Recombinant M2e outer membrane vesicle vaccines protect against lethal influenza A challenge in BALB/c mice

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Points:

- Engineered probiotic *E. coli* Nissle 1917 express and present influenza M2e peptides in outer membrane vesicles (M2e-OMVs)
- M2e-OMVs trigger the innate immune response predominantly through TLR2, TLR4, and TLR5 agonist activity
- BALB/c mice vaccinated with M2e-OMVs are 100% protected against a lethal dose of H1N1 influenza A virus

Abstract

Currently approved influenza vaccines predominantly protect through antibodies directed against the highly variable glycoprotein hemagglutinin (HA), necessitating annual redesign and formulation based on epidemiological prediction of predominant circulating strains. More conserved influenza protein sequences, such as the ectodomain of the influenza M2 protein, or M2e, show promise as a component of a universal influenza A vaccine, but require a Th1-biased immune response for activity. Recently, recombinant, bacterially-derived outer membrane vesicles (OMVs) demonstrated potential as a platform to promote a Th1-biased immune response to subunit antigens. Here, we engineer three M2e-OMV vaccines and show that all elicit strong IgG titers, with high IgG2a:IgG1 ratios, in BALB/c mice. Additionally, the administration of one M2e-OMV construct containing tandem heterologous M2e peptides (M2e4xHet-OMV) resulted in 100% survival against lethal doses of the mouse-adapted H1N1 influenza strain PR8. Passive transfer of antibodies from M2e4xHet-OMV vaccinated mice to unvaccinated mice also resulted in 100% survival to challenge, indicating that protection is driven largely via antibody-mediated immunity. The potential mechanism through which M2e-OMVs initiated the immune response was explored and it was found that the constructs triggered TLR1/2, TLR4, and TLR5. Our data indicate that OMVs have potential as a platform for influenza A vaccine development due to their unique adjuvant profile and intrinsic pathogen-mimetic nature.

Introduction

Influenza remains a significant source of morbidity and mortality throughout the world, despite the availability of seasonal vaccines. Each year, 5-10% of adults and 20-30% of children are infected with seasonal influenza, leading to 3-5 million severe cases and 250,000-500,000 deaths worldwide [1]. Additionally, influenza A viruses have the potential to form pandemic strains via cross-species reassortment, as evidenced by the 1918 (Spanish flu), 1957 (Asian flu), 1968 (Hong Kong flu), and 2009 (swine-origin flu) pandemics, presenting a threat to global health [2]. Current influenza vaccines primarily target the immune response against the immunodominant—but hyper variable—glycoproteins hemagglutinin (HA), and to a lesser extent, neuraminidase (NA), necessitating annual development of strain-specific vaccines [3]. Accordingly, there is great public and scientific interest in the creation of a universal influenza A vaccine to obviate or reduce the need for yearly vaccination and to protect against pandemic strains [4,5].

One promising target antigen for a universal vaccine is M2e, the ectodomain of the influenza A matrix protein 2 (M2), a homotetrameric ion channel with roles in viral entry and budding, first explored as a vaccine target by Richardson et al. in 1985 [6]. M2e demonstrates remarkable conservation of its 23 amino acid sequence, especially among influenza A viruses of the same host restriction. This high conservation has been attributed to M2e's genetic relationship with M1—an integral structural protein—and the lack of selective pressure against M2e due to M2e's low virion presence and immunogenicity [7]. Although steric hindrance from HA and NA limits antibody recognition of M2e upon the virion surface, exposed M2e is abundantly displayed on the membranes of infected epithelial cells [8]. Therefore, while anti-M2e antibodies are unable to neutralize the virus directly, they facilitate clearance of infected cells via antibody dependent cell-mediated cytotoxicity (ADCC) and phagocytosis (ADCP) [9,10].

During influenza infection, ADCC and ADCP are performed by alveolar macrophages and natural killer (NK) cells that recognize the Fc regions of antigen-bound antibodies and display selective efficacy with respect to antibody isotype [11]. It has been previously shown that IgG2a isotype antibody levels, associated with a Th1 type immune response in mice, correlate significantly with enhanced survival against influenza challenge for M2e-based vaccines [10,12]. Therefore, M2e-based vaccines have aimed to generate Th1-biased responses in an effort to improve protective efficacy. However, many methods

that generate a Th1 bias require supplementation with viral components or cost-prohibitive adjuvants [13–16]. Here, we utilize a recently developed adjuvant system, recombinant outer membrane vesicles (OMVs), to elicit preferential production of IgG2a antibodies against M2e without addition of viral components or soluble adjuvant.

OMVs are nanoparticles naturally produced by gram-negative bacteria with complex adjuvant profiles that are parent strain dependent and can promote a robust Th1-biased response when designed accordingly [17,18]. Furthermore, OMVs can be engineered to display recombinant heterologous proteins by transforming hypervesiculating ($\Delta nlpI$) strains of *E. coli* with a plasmid encoding the transmembrane protein ClyA followed by the antigen of interest [19,20]. Previously, we have shown that OMVs derived from the probiotic *E. coli* strain Nissle 1917 (EcN) carrying a model antigen, green fluorescent protein (GFP), promoted a Th1-biased response directed against GFP [18]. Here, we demonstrate that the display of M2e-based peptides on the surface of OMVs derived from EcN generate high levels of IgG2a antibodies—indicative of a Th1 bias—and protect against a lethal challenge with influenza A virus. To ascertain the influence of the innate immune system on the observed response, the agonist activity of OMVs on toll like receptors (TLR) was quantified to help understand the mechanisms through which the OMVs function as an adjuvant.

Methods:

Plasmid and expression vector design: M2e-based genetic sequences were cloned into a pBAD 18-CM plasmid containing transmembrane protein ClyA, as previously described [19]. Briefly, three M2e-based genetic sequences with a terminal histidine-tag were designed and codon optimized for *E. coli* (GeneArt service, [LifeTechnologies, Waltham, U.S.](#)) (Figure 1BC, SI). Gene blocks were inserted into a pBAD18-CM vector directly 3' terminal to the ClyA-encoding region.

OMV production and characterization: OMVs were prepared as previously described [18,20]. Briefly, *E. coli* Nissle 1917 $\Delta nlpI$ (EcN) cultures transformed with appropriate plasmid were grown overnight, subcultured to OD₆₀₀ = 0.08 in Miller LB broth ([ThermoFisher Scientific, Waltham, U.S.](#)), grown to mid-log phase, then induced with 0.2% L-arabinose ([Sigma-Aldrich, St. Louis, U.S.](#)) (Figure 1A). Cultures were

grown at 37°C for 18 hours, centrifuged (5000x g, 4 °C, 15 min) to pellet bacteria, then sterile filtered (0.2 µM). OMVs were isolated from the filtrate by ultracentrifugation (121560x rcf, 4 °C, 3h) and suspended in PBS. Total protein concentration was determined by Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, U.S.) and antigenic content was measured by Western Blot with anti-His6x primary antibody (Sigma-Aldrich, St. Louis, U.S.). Previous work provides detailed analyses of the proteins and sugars present within EcN OMVs [18].

Immunization: Female BALB/c mice (Jackson Laboratories, Bar Harbor, U.S.) were immunized subcutaneously with 100 µL of vaccine at age 8-weeks. Treatment groups M2e1xC-OMV (n=5), M2e1xS-OMV (n=12), and M2e4xHet-OMV (n=12) received 40 µg total protein of OMVs containing ~0.2 µg total M2e antigen in PBS. Group M2e1xS with alum (n=12) received 1 µg M2e1xS peptide (NH₂-SLLTEVETPIRNGSRSNDSSD-COOH, Lifetein, Somerset, U.S.) in PBS adsorbed to 2% Alhydrogel[®] (Invivogen, San Diego, U.S.) at a 1:1 [v/v] ratio. A sham vaccination group received only PBS (n=12). Prime and boost doses were administered 4 weeks apart. Blood was collected 1-2 days before the prime dose and every 2 weeks following. For passive transfer experiments, five 8-week old female BALB/c mice were immunized intraperitoneally (ip) with 100 µL pooled serum diluted to 500 µL in PBS collected 2 weeks prior to challenge from M2e4xHet-OMV (n=5), PR8 pre-exposed (n=5), or sham PBS (n=5) vaccinated mice. Blood was collected 24h post ip injection and analyzed via ELISA to ensure successful antibody transfer. All mouse work was conducted according to protocols approved by Cornell's Institutional Animal Care and Use Committee (IACUC).

Influenza challenge: A/Puerto Rico/8/1934 (PR8) virus (BEI Resources, Manassas, U.S) was diluted in PBS and administered via intranasal injection of 50 µL PBS under isofluorane anesthesia. Mice immunized via PR8 exposure (pre-exposed mice) were administered a sub lethal dose of 5 fluorescence forming units (FFU) PR8 virus at age 8 weeks. M2e1xC-OMV (n=5), M2e1xS-OMV (n=4), M2e4xHet-OMV (n=8), M2e1xS with alum (n=4), sham (PBS) vaccination (n=9), and pre-exposed (n=13) cohorts received a lethal dose (50 FFU) 10 weeks post initial exposure or vaccination. For passive transfer experiments, 8-wk old mice received 50 FFU PR8 24h post passive transfer. Weight loss greater than

30% or signs of severe distress were used as euthanasia criteria.

Viral load quantification via Fluorescence Forming Unit (FFU) assay: Day 6 following PR8 challenge, mice were sacrificed from sham, M2e4xHet-OMV, and pre-exposed groups (n=3/group) and their lungs and trachea isolated and homogenized. Homogenates were centrifuged at 5000x g for 10 minutes at 4°C, filtered (0.2 µm), and stored at -80°C. For FFU assay, sample aliquots were diluted into MEM media containing 0.2% BSA and activated with TPCK Trypsin at 37°C (1 µg/ml) for 20 minutes then deactivated with soybean trypsin inhibitor (8.4 µg/ml) (Corning, Tewksbury, U.S.). Samples were serially diluted, 100 µL loaded into 96-well plates with confluent layers of MDCK cells, and incubated for 24 hours. Cells were stained with mouse anti-nucleoprotein from hybridoma H16-L10-495 (ATCC, Manassas, U.S.) and Hoechst (ThermoFisher Scientific, Waltham, U.S.) then imaged as described previously [21]. FFU/mL was back-calculated from the number of infected cells at the lowest infected dilution.

Enzyme-linked immunosorbent assay (ELISA): ELISAs were conducted on serum samples as previously described, with the modification that plates were coated with 2 µg/mL M2e1xC peptide in PBS at 37 °C overnight [18]. Antibody titer was defined as the reciprocal of the greatest dilution at which the OD₄₅₀ of vaccinated serum exceeded the OD₄₅₀ of pre-vaccinated serum plus 3 standard deviations. The lowest titer detectable by ELISA was 1:500, owing to high background at higher concentrations.

Toll-like receptor (TLR) ligand activity assay: TLR panel using HEK-Blue KD-TLR 5 cells (Invivogen, San Diego, U.S.) was conducted as described for HEK293 cells [18]. Briefly, 1.5x10⁵ cells/well were seeded in 96-well plates and transfected using TransIT-LT1 (Mirus, Madison, U.S.) with plasmids encoding human or mouse TLRs (TLR2, TLR3, TLR4 and MD2, TLR5, TLR7, TLR8, TLR9, mTLR2, mTLR3, mTLR4 and mMD2, mTLR9, mTLR11), CMV-β-galactosidase and 5xNF-κB-luciferase. After 24 h, cells were stimulated with 4xM2eHet-OMVs (10 ng/mL), positive control (TLR1/2: Pam3Cys, 1 µg/mL; TLR3: poly(I:C), 1 µg/mL; TLR4: LPS, 100 ng/mL; TLR5 and TLR11: flagellin from *S. typhimurium*, 100 ng/mL; TLR7: loxoribine, 100 µM, TLR9: CpG 10104, 3 µM (Invivogen, San Diego, U.S.) or PBS for 18h. Media was then removed, reporter lysis buffer, 100 µL/well, (Promega, Madison, U.S.) added, and cells

mechanically lysed. Lysate (20 μ L/well) was added to opaque 96-well plates and relative light units (RLUs) quantified using a Veritas luminometer (Promega, Madison, U.S.) through addition of 100 μ L D-luciferin substrate per well. 4xM2eHet-OMV TLR5 stimulation was further confirmed using the same method with HEK293 cells (Figure S1).

Statistics

Antibody titers are reported as geomean titers (GMT) with error bars representing 95% confidence intervals. Comparisons between IgG1 and IgG2a titers use a paired 2-tailed t-test on log-transformed samples. Luciferase levels of PBS vs. M2e4xHet-stimulated cells in TLR panel are compared using a two-tailed t-test. Kaplan-Meier survival curves analyzed using log-rank test with Bonferroni-corrected alpha to account for multiple comparisons. Anti-M2e titer vs. percent weight loss was analyzed using a linear regression model in which the natural log of the titer was plotted against the maximum percent weight loss each mouse experienced during the challenge. All statistical analyses were conducted using Prism6 software (GraphPad software, La Jolla, U.S.).

Results:

Production of OMVs containing M2e derived peptides

Three M2e-based OMV vaccines were designed and produced to determine the effect of antigen choice upon immunogenicity and protective efficacy (Figure 1BC). Here, we designed OMVs that display the most common human-type influenza A virus M2e antigen (M2e1xC-OMVs) as well as its serine analog (M2e1xS-OMVs). The native cysteines of M2e (C17 and C19) are frequently substituted with serines to prevent disulfide bond formation and aggregation, which may inhibit function or expression, though we did not encounter any difficulties in expression [22]. Additionally, while M2e is highly conserved amongst influenza A strains that infect a common host, M2e is divergent across host restrictions [23]. Recently, the Kang group showed improved immune recognition of broadly divergent influenza A strains by vaccination with a 5xM2e tandem construct containing heterologous host-restricted epitopes [24,25]. Therefore, a 4x multimeric construct (M2e4xHet-OMVs) consisting of the serine analogs of human, swine, and two avian influenza A restricted M2e variants—connected by flexible (G₃SG₃) linkers to provide epitope

separation—was produced (Figure 1BC). Successful expression and localization of ClyA-M2e fusion proteins in OMVs was confirmed via Western blot of isolated vesicles (Figure 1D). TEM images of EcN-derived M2e-OMVs showed they were in the expected size range of 50-100 nm in diameter and consistent with native OMVs (Figure 1E).

M2e-OMV vaccination leads to high IgG titers

M2e1xC-OMV, M2e1xS-OMV, and M2e4xHet-OMV vaccinated mice all developed high and statistically equivalent anti-M2e IgG titers 8 weeks post initial vaccination that were all significantly higher than those of the M2e1xS alum cohort (**** $p < 0.0001$) (Figure 2A). Mice that were pre-exposed to a low dose of PR8 also developed high titers that were significantly higher than the M2e1xS alum cohort (** $p < 0.01$). Despite a 5x mole excess in M2e content compared to M2e1xS-OMVs, the M2e1xS peptide adsorbed to alum was unable to generate a robust immune response.

Serum from vaccinated mice was further assessed to compare the IgG2a:IgG1 ratio. IgG2a isotype antibodies, associated with a Th1 type immune response, are the proposed correlate of protection for M2e-based vaccines and are necessary and sufficient for protection [10,12]. All three M2e-OMV groups showed a significant elevation in IgG2a titers over IgG1 titers (* $p < 0.05$) (Figure 2B), as did the cohort pre-exposed to PR8 (** $p < 0.01$). This elevated IgG2a:IgG1 ratio is of particular significance as it occurred in BALB/c mice, which are naturally biased toward a Th2 response with higher IgG1 levels than IgG2a [26]. Additionally, no supplementation with additional **extrinsic** adjuvants, such as MPL or RNA and viral components, was required to generate this isotype distribution [27–29].

M2e-OMV vaccines protect BALB/c mice from influenza A challenge

M2e4xHet-OMVs provided the best protection against PR8 with 100% survival post challenge (**Figure 3AB**). Additionally, the average lung and trachea viral titer of M2e4xHet-OMV vaccinated mice was approximately 10-fold lower than that of the sham (PBS) control at day 6 post challenge (**Figure 3C**). All sham (PBS) control mice succumbed to infection by day 10 post-challenge, confirming a lethal challenge dose. As titer data predicted, M2e1xS with alum mice fared poorly, with only 25% survival (1/4). Responses to vaccination with M2e1xC-OMVs and M2e1xS-OMVs were comparable, with 40% (2/5)

survival and 50% survival (2/4) respectively. Survival analysis demonstrated that only M2e4xHet-OMVs provided better protection than that of the sham (PBS) vaccine ($p < 0.01$) (Figure 3B).

M2e4xHet-OMV vaccinated mice showed the least morbidity of the vaccinated groups, with an average body weight loss of 14% (Figure 3A). Though the M2e4xHet mice showed greater weight loss than the pre-exposed mice, this result is expected, as M2e-based vaccines are infective permissive, requiring Fc-dependent clearance of infected cells by the innate immune system for protection [10]. Linear regression analysis of log transformed anti-M2e total IgG titers vs. the lowest percent original weight yielded a significant relationship ($r^2 = 0.403$, $p < 0.05$) (Figure 3D). The literature suggests IgG2a is a correlate of protection for M2e; therefore, we next analyzed IgG2a vs. lowest percent original weight and found an even stronger relationship ($r^2 = 0.477$, $p < 0.01$) (Figure 3F), whereas IgG1 titers vs. lowest percent original weight did not have a significant relationship ($r^2 = 0.228$, $p > 0.05$) (Figure 3E) [10,12,30].

M2e-OMVs stimulate multiple toll like receptors (TLRs)

Following the success of M2e4xHet-OMVs in PR8 challenge, their impact on TLR signaling was explored. TLR stimulation is important to drive an appropriate innate immune response against an antigen [31]. HEK293 TLR5 KD cells were transfected with human and murine TLRs and stimulated with M2e4xHet-OMVs. Comparable TLR stimulation patterns were found for both mouse and human TLRs, suggesting these OMVs would have similar adjuvant potency in humans and mice. The most predominantly activated TLRs were TLR1/2, mTLR1/2, TLR4, mTLR4, and TLR5 (Figure 4) (* $p < 0.0001$). TLR9 and mTLR9 were activated to a lesser extent (# $p < 0.01$), suggesting a possible contribution to the innate immune response through these receptors. DNA is commonly found in OMVs, making it likely that the TLR9 stimulation was due to low levels of CpG DNA [32].

Passive transfer of serum protects against influenza A challenge

Consistent with previous reports of M2e mechanism of protection, passive transfer of serum from M2e4xHet-OMV vaccinated mice provided complete protection (100% survival) against subsequent lethal PR8 challenge (Figure 5A) [10,16,33,34]. However, greater weight loss—25% versus 14%—occurred in the passive transfer vaccinated mice vs. M2e4xHet-OMV vaccinated mice. This may be due to the

reduced concentration of antibodies in passively immunized mice, which had an anti-M2e IgG GMT titer of 24,000, as opposed to 102,000 in M2e4xHet vaccinated mice, which is consistent with the anti-M2e IgG titer vs. percent weight loss correlation (Figure 3D).

Discussion:

The ectodomain of influenza A matrix protein 2 (M2e) is a popular target antigen for potential universal influenza A vaccines due to its remarkable conservation over time [35]. To overcome its low immunogenicity, M2e requires a potent adjuvant, and as we and others have demonstrated, peptide-adsorbed alum alone inadequately primes the immune response against influenza [27,36]. Subsequently, a range of subunit fusion vaccines that combine M2e with adjuvanting proteins, such as hepatitis B protein, keyhole limpet protein, and flagellin have been explored and developed, though many require supplementation by additional **extrinsic** adjuvants, such as incomplete Freund's adjuvant or MPL [16,37,38]. Here, we demonstrated that engineering EcN to display M2e peptides upon OMVs resulted in significantly elevated anti-M2e IgG2a titers vs. IgG1 titers and protection against influenza challenge without supplemental adjuvants. Additionally, the IgG2a:IgG1 ratio of 3:1 is comparable to that of M2e vaccines supplemented with viral components [25]. **Future work will evaluate the ability of M2e-OMVs to give heterologous protection against multiple strains of influenza A virus, and thus better determine its potential as a universal influenza A vaccine.**

The proposed mechanism of protection for M2e-based vaccines is clearance of infected cells through antibody dependent cell cytotoxicity and phagocytosis (ADCC and ADCP) and it has been shown that IgG2a antibodies are both necessary and sufficient to provide protection in mice [10,16,33,34]. Our results add further support to this proposed mechanism as we found that protection from weight loss during challenge most significantly correlated with IgG2a antibody levels. Furthermore, our minimum IgG2a antibody titer among passively immunized mice was 8,000, adding strength to the reported minimum protective IgG2a antibody titer of 10,000 previously suggested by Fiers et al. [30]. While only significant to $p < 0.1$, M2e4xHet-OMV IgG2a titers were higher than those of M2exC-OMV and M2eS-OMVs, perhaps contributing to the success of the M2e4xHet-OMV vaccine.

To further elucidate the potent adjuvant mechanisms of M2e-OMVs, we investigated the

stimulation of the toll like receptor (TLR) family, as OMVs naturally contain a complex array of pathogen associated molecular patterns (PAMPS) [39]. The TLR panel indicated that M2e4xHet-OMVs trigger multiple bacteria-associated TLRs, predominantly TLR1/2, TLR4, and TLR5, and, to a lesser extent, TLR9. These TLRs signal through both the MyD88 (TLR1/2, TLR4, TLR5, TLR9) and TRIF/TRAF (TLR4) pathways, leading to NF- κ B activity and type I interferon production, which strongly promote a Th1 bias [40]. Previous work characterized the response of murine TLRs to EcN OMV stimulation, but the work was conducted in HEK293 cells that endogenously express TLR5 [18]. Here, we use HEK293 TLR5 KD cells to provide an enhanced perspective of OMV stimulation and to confirm that M2e4xHet-OMVs stimulate mouse and human TLRs similarly, suggesting translational possibilities. One current limitation of this platform is that while the bacterial components of OMVs elicit potent immune responses through high levels of TLR stimulation, these components also limit the maximum tolerable dose which may be administered. Lipopolysaccharide (LPS), a molecule present in bacterial outer membranes, is a strong TLR4 agonist, but also a causative agent of sepsis. M2e-OMV dose size was kept consistent with previous work involving OMV-based vaccines in which OMV LPS levels were measured and safe OMV dose sizes established [18,20]. However, further work in attenuating the LPS load in OMVs is ongoing and will permit larger doses to be administered. Additionally, future work will explore other innate immune pathways, such as nucleotide-binding oligomerization domain containing protein 1 (NOD1) signaling, to develop a more nuanced perspective on the signaling mechanisms of OMVs [31].

In summary, M2e antigens presented on *E. coli* Nissle 1917 OMVs demonstrate protection against influenza A strain PR8. The three OMV vaccines tested, M2e1xC, M2e1xS, and M2e4xHet, all yielded high anti-M2e IgG titers with significantly elevated IgG2a:IgG1 ratios, and had improved survival over the peptide M2e1xS with alum formulation. The heterologous M2e4xHet vaccine was most protective, resulting in 100% survival following challenge. TLR studies demonstrated that the OMV adjuvancy could be partly attributed to the OMVs triggering TLR2, TLR4, TLR5, which was consistent for both human and mouse TLRs. Naïve mice that passively received antibodies from M2e4xHet-OMV vaccinated mice showed 100% survival following challenge. Overall, the data support the premise that OMVs represent a promising adjuvant platform for development of an M2e-based universal influenza vaccine.

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Conflicts of interest

The authors disclose no conflicts of interest, real or apparent.

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Figure 1: **A:** Hypervesiculating *E. coli* strain Nissle 1917 Δnlp was transformed with a pBAD plasmid that encodes transmembrane protein ClyA followed by an M2e-based antigen, resulting in production of outer membrane vesicles (OMVs) that display the desired antigen. **B.** Three ClyA-M2e constructs terminated by a 6x histidine-tag were expressed in *E. coli*: M2e1xC, M2e1xS, and M2e4xHet. M2e1xC contains a common human restriction M2e variant, M2e1xS contains the serine analog to M2e1xC, and M2e4xHet contains the serine analog to M2e1xC, along with the serine analogs to a common swine restricted M2e variant and two avian restricted variants. **C.** M2e contains several variants, largely dependent on host restriction. The sequence of M2e in the virus used for challenged, A/Puerto Rico/8/1934 (PR8), along with the sequences of M2e used to create M2e-based constructs (Human (C), Human (S), Swine, Avian (1), and Avian (2)) are listed. **D.** Western blot of M2e-OMV constructs demonstrates that M2e1xC, M2e1xS, and M2e4xHet are present in OMVs (5 μ g total protein/well, anti-His antibody). **E.** Transmission electron microscopy (TEM) images of OMVs show polydispersed particles ranging approximately 50-100 nm in diameter (uranyl acetate staining).

Figure 2: Mouse serum was analyzed for anti-M2e titers 8 weeks post initial vaccination. Dotted line represents lowest titer detectable above background. **A.** All three M2e-OMV vaccines yielded an IgG titer higher than that of M2e1xS peptide with alum (**** $p < 0.0001$). IgG titers from M2e1xC-OMVs, M2e1xS-OMVs, and M2e4xHet-OMVs did not have any statistical difference ($p > 0.05$). Additionally, pre-exposed mice developed titers that were significantly higher than M2e1xS + alum (** $p < 0.01$). Log transformed titers were analyzed using a one-way ANOVA followed by multiple comparisons that were corrected by Tukey's test. **B.** All M2e-OMV vaccines resulted in statistically elevated titers of IgG2a relative to IgG1, indicating a Th1 bias (* $p < 0.05$). As expected the Th1 bias from mice pre-exposed to PR8 was especially robust (** $p < 0.01$). The serum from mice vaccinated with M2e1xS with alum could not be analyzed for IgG1 vs. IgG2a titers as it was beneath the range of the ELISA assay. Log transformed IgG1 and IgG2a titers were compared in paired, 2-tailed t-tests. All graphs display geometric titers (GMT) with error bars that represent 95% confidence intervals. Data analyzed using Prism6 software.

Figure 3: Mice were challenged with a lethal dose (50 FFU) of mouse adapted influenza A virus PR8 10 weeks after prime vaccine dose. Mortality (**A**) and morbidity (**B**) graphs show that different vaccines resulted in different levels of protection. As expected, all pre-exposed mice survived challenge and all sham vaccinated (PBS) mice died/required euthanasia. Mice that received the M2e4xHet-OMV vaccine fared best, with the highest survival rate (100%) and the least weight loss (apart from pre-exposed). Mice vaccinated with M2e1xS with alum fared worst, 25% survival, and M2e1xS-OMV and M2e1xC-OMV vaccinated mice fared intermediately, 50% and 40% survival respectively. Error bars represent standard error of the mean. **C.** PR8 viral titer from lungs and tracheas excised on day 6 of challenge for M2e4xHet-OMV, exposure-immunized, and sham PBS injection. **D-E.** Linear regression of anti-M2e IgG (**D**) IgG1 (**E**) and IgG2a (**F**) log transformed titers vs. minimum percent original weight of OMV-immunized mice during PR8 challenge. The highest significance was found for IgG2a, followed by that for IgG. IgG1 did not correlate significantly with weight loss.

Figure 4: The toll like receptor (TLR) response to M2e4xHet-OMVs was analyzed using both human (A) and murine (B) TLRs. OMVs primarily stimulated TLR1/2, TLR4, TLR5, mTLR1/2 and mTLR4 (* $p < 0.0001$), and to a lesser extent, TLR9 and mTLR9 (# $p < 0.01$). Media vs. M2e4xHet-OMV stimulated luciferase production was compared using a 2-tailed t-test. Error bars represent standard deviation of the mean.

Figure 5: Mortality (**A**) and morbidity (**B**) of mice immunized through passive transfer of 100 µL of serum (Ab) from PR8 pre-exposed, M2e4xHet-OMV vaccinated, or sham vaccinated (PBS) mice 8 weeks following initial vaccination or exposure. Mice were vaccinated with serum 24h prior to challenge with 50 FFU of PR8. Cohorts that received serum from the pre-exposed or M2e4xHet-OMV vaccinated mice had 100% survival during challenge. All mice that received PBS serum died during challenge. Though all mice passively immunized with serum from donor M2e4xHet-OMV vaccinated mice survived, they showed higher levels of morbidity than the M2e4xHet-OMV vaccinated donor mice in equivalent PR8 challenge.