assay

Serum antibodies against the surface proteins of HK68 virus and antibody against NP protein were detected by ELISA as previously published.[24] Briefly, 0.3 µg of HK68 subunit vaccine prepared as before or 0.3 µg of his-tagged NP protein was coated on 96-well plates overnight in 100 µl of coating buffer (0.05 M carbonate bicarbonate, pH 9.6–9.8). Then, plates were blocked with 200 µl of 2.5% milk powder solution in coating buffer for 45 min at 37 °C. A series of twofold diluted serum samples was then added to the plate and incubated for 1.5 h for 37 °C. Subsequently, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody was added and incubated for 1 h at 37 °C. O-phenylene-diamine(OPD) was used as a substrate to react with HRP. The reaction was stopped by adding 50 µl of 2 M H_2SO_4. The plates were then read at 492 nm. IgG titers were calculated as the log value of the reciprocal of the dilution of serum sample corresponding to an OD492nm of 0.2. Microneutralization (MN) assay was performed as described previously.[25]

2.12. Statistics

Differences between read-outs of two different vaccination groups were analyzed using Mann–Whitney *U*-test. $p < 0.05$ was considered as significantly different.

3. Results

3.1. Incorporation of MPLA Enhances the APC-Activating Capacity of Modified Virosomes

MPLA has been tested as adjuvant because it can activate APCs through engagement with TLR4 expressed on the cell surface. We first determined whether the modified influenza virosomal vaccines can activate APCs utilizing the RAW-Blue™ cell line which stably expresses all TLRs (except TLR5) and contains a reporter gene, secreted embryonic alkaline phosphatase (SEAP), inducible by NF-κb and AP-1. RAW-Blue™ cells were cultured with either influenza virosomes only (V), virosomes with incorporated MPLA (M/V), virosomes with incorporated DOGs-NTA-Ni (V_{Ni+}), or the TLR agonists CpG or LPS. As shown in **Figure 1**, the TLR ligands LPS and CpG activated the NF-κb signaling pathway resulting in the production of SEAP. M/V also strongly activated the RAW-Blue™ cells. In contrast, virosomes without MPLA (V, V_{Ni+}) failed to activate NF-κb signaling. These results demonstrate that MPLA was successfully incorporated into virosomes enabling the virosomes to activate APCs in vitro. Moreover, V_{Ni+} did not activate RAW-Blue™ cells indicating that presence of DOGs lipids alone is insufficient for activation of APCs.

3.2. His-Tagged Protein is Conjugated to Modified Virosomes

To assess the conjugation of his-tagged protein to modified virosomes we incubated his-tagged EGFP with different types of modified virosomes. The presence of the histidine residues provides a tag that can bind with Ni^+ chelating lipid. After mixing his-tagged EGFP with modified influenza virosomes, including V_{Ni-}, V_{Ni+}, and M/V_{Ni+}, the conjugation of his-tagged protein to these modified virosomes was determined by sucrose density gradient centrifugation. When incubating his-tagged EGFP with

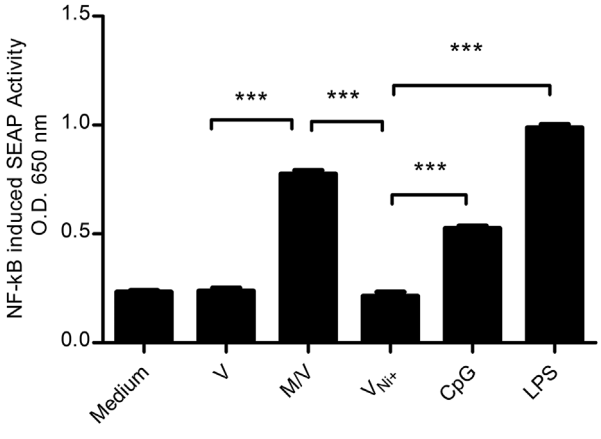

Figure 1. Bioactivity of modified virosomes in vitro. RAW-Blue cells were co-cultured with the indicated virosome preparations or with TLR ligands. After overnight incubation, SEAP activity in the supernatant was detected by QUANTI-Blue. Representative of two independent experiments. Data represent mean O.D. values ± SEM. $^{***}p < 0.0001$, Mann–Whitney *U*-test.

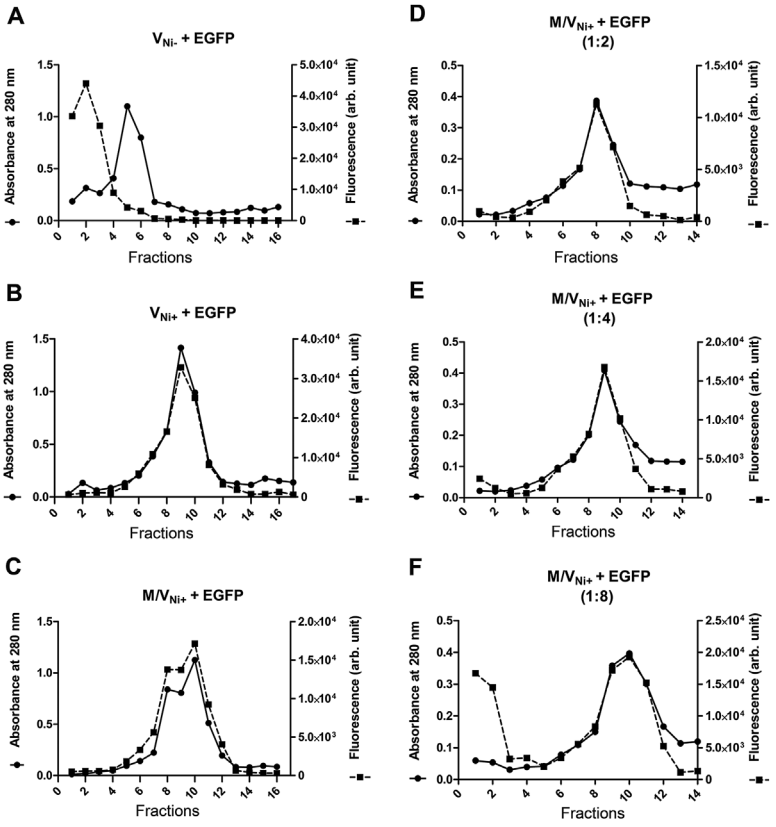

Figure 2. Conjugation ability and binding capacity of modified virosomes for his-tagged EGFP. A–C) Modified virosomes (100 µg), including V_{Ni-} (A), V_{Ni+} (B), and M/V_{Ni+} (C), were mixed with his-tagged EGFP (100 µg) at room temperature for 30 min. Subsequently, free his-tagged EGFP protein was separated from conjugated EGFP on a 10–30–50% sucrose gradient by ultracentrifugation. D–F) M/V_{Ni+} (100 µg) were mixed with various amounts (D: 200 µg, E: 400 µg, F: 800 µg) of his-tagged EGFP at room temperature for 30 min. Subsequently, free his-tagged EGFP protein was separated from conjugated EGFP on a 10–30–50% sucrose gradient by ultracentrifugation. The absorbance at 280 nm and the fluorescence of each fraction were determined by ELISA reader as described in Section 2. The solid line represents the absorbance of protein in each fraction at 280 nm while the dashed line represents the fluorescence of his-tagged EGFP. Data representative of three independent experiments.

virosomes without Ni (V_{Ni-}), the fluorescence of his-tagged EGFP was mainly detected in the top fraction while virosomal protein was mainly detected in the middle fraction of the gradient (**Figure 2**A). In contrast, virosomal and fluorescent protein migrated to the same fractions of the gradient when his-tagged EGFP was incubated with V_{Ni+} (Figure 2B). This indicates that the presence of Ni^{+} is crucial for the conjugation of his-tagged protein to virosomes. Moreover, as shown in Figure 2C, after incubating his-tagged EGFP with M/V_{Ni+}, protein and fluorescence signal also migrated together to the same fractions of the gradient, which demonstrates that incorporation of MPLA into virosome did not interfere with the conjugation of his-tagged protein to membrane-incorporated DOGS-NTA-Ni.

To determine the binding capacity of M/V_{Ni+} for his-tagged protein, a fixed amount of M/V_{Ni+} virosomes was incubated with different amounts of his-tagged EGFP as indicated in Section 2. The conjugation of his-tagged protein to the virosomes was then analyzed by sucrose density gradient centrifugation. Protein and fluorescent signals from different fractions of the sucrose gradient showed that all the his-tagged EGFP co-migrated with the virosomal protein to the same fraction of the sucrose gradient when the ratio of virosomal protein to his-tagged EGFP was 1:2 or 1:4 (Figure 2D and E). In contrast, a strong fluorescent signal was found in the top fractions of the gradient when the ratio of virosomal protein:EGFP was 1:8, indicating that only part of the his-tagged EGFP was conjugated to M/V_{Ni+} under those conditions (Figure 2F). M/V_{Ni+} showed similar conjugation capacity for his-tagged NP protein. At virosomal protein:NP ratios of 1:2 and 1:3 all NP was bound to the virosomes but at a ratio of 1:5 NP started to appear in the top fraction of the gradient (Figure S2, Supporting Information). Taken together, M/V_{Ni+} showed high conjugation efficacy for his-tagged proteins.

3.3. Modified Virosomes Facilitate the Uptake of Conjugated His-Tagged Protein by APCs

We hypothesized that conjugation of a protein to influenza virosomes will facilitate its uptake by APCs. The uptake of his-tagged protein by APCs was determined by incubating M/V_{Ni+}-EGFP or free EGFP protein with RAW-Blue™ cells and quantification of internalized EGFP by flow cytometry. As shown in **Figure 3**, after 1 h incubation, free EGFP was not internalized efficiently by RAW-Blue™ cells. However, EGFP conjugated to virosomes was readily taken up by RAW-Blue™ cells, as indicated by a marked increase in mean fluorescence intensity. Moreover, higher amounts of the M/V_{Ni+}-EGFP

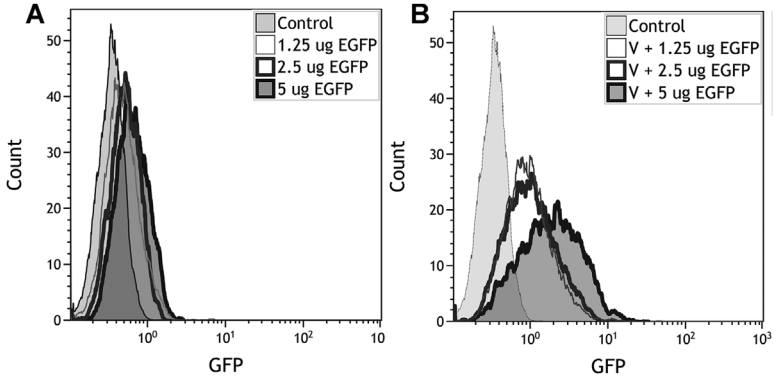

Figure 3. Uptake of free EGFP or conjugated EGFP by APCs in vitro. RAW-Blue™ cells were incubated with various amounts of free EGFP (A) or modified virosomes (M/V_{Ni+}) with conjugated EGFP (B) at 37 °C for 1 h and then the uptake of EGFP was analyzed by flow cytometry.

resulted in more uptake by APCs. These results indicate that conjugation of his-tagged protein to virosomes enhances antigen uptake by APCs.

3.4. Virosomes with Associated NP Induce Cytotoxic T Lymphocytes and NP-Specific Antibodies

We next evaluated the immunogenic properties of the modified virosomes in vivo. We hypothesized that attachment of NP and incorporation of MPLA into virosome would facilitate the cross-presentation of NP thereby allowing induction of NP-specific CTLs which could aid in protection from virus challenge. Moreover, immunization with modified virosomes with NP was expected to induce NP-specific antibodies.

Modified virosomes were prepared from NC99 (H1N1) while NP protein was derived from HK68 (H3N2). C57BL/6 mice were primed and boosted with either M/V_{Ni-}+NP, M/V_{Ni+}-NP, free NP mixed with MPLA or M/V on day 0 and day 21. Control animals received PBS.

To identify NP-specific IFN-γ producing cells against HK68 NP, the $NP_{366–374}$ epitope (ASNENMDAM) present in HK68 NP but absent from NC99 virus was used to stimulate mouse splenocytes. As indicated in **Figure 4**A, only M/V_{Ni+}-NP induced significant numbers of NP-specific IFN-γ producing T cells while the number of NP-specific CTLs found after immunization with M/V_{Ni-}+NP, M/V_{Ni+}, or free NP protein mixed with MPLA was not significantly different from the number observed in PBS control mice.

To detect NP-specific antibody responses, mouse serum samples taken 3 days post challenge were analyzed by ELISA. As shown in Figure 4B, NP-specific antibody titers in each of the NP-vaccinated groups (M/V_{Ni-}+NP, M/V_{Ni+}-NP, and NP+MPLA), were significantly higher than in the PBS control group. Moreover, M/V_{Ni-}+NP and M/V_{Ni+}-NP vaccination induced higher levels of NP-specific antibodies compared to vaccination with MPLA-adjuvanted free NP.

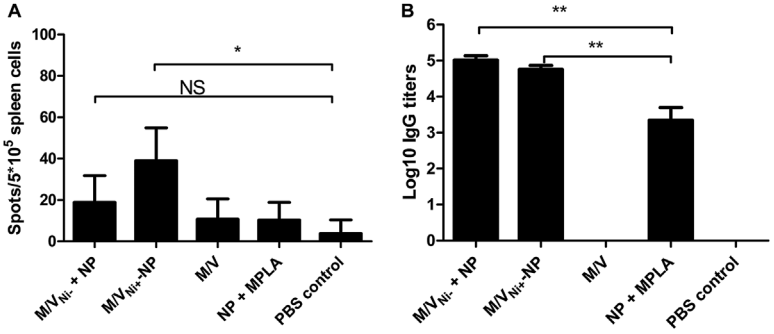

Figure 4. Immunogenicity of modified virosomes in vivo. C57BL/6 mice were immunized either with the mixture of free NP with virosomes (M/V_{Ni-}+NP), NP conjugated virosomes (M/V_{Ni+}-NP), MPLA conjugated virosomes without NP (M/V), the mixture of NP with MPLA, or PBS on day 0 and day 21. On day 28, mice were challenged intranasally with 10^3 $TCID_{50}$ of HK68. On 3 days post challenge (day 31), NP-specific CD8 T cell immunity in splenocytes was determined ex vivo by ELISPOT (A). NP-specific IgG in serum was analyzed by ELISA (B). Data represent mean ± SEM ($n = 4$). $^{*}p < 0.05$, $^{**}p < 0.005$. NS, not significant.

3.5. Virosomal Vaccines Induce Cross-Protective Immunity Against Virus Infection

Next, the protective potential of the modified virosome vaccines against heterosubtypic virus infection was determined. Mice were vaccinated twice as described above with modified NC99 (H1N1)-derived virosomes with admixed or attached HK68 NP, with HK68 NP+MPLA or with plain virosomes and were subsequently challenged with a lethal dose of HK68 (H3N2) virus.

After virus challenge, five out of six mice in the PBS control group developed severe symptoms and lost more than 20% weight (humane endpoint) necessitating euthanasia on day 7 or 8 post infection (**Figure 5**A and B). Mice that were immunized with the mixture of NP protein and MPLA showed more rapid weight loss than mice in the PBS control group, but three mice in this group started to recover from day 5 post infection onwards. In the M/V_{Ni-}+NP vaccinated group, four out of six mice showed rapid weight loss as observed in the NP+MPLA group. However, these mice started to recover from day 7 post infection and none of the mice needed to be sacrificed. In the M/V_{Ni+}-NP vaccinated group, one out of six mice had to be sacrificed on day 5 post infection. The other five mice started to recover by day 6 or day 7 post infection after a period of moderate weight loss. Surprisingly, in the M/V vaccinated group, mice only showed mild weight loss (around 10%) and all mice recovered quickly.

Determination of virus in lung tissue on day 3 post-infection revealed almost identical mean virus titers of about $_{10}$log 4.5 for all experimental groups (Figure S3, Supporting Information). Thus, infection and initial virus growth were not affected by any of the vaccines.

To explore the possible mechanisms responsible for cross-protection, sera collected on day 3 post infection from vaccinated mice were evaluated for antibodies recognizing HK68 virus. As shown in **Figure 6**A, MPLA-adjuvanted virosomes derived from NC99 virus readily induced IgG cross-reactive with HK68 surface proteins (HA+NA) in an ELISA assay. However, these antibodies were unable to neutralize HK68 virus particles (Figure 6B).

Taken together, these results indicate that virosomes with conjugated NP could elicit the production of NP-specific CTLs and NP-specific antibodies. However, NP-specific immunity was not required for protection from severe disease symptoms upon challenge and might even have resulted in immunopathology. Thus, immunization with (NP) virosomes protected mice against infection with heterosubtypic HK68 virus by a so far non-identified mechanism.

4. Discussion

In this study, we developed a novel virosomal influenza vaccine, aiming to enhance the generation of influenza-specific CD8 T cell responses against the NP antigen. To this end, MPLA and DOGs-NTA-Ni lipid were incorporated into the lipid bilayers of influenza virosomes. In vitro, M/V strongly activated

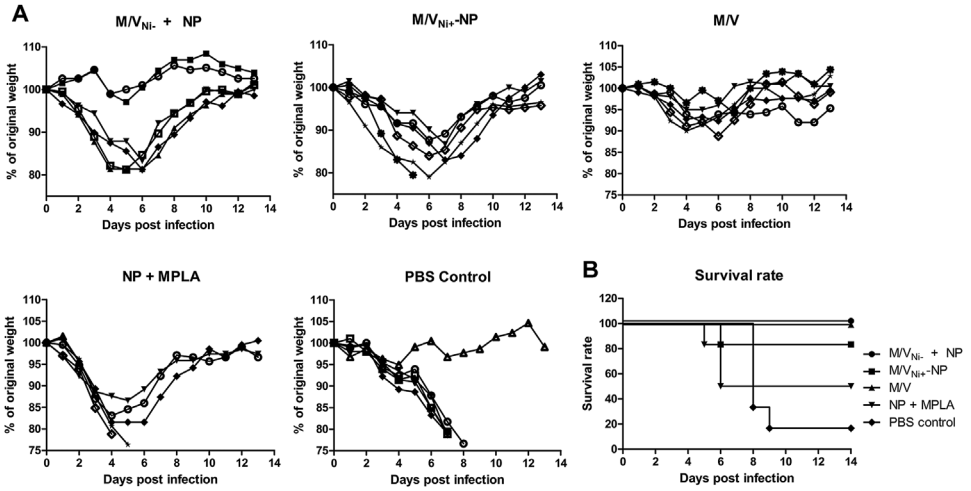

Figure 5. Weight loss and survival rate after heterosubtypic challenge. Mice ($n = 6$) were vaccinated and challenged as described in **Figure 4**. After challenge, mice were monitored daily for weight loss (A) and survival rate (B). Loss of more than 20% of original weight was considered as humane endpoint.

RAW-Blue^{TM} cells. M/V$_{Ni+}$ exhibited high conjugation ability to his-tagged EGFP or NP protein and significantly enhanced the uptake of his-tagged protein by APCs. Furthermore, while virosomes with admixed or conjugated NP induced equal levels of NP-specific antibodies, only virosomes with conjugated NP mounted an NP-specific CD8 T cell response. Yet, upon live virus challenge NP-specific immunity was not required for protection since MPLA-adjuvanted virosomes without NP provided the best protection.

Activation of APCs is a prerequisite for efficient induction of CTLs by cross-presentation. We found that virosomes with incorporated MPLA exhibited a stronger ability to activate RAW-Blue^{TM} cells than plain virosomes. Incorporation of MPLA into membranous delivery systems has been described before. A study reported that incorporation of MPLA into virosomes derived from respiratory syncytial virus (RSV) strongly enhanced the ability of the virosomes to activate mouse APCs.[19] Another study reported that incorporation of MPLA into glycoliposomes not only induced DC maturation, but also enhanceds cross-presentation of liposome-conjugated tumor antigen to CD8 T cells.[26] Activation of APCs by membrane-incorporated MPLA induces the expression of co-stimulatory molecules on the cell surface and increases cytokine release,[19,26,27] thereby enabling efficient generation of CD8 T cell immunity.

Next to proper activation of APCs, an important prerequisite for the induction of CTLs by non-living vaccines is the delivery of sufficient antigen to the cells. Antigen delivery depends on adequate loading of antigen in or on the delivery device and efficient uptake of the loaded delivery device by APCs. We show that incorporation of DOGS-NTA-Ni into influenza virosomes significantly enhanced the conjugation of his-tagged EGFP or NP to virosomes (Figure 2). DOGS-NTA-Ni was earlier shown to significantly increase the association of therapeutically active his-tagged peptides to liposomes without influencing the biological function of these peptides.[18] Another study reported by Masek et al. demonstrates that DOGS-NTA-Ni facilitates the attachment of his-tagged heat shock protein from *Candida albicans* (hsp90-CA) onto liposome.[28] We used DOGS-NTA-Ni lipid for the first time with virosomes which have a membrane that is densely covered with viral surface proteins. Nevertheless, presence of DOGS-NTA-Ni allowed the conjugation of large amounts of protein to the virosomes indicating that the presence of the viral surface proteins did not sterically hinder binding of his-tagged protein. Originally, passive encapsulation was used to generate protein- or peptide-carrying influenza virosomes.[16,17] However, only approximately 225 ovalbumin (OVA) molecules could be encapsulated per virosomal particle using this technique.[16] In contrast, we estimate that one particle of DOGS-NTA-Ni-containing virosomes could conjugate with about 27 500 molecules of EGFP (equal to 4 μg EGFP/μg

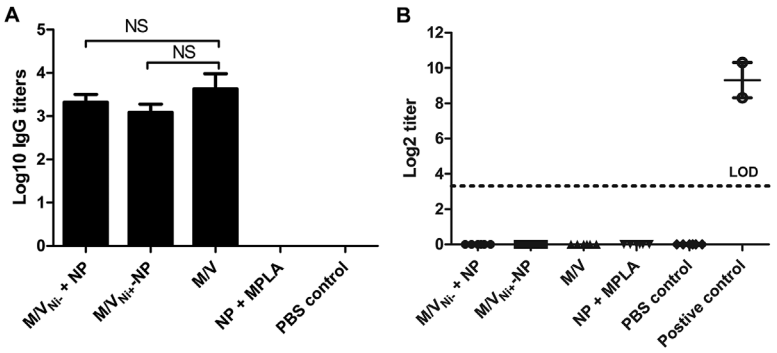

Figure 6. Cross-reactive activity of vaccination-induced antibodies against HK68 virus. Mice ($n = 6$) were vaccinated and challenged as described in **Figure 4**. On day 3 post challenge (day 31), serum antibodies against surface protein (HA+NA) of HK68 virus were determined by ELISA (A). Neutralizing antibodies against HK68 virus were determined by MN assay (B). Data represent mean ± SEM. NS, not significant. LOD, limit of detection.

virosomal protein) or 12 000 molecules of NP (equal to 3 μg NP/μg virosomal protein). Thus, the incorporation of DOGS-NTA-Ni facilitated binding of approximately 50 times more NP protein than could have been achieved by passive encapsulation.

Interaction of antigen and delivery system is important for the uptake of antigen by APCs and induction of antigen-specific CTLs. We demonstrate that the conjugation of his-tagged EGFP to the membrane of M/V_{Ni+} significantly enhanced the uptake of the protein by APCs. Our results are in line with those reported by Soema et al. who show that encapsulation of M1 peptide in virosomes strongly increased the uptake of the peptide by DCs in vitro.[17] Another study demonstrates that loading melanoma tumor antigen gp100 peptide to MPLA-incorporating glycoliposomes efficiently enhanced the cross-presentation of peptide to CD8 T cells.[26]

In vivo experiments confirmed our concept that modified virosomes combining activation of APCs with efficient antigen delivery have the potential to effectively activate CD8 T cells. Indeed, we observed that M/V_{Ni+}-NP induced NP-specific CD8 T cells while free NP with MPLA or M/V_{Ni-}+NP did not or to a much lower extent. In contrast, induction of NP-specific antibodies was independent of conjugation of NP to virosomes.

Different vaccination strategies have been developed to generate influenza-specific CD8 T cells. These strategies include DNA vaccine, viral vector vaccine, peptide vaccine and others (for review see Ref. [29]). Conserved T cell epitopes present in peptide vaccines or expressed by DNA vaccines or viral vector vaccines could induce influenza-specific CD8 T cells in vivo. Yet, so far the number of CD8 T cells that could be induced by these vaccines in humans was very moderate (reviewed in Ref. [30]). Accordingly, in some cases, e.g., the MVA-NP+M1 vaccine and the peptide vaccine Multimeric-001, the vaccine is not used alone but rather in combination with or as priming for immunization with conventional inactivated influenza vaccine (https://clinicaltrials.gov/ct2/show/results/NCT03300362).[31] Compared with these vaccination strategies, M/V_{Ni+}-NP, consisting of virosomes which present the viral surface proteins and harbor conserved NP, have the potential to induce not only influenza-specific CD8 T cells but also cross-reactive antibodies.

In line with others working on CD8 T cell inducing influenza vaccines, we hypothesized that CTL induction by virosomes would result in mitigation of disease symptoms upon infection by allowing rapid virus clearance. M/V_{Ni+}-NP immunization indeed provided a certain level of cross-protective immune response against heterosubtypic (HK68) virus infection. Yet, a similar level of cross-protection was achieved with M/V_{Ni-}+NP which did not induce significant numbers of NP-specific CD8 T cells. This result implies that NP-specific CD8 T cells were not crucial for cross-protective immunity afforded by the virosomes. Moreover, immunization with M/V without NP provided the best cross-protection against heterosubtypic virus infection, suggesting that antibodies or T helper cells recognizing HA or NA were responsible for the observed mitigation of disease symptoms. No neutralizing antibodies against the challenge virus were detected, but non-neutralizing antibodies against the surface proteins (HA+NA) and NP protein were present in the vaccinated animals. It is possible that these non-neutralizing antibodies provided cross-protection for example via antibody-dependent cellular cytotoxicity (ADCC).[32]

It is striking that the presence of NP-specific immunity seemed to have a negative rather than a positive effect during the initial phase of the infection: mice immunized with NP/MPLA showed a more rapid decline in weight than PBS control mice and mice immunized with virosomes with admixed or conjugated NP were less well protected than mice receiving virosomes without NP. The reason for this phenomenon is not clear. We consider it unlikely that the observed immunopathology was caused by NP-specific CTLs as CTLs recognizing the immuno-dominant $NP_{366-374}$ epitope were not induced by NP+MPLA and M/V_{Ni-}+NP. It remains elusive whether NP-specific antibodies or T helper cells or both were responsible for the observed enhanced disease symptoms.

In conclusion, we developed "all-in-one" influenza virosomes, with MPLA and DOGS-NTA-Ni incorporated in the membrane and NP protein conjugated on the surface. These "all-in-one" virosomes could activate APCs and enhance the uptake of NP protein by APCs in vitro and favored the generation of NP-specific CD8 T cell responses in vivo. Although virosome-induced NP-specific CTLs were not involved in heterosubtypic cross-protection in this study, our results provide a basis for the use of MPLA-adjuvanted influenza virosomes containing DOGS-NTA-Ni lipid as platform for a universal influenza vaccine.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Abbreviations

APCs, antigen presenting cells; CTLs, CD8 cytotoxic T cells; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; MPLA, monophosphoryl lipid A; NA, neuraminidase; NP, nucleoprotein; TLR4, toll-like receptor 4.

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Conflicts of Interest

The authors declare no financial or commercial conflict of interest.

Keywords

CD8+ T cells, cross-protection, MPLA, NP, Virosomes

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