Prokaryote-expressed M2e protein improves H9N2 influenza vaccine efficacy and protection against lethal influenza a virus in mice

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Abstract

Background: Influenza vaccines are prepared annually based on global epidemiological surveillance data. However, since there is no method by which to predict the influenza strain that will cause the next pandemic, the demand to develop new vaccination strategies with broad cross-reactivity against influenza viruses are clearly important. The ectodomain of the influenza M2 protein (M2e) is an attractive target for developing a vaccine with broad cross-reactivity. For these reasons, we investigated the efficacy of an inactivated H9N2 virus vaccine (a-H9N2) mixed with M2e (1xM2e or 4xM2e) proteins expressed in Escherichia coli, which contains the consensus of sequence the extracellular domain of matrix 2 (M2e) of A/chicken/Vietnam/27262/09 (H5N1) avian influenza virus, and investigated its humoral immune response and cross-protection against influenza A viruses.

Results: Mice were intramuscularly immunized with a-H9N2, 1xM2e alone, 4xM2e alone, a-H9N2/1xM2e, or a-H9N2/4xM2e. Three weeks post-vaccination, mice were challenged with lethal homologous (A/chicken/Korea/ma163/04, H9N2) or heterosubtypic virus (A/Philippines/2/82, H3N2 and A/aquatic bird/Korea/maW81/05, H5N2). Our studies demonstrate that the survival of mice immunized with a-H9N2/1xM2e or with a-H9N2/4xM2e (100% survival) was significantly higher than that of mouse-adapted H9N2 virus-infected mice vaccinated with 1xM2e alone or with 4xM2e alone (0% survival). We also evaluated the protective efficacy of the M2e + vaccine against infection with mouse-adapted H5N2 influenza virus. Protection from death in the control group (0% survival) was similar to that of the 1xM2e alone and 4xM2e alone-vaccinated groups (0% survival). Only 40% of mice vaccinated with vaccine alone survived challenge with H5N2, while the a-H9N2/1xM2e and a-H9N2/4xM2e groups showed 80% and 100% survival following mouse-adapted H5N2 challenge, respectively. We also examined cross-protection against human H3N2 virus and found that the a-H9N2/1xM2e group displayed partial cross-protection against H3N2 (40% survival), whereas vaccine alone, 1xM2e alone, 4xM2e alone, or H9N2/1xM2e groups showed incomplete protection (0% survival) in response to challenge with a lethal dose of human H3N2 virus.

Conclusions: Taken together, these results suggest that prokaryote-expressed M2e protein improved inactivated H9N2 virus vaccine efficacy and achieved cross-protection against lethal influenza A virus infection in mice.

Keywords: Influenza A virus, M2e protein, Escherichia coli, Inactivated vaccine
Background
Influenza A virus is an important human pathogen that causes occasional pandemics and has a huge impact on global health. Vaccination is the most economical and effective strategy by which to control the emergence and spread of influenza pandemics [1,2]. There are several influenza vaccines that have been licensed for use in humans, such as inactivated or live-attenuated whole virus vaccines, split vaccines, and subunit vaccines [1,3]. Inactivated seasonal vaccines include antigens from at least three different influenza strains. They are prepared annually in an effort to match vaccine composition with the global epidemiological surveillance data for a particular year [4,5]. Unfortunately, these vaccines are mainly designed to induce subtype-specific neutralizing antibodies and do not protect against infection with other influenza subtypes or with antigenic variants [4,6]. Additionally, because the influenza virus strain that will cause the next epidemic or pandemic cannot be predicted, new vaccination strategies that will result in broad cross-reactivity against influenza viruses need to be developed. The use of the ectodomain of the influenza virus matrix 2 protein (M2e) as an attractive target for developing broadly cross-reactive, universal influenza virus vaccines has been conceptualized and tested for several decades [7]. The M2e sequence is highly conserved across influenza virus subtypes (Table 1), and induced humoral anti-M2e immunity protects against lethal influenza virus infection in animal models [8]. M2 vaccine candidates that have been explored included peptide-carrier conjugates [9], baculovirus-expressed M2e [10], fusion proteins [11,12], multiple antigenic peptides [13,14], and M2e DNA constructs that potentially express M2e [15,16]. In this regard, previous studies of M2e conjugate vaccines used various adjuvants such as Freund’s adjuvants [17], cholera toxin [18], heat labile endotoxins derivatives, flagellin [19], or bacterial protein conjugates [20]. These adjuvants or conjugates (viral particles or carrier molecules) [21], even combined with inactivated vaccine, were not completely protective against influenza virus infection as immunized animals still showed disease symptoms such as weight loss.

In this study, we investigated the efficacy of inactivated H9N2 virus vaccine (a-H9N2) developed a considerable antibody response against H9N2 virus, but not against H5N2 or H3N2 virus. In particular, HI titer specific only for homologous virus (ma163/04, H9N2) was noted in the mice immunized with a-H9N2 alone (245.11 HI titers) and a-H9N2/1×M2e (375.5 HI titers), and a-H9N2/4×M2e (929.55 HI titers) but not against heterosubtypic (maW81/05, H3N2 and Phil/82, H3N2) influenza viruses (Table 2); more appreciable homologous antibody titers were induced by a-H9N2/4×M2e (929.55 HI titers). To evaluate whether the noted antibodies could neutralize influenza virus, serum samples were tested by microneutralization assay (Figure 2). Among the M2e protein-mixed vaccines, receipt of the a-H9N2/4×M2e vaccine preparation induced neutralization titer relative to a-H9N2 alone against the H9N2 (4.16 versus 4.8 log2HAU) (p = 0.085) and H5N2

Results
Vaccines containing M2e protein induced cross-reactive humoral immune response in mice.

Two plasmid constructs bearing monomer or polymer of the viral M2e protein derived from A/chicken/Vietnam/27262/09 (H5N1) avian influenza virus (1×M2e and 4×M2e, respectively) were expressed in prokaryotic cells (BL21). Upon confirmation of protein expression and subsequent purification (Figure 1), groups of nineteen mice were intramuscularly (i.m.) immunized with 2 μg of inactivated whole-virus H9N2 vaccine (a-H9N2), only 1×M2e (15 μg), only 4×M2e (15 μg), inactivated H9N2 + 1×M2e (a-H9N2/1×M2e) and inactivated H9N2 + 4×M2e (a-H9N2 vaccine/4×M2e) with two doses at three week intervals. Polyclonal sera from immunized mice, taken three weeks after the first and second administration, were analyzed by hemagglutination inhibition (HI) test to identify IgG antibodies (Abs) directed against influenza A virus. Table 2 shows mice immunized with inactivated H9N2 vaccine (a-H9N2) developed a considerable antibody response against H9N2 virus, but not against H5N2 or H3N2 virus. In particular, HI titer specific only for homologous virus (ma163/04, H9N2) was noted in the mice immunized with a-H9N2 alone (245.11 HI titers) and a-H9N2/1×M2e (375.5 HI titers), and a-H9N2/4×M2e (929.55 HI titers) but not against heterosubtypic (maW81/05, H3N2 and Phil/82, H3N2) influenza viruses (Table 2); more appreciable homologous antibody titers were induced by a-H9N2/4×M2e (929.55 HI titers). To evaluate whether the noted antibodies could neutralize influenza virus, serum samples were tested by microneutralization assay (Figure 2). Among the M2e protein-mixed vaccines, receipt of the a-H9N2/4×M2e vaccine preparation induced neutralization titer relative to a-H9N2 alone against the H9N2 (4.16 versus 4.8 log2HAU) (p = 0.085) and H5N2

Table 1: Comparison of M2e sequence among vaccine and challenge strains

| M2e sequence homology | Vaccine strain | M2e sequence |
|-----------------------|---------------|--------------|
| M2e Protein           | A/chicken/Vietnam/27262/09 (H5N1) | MSLTEVTPTINWECRCSDSSD |
| Inactivated vaccine   | A/chicken/Korea/04163/2004 (H9N2) | MSLTEVTPTINWECRCSDSSD |
|                       | A/chicken/Korea/ma163 (H9N2)      | MSLTEVTPTINWECRCSDSSD |
| Challenge virus       | A/aquatic bird/Korea/maW81/05 (H5N2) | MSLTEVTPTINWECRCSDSSD |
|                       | A/Philippines/82 (H3N2)           | MSLTEVTPTINWECRCSDSSD |

Amino acids in bold are the variant residues.
Interestingly, a-H9N2/4×M2e was able to neutralize the human Phil82/H3N2 virus although the polymer vaccine preparation remained most efficient (Figure 2C). None of the other vaccine groups could elicit detectable titers beyond the limit of detection.

1×M2e or 4×M2e mixed with inactivated a-H9N2 vaccine induced protection against a mouse-adapted H9N2 avian influenza virus

To determine whether prokaryote-expressed 1×M2e or 4×M2e proteins could improve the efficacy of the inactivated a-H9N2 vaccine derived from A/chicken/Korea/ma163/04 and confer protection against infection from a virulent mouse-adapted homologous variant virus, immunized mice were challenged with a lethal dose of A/chicken/Korea/ma163/04 (ma163/H9N2) virus two weeks after the last vaccination. Protective efficacy and morbidity (measured by survival rates and weight losses, respectively) were monitored every other day for 14 days post-infection (dpi); mice were euthanized and considered dead if the original body weight is reduced by >25%. Groups of mice administered with the purified protein alone (1×M2e vaccine groups), as well as mock-immunized group, showed weight losses (>25%) at 6 to 7 dpi resulting in a survival rate of 0% by 14 dpi. Some of 4×M2e alone-immunized mice had slightly extended mean survival relative to mice immunized with 1×M2e or mock-immunized animals (p = 0.39). In contrast, receipt of the a-H9N2 vaccine demonstrated moderate (14%) weight loss but conferred 100% survival in mice at 14 dpi. Interestingly, mice that were immunized with a-H9N2/1×M2e or with a-H9N2/4×M2e vaccine all survived (100%) until 14 dpi but were accompanied with very modest loss of body weight (3-7%) (Figure 3). These results demonstrate that the a-H9N2 vaccine itself could protect mice from lethal H9N2 virus infection whereas combination with the prokaryotic-expressed 1×M2e or 4×M2e protein moderated signs of morbidity and clinical disease.

M2e protein vaccination has been considered as a method to enhance cross protection against antigenic
variants and even hetero-subtypes of influenza A viruses [20]. To better understand the degree of cross-protection of E. coli-expressed M2e protein in the context of the a-H9N2 vaccine, groups of vaccinated mice were lethally challenged by i.n. infection with a mouse-adapted avian H5N2 A/aquatic bird/Korea/maW81/05 (maW81/H5N2) virus at two weeks after the last vaccination. Immunization with a-H9N2/1×M2e and a-H9N2/4×M2e induced high survival rates at 80% and 100%, with only 15-17% mean weight losses (Figure 4A and 4B). On the other hand, the group vaccinated with the inactivated a-H9N2 vaccine alone exhibited up to 20% reduction in body weight and at 7 dpi, only four out of ten mice survived the lethal infection (40% survival rate). The mock-vaccinated control, 1×M2e, and 4×M2e only vaccine groups displayed the highest weight losses (>25%) and all mice eventually succumbed to death within 6 to 10 dpi (Figure 4A and 4B). To further illustrate the breadth of cross-protection induced by the M2e protein, we also lethally challenged additional groups of immunized mice with a human H3N2 A/Philippines/82 virus (Phil82/H3N2) at two weeks post-vaccination. All mice in the control, 1×M2e only, 4×M2e only, and a-H9N2 vaccine groups became severely ill, lost weight (>25% from baseline) starting at 4 dpi, and all mice eventually died by 9 dpi (Figure 4C and 4D). Receipt of a-H9N2/1×M2e vaccine mix extended survival but could not completely protect immunized mice during the course of experiment. In contrast, the a-H9N2/4×M2e immunized group experienced 19% loss in body weight within 4 to 7 dpi, but started to recover thereafter (Figure 4C); 40% of the vaccinated mice survived the H3N2 virus challenge. Altogether, these results suggest that addition of the bacterially expressed M2e protein stimulated enhanced heterosubtypic protection even against a human Phil82/H3N2 virus despite some accompanying morbidity and mortality as reflected by body weight loss; improved results were

Figure 2 M2e protein induces neutralization of influenza virus in mice. 1×M2e, 4×M2e, a-H9N2, a-H9N2/1×M2e, or a-H9N2/1×M2e was used to immunize mice. Three weeks after boost vaccination, sera were collected. The samples were serially diluted two-fold. Serum neutralization activity was tested against 10^2 TCID<sub>50</sub>/ml of respective viruses. (A) A/chicken/Korea/ma163/04 (ma163/H9N2), (B) A/aquatic bird/Korea/maW81/05 (maW81/H5N2), or (C) A/Philippines/2/82 (Phil82/H3N2) virus for 30 min, followed by incubation with MDCK cells for 48 h. Data are representative of three independent experiments with three replicate wells per group. The lower limit of detection (0.5 10 log<sub>2</sub>TCID<sub>50</sub>) is indicated by a dotted line.
markedly observed with the multimer 4×M2e protein mixture.

1×M2e and 4×M2e proteins induced viral clearance in mice challenged with lethal dose of influenza viruses

We also assessed the ability of vaccines containing M2e proteins to inhibit viral growth in lungs of immunized and subsequently infected animals. Two weeks after the second administration, groups of immunized mice were intranasally challenged with a lethal dose (2LD_{50}) of A/chicken/Korea/ma163/04 (ma163/H9N2) influenza virus three weeks after boost vaccination. (A) Body weight changes and (B) survival were recorded for 14 days post-challenge. 1×M2e or 4×M2e mixed with inactivated H9N2 vaccine induced cross-protection against heterosubtypic avian H5N2 and human H3N2 influenza virus.

Discussion

During the last decade, H9N2 avian influenza viruses circulated worldwide in poultry populations causing mild respiratory disease and reductions in egg production [23-26]. However, H9N2 viruses do not appear to replicate efficiently or cause severe disease until in April 1999 when two World Health Organization (WHO) reference laboratories independently confirmed the isolation of avian H9N2 influenza A (A/HK/1073/99) viruses for the first time in humans [27]. Following that year, another strain of H9N2 virus has been isolated repeatedly from the human population in mainland China [27,28]. Other reports also indicated continuous interspecies transmission of H9N2 avian influenza virus from avian to mammalian hosts [27,29]. Therefore, WHO declared H9N2 influenza virus as a potential candidate for the next influenza pandemic [30]. Currently available influenza virus vaccines only induce humoral immunity by boosting anti-influenza antibodies whose targets are limited to the surface glycoproteins, HA and NA [31]. Accordingly, contemporary universal influenza vaccines were developed mainly based on conserved sequences in M2, HA1, HA2, and NP proteins of the influenza virus [32]. Because it is highly conserved in all types of influenza A viruses, M2e has been studied as a universal influenza vaccine target. A number of studies with M2e vaccines have already been conducted [17,20,32-34] and recently, phase I clinical studies have been carried out with chemically or genetically produced M2e fusion proteins, indicating promising results.
proteins [35]. Tompkins et al. [36] proposed that various M2e sequences of M2 expression constructs could be used as vaccines. Despite substantial sequence divergence, H5-derived vaccines might also protect against circulating H1N1 and H3N2 subtypes. Here, we investigated the potential of vaccines containing prokaryotic expressed monomer or polymer of M2e proteins (1×M2e and 4×M2e, respectively) without adjuvant, to contribute to cross-protective immunity against several influenza virus subtypes. 1×M2e and 4×M2e clones were generated by using consensus M2e gene from an H5N1 avian virus without its trans-membrane domain (Table 1). In contrast to adjuvanted M2e vaccine studies, our serologic assays revealed that receipt of the prokaryotic cell-expressed M2e protein alone did not exhibit neutralizing activity against homologous or heterologous viruses indicating that our M2e formulation might not be sufficient to prevent morbidity. Similar results were also observed in a report that utilized baculovirus-expressed M2 VLPs [20]. Surprisingly, apart from providing homologous protection, an inactivated H9N2 (a-H9N2) vaccine in combination with the 4×M2e protein elicited enhanced cross-protection against a mouse-adapted H5N2 avian virus A/aquatic bird/Korea/maW81/05 and appeared to extend against a human H3N2 (A/Philippines/2/82) virus. Although sterile immunity was not achieved in any of our vaccination strategies, our data demonstrated potentially interesting enhancement in cross-protection.

Neutralization of influenza viruses has been primarily attributed as a function of antibodies directed against the HA surface glycoprotein antigen. However, anti-NA antibodies could also produce apparent neutralization by steric inhibition of virus adsorption and by interfering with viral release [37,38]. Comparison of the deduced N2 amino acid sequences of the three viruses showed 91.9% and 83.8% homology between H9N2 and the H5N2 and H3N2 viruses, respectively. Therefore, we could not completely rule out the role of N2-derived antibodies in the cross-neutralization and protection observed in Figures 2 and 4. Apparently though, mixture of the a-H9N2 vaccine with monomer and polymer M2e exhibited improved serologic and survival values particularly those with the a-H9N2/4×M2e vaccine group. M2e-specific antibodies have been shown to induce humoral immunity and mediate protection against influenza infection in vivo [39,40]. Furthermore, M2e-specific
antibodies could promote antibody-dependent cell-mediated cytotoxicity (ADCC) and/or complement-mediated cytotoxicity (CDC) [41,42]. Therefore, we speculate that the difference in cross-protectivity afforded by the 1×M2e and 4×M2e, albeit administration of similar antigen concentrations, was mediated by the multiple copies of the M2e proteins that induced more robust cross-reactive antibodies.

Development of influenza M2e vaccines based on prokaryotic expression system without adjuvant is significant since E. coli-expressed M2e can be easily produced, safe and practical for animal and public health use. One concern about M2-based vaccines is the possibility that escape mutants may arise. However, a study of forced escape mutants found limited diversity [43] indicating that structural constraints, perhaps due to the requirements

Figure 5 Vaccine containing M2e protein showed a reduction in viral load during the course of influenza A virus infection. BALB/c mice (9 heads/group) were infected with A/chicken/Korea/ma163/04 (ma163/H9N2), A/aquatic bird/Korea/maW81/05 (maW81/H5N2), or A/Philippines/2/82 (Phil82/H3N2) influenza virus at 2LD50 per mouse. Mice were sacrificed 3, 5, or 7 days post-challenge and samples were collected for lung virus titer. Lung (A) ma163/H9N2, (B) maW81/H5N2, and (C) Phil82/H3N2 influenza virus titers were detected at 3, 5, and 7 days post-challenge. The data are presented as GMT ± SD of 3 mice per group. † indicates p < 0.01 compared to the control group; ‡, p < 0.001 compared to the control group; §, p < 0.05 relative to the control group; mice died are indicated by *. The lower limit of detection (0.75 log10TCID50) is indicated by a dotted line.
of the M1 structure encoded by the same segment, may limit drift [36]. It is noteworthy that our vaccination strategy showed that H9N2/4×M2e could protect the immunized host against a range of the viruses containing mismatched amino acid sequence (ranging from 0 to 3 out of 24 amino acids) of the M2e protein from A/chicken/Vietnam/27262/09 (H5N1) strain (Table 2). A previous report has shown that the monoclonal anti-M2e 14C2 (IgG1) antibody inhibits plaque growth of some influenza strains in vitro [10]. In addition, another study showed that M2 VLPs (eukaryotic expression system) provides complete cross protection against influenza A virus [20]. However, producing the VLP-based M2 proteins is relatively tedious and expensive compared to prokaryote-expressed ones. In addition, most of the studies conducted so far used M2e proteins in combination with various adjuvants. Therefore such reports may not have appreciated the additive effect of the M2e proteins (alone) which we observed when combined with an inactivated whole-virus vaccine.

Conclusion
Recently, there have been some concerns regarding the possible emergence of a new influenza pandemic by avian H5N1, H9N2, and H3N2 variants. Furthermore, the number of reported cases of human infections with a novel triple reassortant A (H3N2)v (isolated from North American swine) [44] has been increasing since July 2012 [45,46] indicating a potential public health risk. Therefore, the development of universal influenza vaccines against various subtypes is urgently needed. In this study, we have demonstrated the efficacy of E. coli-expressed M2e proteins in providing cross-protection against lethal influenza virus infection. We provide evidence that an inactivated a-H9N2 vaccine containing M2e proteins could be potential candidate for inducing cross-protection, as shown against avian A/chicken/Korea/ma163/04(H9N2) and A/Aquatic bird/Korea/maW81/05(H5N2) and human A/Philippines/2/82(H3N2) influenza viruses. The cross-reactivity and protective efficacy of the M2e protein suggests that polymer M2e protein, which in our case 4×M2e, could potentially promote protection against other influenza viruses.

Overall, our results demonstrate that prokaryote-expressed 1×M2e and 4×M2e protein immunization with an inactivated vaccine are efficacious against influenza A virus in mice. Although sterile immunity was not achieved in any of our vaccination strategies, our data demonstrated potentially interesting enhancement in cross-protection. These findings may offer an approach to control epidemic and pandemic influenza viruses.

Materials and methods
Construction of plasmids expressing 1×M2e or 4×M2e protein
The M2e protein from A/chicken/Vietnam/27262/09 (H5N1) was amplified and inserted into pRSETA vector (Invitrogen, CA, USA). For the 1×M2e gene cloning, M2e was amplified using a forward primer containing the Nhe I enzyme recognition site (Primer 1) and a reverse primer (Primer 3) bearing the BamH I and Hind III sites with stop codons (TAATGA) in between (Figure 1 and Table 3). To create the M2e polymer construct, a forward primer containing the Bgl II enzyme recognition site (Primer 2) was used and paired with Primer 3. Amplicons from Primer 1/3 and Primer 2/3 were digested with corresponding Nhe I/BamH I (Fragment 1) and Bgl II/ Hind III restriction enzymes (Fragment 2), respectively. The fragments were then ligated together with a T4 DNA ligase (Invitrogen, CA, USA) and inserted into a T-easy vector (Promega, Wisconsin, USA). Cloned product is further digested with BamH I and then fused with Fragment 2. The process was repeated until a construct bearing four copies of the M2e protein (4×M2e) was produced with linker DNA sequences (21 nucleotide bases) in between the polymer. Upon confirmation, the two proteins (1×M2e and 4×M2e) were expressed in E. coli BL21 (DE3) bacterial cells and then purified by Ni-NTA beads through the His–tag affinity purification. The purified proteins were further processed to remove potential bacterially-derived endotoxin as previously described [34]. Briefly, the purified 1×M2e and 4×M2e proteins containing endotoxin were filtered through Polymixin B column kit (GenScript, USA). The endotoxin level of each protein was measured by the toxinsensor™ chromogenic limulus amebocyte lyase (LAL) endotoxin assay kit according to the manufacturer’s instructions (GenScript, USA). Endotoxin levels of the proteins were less than 0.18. Concentrations of eluted proteins were determined by Bradford Protein Assay Kit (Bio-rad). Thirty micrograms of the purified proteins were electrophoresed on a 10-15% SDS-PAGE and were visualized by Coomassie brilliant blue staining (Figure 1B and 1C). Purified proteins were stored at −80°C until use.

Mice and viruses
Five-week-old female BALB/c (H-2b) mice were purchased from SAMTAKO (Pyungteack, Korea). The A/chicken/Korea/ma163/04 (ma163/H9N2), A/aquatic bird/Korea/maW81/05 (maW81/H5N2), and A/Philippines/2/82 (Phil82/H3N2) were grown for two days at 37°C in the allantoic cavities of 10-day-old fertile chicken eggs. Clarified allantoic fluids were aliquoted and then stored at −70°C.
Table 3 The list and sequence of primers used for PCR analysis

| Primer number | Sequence | Length (base) |
|---------------|----------|---------------|
| 1             | 5'-CTAGCTAGCTAGATTCATTATTAACA-3' | 24 |
| 2             | 5'-GAAGATACTATGCTATTATAACA-3' | 23 |
| 3             | 5'-AGCTTTAATGAGATCCACCTGACCCACTGAAACCACCATCAGTTC-3' | 57 |

Nucleotide bases in bold are the stop codons. Sequences of the used restriction enzyme sites are underlined.

Cell line
Madin-Darby Canine Kidney (MDCK) cells obtained from ATCC were maintained in EMEM (LONZA, Inc., Allendale, NJ) supplemented with 5% fetal bovine serum (LONZA, Inc.), 1% penicillin/streptomycin (Gibco-Invitrogen, Inc., Carlsbad, CA), and 1% non-essential amino acids (Gibco-Invitrogen, Inc.).

Vaccination and challenge study
Five-week-old female inbred BALB/c mice were used for all experiments. Groups of 19 mice were intramuscularly (i.m.) immunized with 2 μg of inactivated H9N2 vaccine (a-H9N2), only 1×M2e (15 μg), only 4×M2e (15 μg), inactivated H9N2 + 1×M2e (a-H9N2/1×M2e) and inactivated H9N2 + 4×M2e (a-H9N2 vaccine/4×M2e) with two doses at three week intervals. Two weeks after the final immunization, mice were lightly anaesthetized and challenged intranasally (i.n.) with 2LD₅₀ of A/chicken/Korea/ma163/04 (ma163/H9N2), A/aquatic bird/Korea/maW81/05 (maW81/H5N2), or A/Philippines/2/82 (Phil82/H3N2) in a volume of 30 μl. Following infection, three mice were sacrificed 3, 5, and 7 dpi for lung viral titrations whereas the remaining ten mice were monitored daily for morbidity assessed by measuring body weight loss and survival for up to 14 dpi. Individual body weights were recorded for each mouse on various days post-infection.

Hemagglutination inhibition (HI) test
Total lung homogenate samples were treated with receptor-destroying enzyme (RDE, Denka Seiken, Japan) at 37°C overnight, followed by heat-inactivation at 56°C for 30 min. RDE-treated lung samples were serially diluted two-fold and incubated with 25 μl of ma163/H9N2, maW81/H5N2, or Phil82/H3N2 virus in U-bottom microtiter plates (Nunc, Corning, NY) in a volume of 30 μl. Following infection, three mice were sacrificed 3, 5, and 7 dpi for lung viral titrations whereas the remaining ten mice were monitored daily for morbidity assessed by measuring body weight loss and survival for up to 14 dpi. Individual body weights were recorded for each mouse on various days post-infection.

Neutralizing assay
Twenty-five microliters of Phosphate buffer saline (PBS) was dispensed in a 96-well microplate. Heat-inactivated serum samples (at 56°C for 30 min) were serially diluted two-fold and incubated with 25 μl of ma163/H9N2, maW81/H5N2, or Phil82/H3N2 virus in a volume of 30 μl. Following infection, three mice were sacrificed 3, 5, and 7 dpi for lung viral titrations whereas the remaining ten mice were monitored daily for morbidity assessed by measuring body weight loss and survival for up to 14 dpi. Individual body weights were recorded for each mouse on various days post-infection.

Virus titers in lung tissues
To determine titers of infectious virus in lungs of infected mice, lung samples from three mice per group were collected 3, 5, or 7 dpi. Lung tissues from euthanized mice were aseptically extracted and homogenized in minimal essential medium (MEM). Antibiotics were added to achieve 10% (w/v) suspensions of lungs. Ten-fold serial dilutions of samples were added in quadruplicate to a monolayer of MDCK cells seeded in 96-well cell culture plates 18 h before infection, and allowed to absorb for 2 h at 37°C. Fresh medium was then added to the cells, which are incubated back at 37°C for 48 h. Virus cytopathic effect (CPE) was observed daily and the viral titer was determined by the hemagglutinin (HA) test as follows. Fifty μl of 0.5% tRBCs were added to 50 μl of cell culture supernatant and incubated at room temperature for 30 min. Wells showing HA activity were scored as positive. The virus titer was calculated by the Reed and Muench method [47] and expressed as log₁₀TCID₅₀/ml of lung tissue.

Statistical analysis
The data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA). p values of less than 0.05 (p < 0.05) were considered to be statistically significant.

Ethics statement
The research protocol for the use of mice in this study was conducted in strict accordance and adherence to relevant policies regarding animal handling as mandated under the Guidelines for Animal Use and Care of the Korea Center for Disease Control (K-CDC) and was approved by the Medical Research Institute (approval number CBNM-IRB-2012-GM01). Animal care and use in an enhanced biosafety level 3 containment laboratory
was approved by the Animal Experiment Committee of Bioleaders Corp. (permit number BLS-ABSL-12-010).

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
EHK, PNP, AD and YKC conceived the study and wrote the paper. EHK, YHB, JHL, HIK, SJP, GIL, MYEC and SKS performed the experiments. EHK, MSS, MKS, CJK and YKC analyzed the data. All authors have read and approved of the final manuscript.

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