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## Delivery of Woodchuck Hepatitis Virus-like Particle Presented Influenza M2e by Recombinant Attenuated *Salmonella* Displaying a Delayed Lysis Phenotype

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### Keywords

*Salmonella*; virus-like particle; influenza; RASV; M2e

## 1. Introduction

Influenza virus is one of the most widespread respiratory viral pathogens currently known to man. It has three transmembrane proteins expressed on the surface of both virions and infected cells. Two of these, hemagglutinin (HA) and neuraminidase (NA), are glycoproteins and are highly variable whereas the third, the matrix 2 protein (M2), has remained relatively conserved. Current influenza vaccines depend on HA as the primary antigen but researchers are exploring using both the NA and M2 as potential alternate antigens for vaccine development.

Yearly epidemics and the more infrequent pandemics occur due to two separate mechanisms, both relating to the potential for changes in HA and NA. Yearly epidemics are caused by antigenic drift, which is the accumulation of mutations in the hemagglutinin or neuraminidase genes in the currently circulating virus allowing it to escape the immunity generated by the original virus. With current egg-based technologies the process of producing a matching vaccine takes 6–8 months. Because of this delay vaccine

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manufacturers must predict the antigenic features of the strain(s) that will predominate the next season. Therefore, the vaccine's effectiveness depends heavily on the accuracy of this prediction. The more infrequent pandemics can be caused by antigenic shift, the replacement of either HA or NA subtypes with novel ones, resulting in influenza subtypes to which the general population may be totally naive [1]. Even with new technologies such as cell based influenza vaccines and chimeric virus-like particles (VLPs) [2] immunity will still be dependent upon the similarity of the HA between the circulating virus and the formulation of the vaccine. In light of this, conserved proteins such as the extracellular domain of the matrix 2 (M2) protein have been investigated as a potential "universal" vaccine for influenza [3].

The M2 protein of influenza A virus is a homotetramer which forms a proton channel and is required for viral entry into the cell [4] as well as for packaging viral genomes into newly formed virions [5,6]. Although it is expressed only at low levels on viral particles, it is expressed at much higher levels on infected host cells [3,7]. It is for this reason, in addition to its highly conserved nature, that the extracellular domain of the M2 protein (M2e) has been studied as a target for effective vaccine development for the past twenty years. Monoclonal antibodies against M2e have been demonstrated to reduce viral titers [3], providing the basis for the use of this protein as a vaccine candidate. The entire M2 protein produced in baculoviruses provided heterosubtypic protection from lethal challenge [8], bacterially-produced M2 lacking the transmembrane region was found to decrease lung titers across virus subtypes [9], and a DNA vaccine construct causing expression of the M proteins in host cells prevented mortality in mice [10]. The M2e portion of the molecule has been used in various forms including single or multiple peptide carrier conjugates or fusions [11–13], liposome encapsulated multiple antigens [14], fusions with TLR ligands [15], oligomerized M2e domains [16] and fusion with VLPs derived from the Q $\beta$  RNA phage [17], hepatitis B virus (HBV)[18,19], papaya mosaic virus (PapMV) [20], and human papillomavirus (HPV) [21], all resulting in induction of both immunity and protection from lethal influenza virus challenge. Recently, the core antigen from the woodchuck hepatitis virus (WHc) has been developed as an epitope carrier platform [22] with specific advantages over the HBV platform such as ease of insertion of sequences and antigenic dissimilarity to the human hepatitis virus thereby avoiding any pre-existing immunity to the carrier [23,24].

Without exception the above vaccine platforms are designed for either intranasal or needle assisted delivery (subcutaneous or intra-muscular) and require purification of the antigen or particle prior to delivery [15,17–21,23,24]. With the exception of the flagellin linked M2e epitope [15] the above methods also require some form of adjuvant to be fully effective. Orally administered recombinant attenuated *Salmonella* vaccines (RASVs) are able to colonize the gut-associated lymphoid tissue (GALT) and the secondary lymphatic tissue, eliciting mucosal, humoral, and cellular immune responses against both itself and heterologous antigens [25,26]. Over 50 different bacterial, viral, and protozoan antigens have been expressed in these RASVs in preclinical and clinical trials [25–27] making RASVs an attractive and a reliable platform for delivering antigens against various pathogens.

A number of factors influence the immunogenicity of antigens delivered by *Salmonella*, the most important being the method of attenuation [28] and the intracellular location of antigens [29]. Previous work has demonstrated that regulated delayed attenuation can increase antigen specific antibodies as well as protection from a lethal challenge [30] and that secretion or release of the antigen also improves the immune response [31]. As macromolecules such as VLPs cannot be secreted by *Salmonella* we took the advantage of the recently developed regulated lysis system to deliver VLPs to the host. [32].

The regulated lysis system consists of two components, the strain and the plasmid. The first component is *Salmonella* Typhimurium strain  $\chi$ 8937, with deletion of *asdA* and insertion of arabinose-regulated expression of *murA*. The *asdA* and *murA* genes are required for peptidoglycan synthesis. Additional mutations are also present to enhance complete lysis and antigen delivery. The second component is a derivative of plasmid pYA3681, which encodes arabinose regulated *murA* and *asdA* expression, and C2-regulated synthesis of antisense *asdA* and *murA* mRNA transcribed from the P22 P<sub>R</sub> promoter [32]. To create a strain exhibiting a delayed lysis phenotype  $\Delta$ *araBAD* and  $\Delta$ *araE* mutations were included in the original lysis strain  $\chi$ 8937. The  $\Delta$ *araBAD* denotes the deletion of structural genes for catabolism of arabinose thereby preventing the use of arabinose retained in the cell cytoplasm at the time of immunization. The  $\Delta$ *araE* mutation, which deletes the gene for arabinose transport and thus enhances retention of arabinose, precludes leakage of internalized arabinose. This inability to use arabinose prolongs time to lysis in vivo by one to two cell divisions increasing cell numbers and thus enhancing antigen delivery [33]. *S.* Typhimurium strain  $\chi$ 8888 (Table 1) used in the current study is the prototype strain exhibiting all the above mentioned features and we hypothesize that lysis of the vaccine strain would result in better accessibility of the target antigen.

In contrast to  $\chi$ 8888 the strain  $\chi$ 8025 (Table 1) is constitutively attenuated in vivo through the addition of the  $\Delta$ *cya-27* and  $\Delta$ *crp-27* mutations which together eliminate the catabolite repressor protein. The  $\Delta$ *asdA16* mutation prevents synthesis of diaminopimelic acid (DAP), a unique essential constituent of the peptidoglycan layer of the cell wall. In the absence of DAP, cells undergo cell wall-less death and lyse. The mutation can be complemented either by an external supply of DAP in the growth medium or by complementing with the wild-type copy of *asd* on a plasmid, resulting in a balanced-lethal system [34,35].

Attenuated *S.* Typhimurium strains have been used to deliver both viral and protozoan epitopes on a hybrid Hepatitis B virus (HBV) core antigen (HBc) [36]. In the present work we demonstrate that the WHcAg platform can be expressed in *S.* Typhimurium and when fused to an immunogenic viral epitope, can induce a strong immune response in mice. We further examine this model by comparing the effectiveness of delivery by the above two RASV strains as well as multiple routes of administration of the RASV.

## 2. Materials and Methods

### 2.1. Bacterial strains, plasmids, media, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* Top10 strain was used for all cloning experiments. *E. coli* or *S.* Typhimurium cultures were grown at 37°C in LB broth or on LB agar plates [37]. When required, antibiotics were added to growth media at the following concentrations: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; and tetracycline, 12.5 µg/ml. Diaminopimelic acid (DAP) (50 µg/ml) was added for the growth of  $\Delta$ *asdA* strains [34] and 0.2% arabinose was added in addition to DAP for the growth of strain  $\chi$ 8888 [32].

### 2.2. General DNA procedures

DNA manipulations were carried out as described by Sambrook *et al.* [38]. Transformations into *E. coli* or *S.* Typhimurium were done by electroporation (Bio-Rad, Hercules, CA). Transformants containing Asd<sup>+</sup> plasmids were selected on LB agar plates without DAP. *S.* Typhimurium strain  $\chi$ 8888 transformants containing derivatives of pYA3681 were selected on LB agar plates without DAP and with 0.1% arabinose. Only clones containing the recombinant plasmids were able to grow under these conditions [32,34,39].

### 2.3. Construction of plasmids pYA3664, pYA4037, and pYA4038

Plasmid pUCHyW2 AM2A19 78 (Table 1), which codes for an altered WHc lacking DNA/RNA binding site at its C-terminus, was used as the template for construction of WHc-M2e hybrid particles. The M2e sequence MSLLETVETPTRNGWECSASDSSD was inserted at amino acid position 76 of the original sequence with the following changes: we deleted the leading methionine (M1) and the cysteine in position 19 was changed to alanine (C19A) to prevent formation of disulfide bonds [22]. To improve expression of the heterologous gene in *Salmonella*, we replaced certain rare codons in the gene with codons that are compatible with the tRNA pool in *S. Typhimurium* [40] specifically those encoding aa 3 (ATA to ATT), aa 5 (CCC to CCG), aa 27 (CTT-CTG), aa 42 (CTA to CTG), aa 45 (AGG to CGC), aa 56 (AGA to CGT), aa 76 (ATA to ATC), aa 81 (CTA to CTG), aa 83 (CTA to CTG), aa 84 (CTA to CTG), aa 92 (AGG to CGC), aa 109 (AGA to CGC), aa 125 (AGA to CGC), aa 153 (ATC to ATT), aa 154 (AGG to CGT), aa 160 (AGA to CGT), aa 165 (CCC to CCG), and aa 180 (AGG to CGT) resulting in pYA4036. The intermediate plasmid pYA4036 was digested with the restriction enzymes *NcoI* and *BamHI* and the insert was sub-cloned individually into expression vectors pYA3341 (pUC *ori*) to obtain pYA4037; pYA3342 (pBR *ori*) to obtain pYA4038 and into the lysis vector pYA3681 (pBR *ori*) to obtain pYA3664 (Fig. 1).

Nucleotide sequencing reactions were performed by the sequencing facility at Arizona State University, using ABI Prism fluorescent BigDye terminators according to the instructions of the manufacturer (PE Biosystems, Norwalk, CT).

### 2.4 SDS and native PAGE gels and immunoblot analysis

To evaluate expression of hybrid core monomers and assembled particles, cells were grown in LB medium, with 0.1% arabinose added for the growth of the strain  $\chi$ 8888(pYA3664) overnight. The supernatant was removed and cultures were lysed in PBS with complete protease inhibitors (Roche, Indianapolis, IN) at 10 ml per liter by passing the culture twice through a French Pressure Press Cell (Thermo Scientific, Waltham, MA) at a pressure of 13,000 psi. Cell lysates were then cleared by centrifugation at  $10,000 \times g$  for 30 min and the soluble protein was normalized for protein concentration using absorbance at 280 nm. Samples for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were mixed with 1/5 volume of  $5 \times$  sodium dodecyl sulfate (SDS) loading buffer, boiled for 10 min and centrifuged at  $10,000 \times g$  for 10 min. Samples were loaded on a 12.5% SDS-PAGE gel and separated by electrophoresis as previously described [41]. Samples for native PAGE electrophoresis were mixed with 1/5 volume native loading buffer loaded onto a NativePAGE™ Novex® 3–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA). Reduced samples were transferred to nitrocellulose membranes and native samples to polyvinylidene (PVDF) membranes. The membranes were blocked with 5% skim milk in PBS for 1 h at room temperature and incubated with a 1:5000 dilution of rabbit polyclonal antibody specific for the WHcAg overnight at 4°C. Membranes were then washed three times with PBS- Tween 20. Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) (Southern Biotech, Birmingham, AL) diluted 1:10,000 was then added in PBS-milk for 1 h. After washing with PBS-T three times immunoreactive bands were visualized by the addition of nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma, St. Louis, MO). The reaction was stopped after 5 min by washing with several large volumes of deionized water.

### 2.5. Purification and electron microscopy of core particles

Crude lysates were semi-purified by ultracentrifugation through a 30 % (v/v) sucrose cushion in an Airfuge (Beckman Coulter, Brea, CA) at  $95,000 \times g$  for 15 min. The pellet was washed once with PBS (pH 7.2) and centrifuged at  $95,000 \times g$  for 15 min. The pellet was

resuspended in 10  $\mu$ l PBS and stained with an equal volume of 1% phosphotungstic acid (PTA) for 10 min and then adsorbed on carbon-coated formvar grids. Excess liquid was removed and the grids were air dried and observed by electron microscopy (Philips CM12, Hillsboro, OR).

## 2.6. Immunization of Mice and Sample Collection

All animal experiments were conducted as per protocols approved by the ASU IACUC. Mice were kept 1 week after arrival to acclimate them to our animal facility prior to immunization. Each group of 5 or 10 inbred 7 week old female BALB/c mice (Charles Rivers Laboratories, Wilmington, MA) was deprived of food and water for 6 hours before oral immunization. Recombinant *S. Typhimurium* strains  $\chi$ 8025(pYA3341) and  $\chi$ 8025(pYA4037) were grown in LB and strains  $\chi$ 8888(pYA3681) and  $\chi$ 8888(pYA3664) were grown in LB with 0.1% arabinose to an optical density at 600 nm of 0.9. The cultures were centrifuged at  $4,000 \times g$  at room temperature and suspended in buffered saline containing 0.01% gelatin (BSG) [42] to give a final concentration of  $5 \times 10^{10}$  or  $1 \times 10^8$  CFU/ml. Then, groups of mice were inoculated orally with  $1 \times 10^9$  CFU of bacteria in 20  $\mu$ l or intranasally with  $1 \times 10^7$  CFU of bacteria in 10  $\mu$ l. Food and water was returned to the orally immunized mice 30 min after immunization. A separate group of mice was immunized subcutaneously with 50  $\mu$ g of purified WHc antigen mixed 1:1 with Imject® Alum Adjuvant (Pierce, Rockford, IL). Booster immunization was given to all immunization groups three weeks later. Blood was drawn by cheek pouch bleeding prior to immunization and then on three separate occasions at 2 week intervals after the booster immunization and stored at room temperature overnight. Following centrifugation at  $4,000 \times g$  for 5 minutes the serum was removed and stored at  $-20^\circ\text{C}$ . Vaginal washes were also collected at the same time points in 50  $\mu$ l PBS and stored at  $-20^\circ\text{C}$  [43].

## 2.7. Influenza A strain

The extracellular domain of the M2 protein of A/WSN/33 differs from that found in the avian influenza virus A/Weybridge (H7N7) at three positions: I11T, E14G, and N20S. PCR-mediated mutagenesis was utilized to change the DNA coding sequence of a plasmid encoding the M segment from A/WSN/33 [44] to that found in A/Weybridge at these three positions. The DNA sequence of the mutagenized plasmid was determined to confirm the presence of the introduced nucleotide changes and the absence of any other nucleotide changes within the M segment coding region. The recombinant influenza A virus (rWSN M2 avian) was rescued via plasmid based reverse genetics as described previously [5,44]. Virus stocks were propagated on Madin Darby canine kidney (MDCK) cells, sequenced via RT-PCR to confirm the M2 extracellular domain sequence. Infectious virus titers were determined via the median tissue culture infectious dose (TCID<sub>50</sub>) method or plaque assay as described previously [5,6].

## 2.8. ELISA

IgG, IgG1, IgG2a and IgA responses against each of M2e, LPS, and WHcAg in mouse sera or in vaginal secretions were determined by ELISA. Polystyrene 96-well flat-bottom microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with synthetic M2e peptide (Sigma Genosys) suspended in PBS, purified WHcAg, or LPS suspended in carbonate coating buffer. Plates were coated with 100 ng/well of each antigen and incubated at  $4^\circ\text{C}$  overnight. Free binding sites were blocked with PBS-0.05% Tween 20 containing 0.5% bovine serum albumin. Sera were serially diluted from an initial dilution of 1:50 and the vaginal washes were serially diluted from an initial dilution of 1:10. A 50- $\mu$ l volume of diluted sample was added to duplicate wells and incubated for 1 hour at room temperature. Plates were treated with biotinylated goat anti-mouse IgG, IgG1, IgG2a, or IgA (Southern Biotechnology Inc., Birmingham, AL.). Wells were developed with streptavidin-alkaline

phosphatase conjugate (Southern Biotechnology Inc., Birmingham, AL.) followed by *p*-nitrophenyl phosphate (pNPP) (Sigma). Color development (absorbance) was recorded at 405 nm using an automated ELISA plate reader (SpectraMax, Molecular Devices, Sunnydale, CA). Endpoint titers were expressed as the reciprocal log<sub>2</sub> values of the last positive sample dilution. Absorbance reading two times higher than preimmune serum baseline values was considered positive.

## 2.9. Challenge experiments

To assess vaccine efficacy groups of 5 or 10 mice were lightly anesthetized with 0.05 ml/ 20 gram body weight of a ketamine cocktail (21.0 mg ketamine, 2.4 mg xylazine, and 0.3 mg acepromazine) administered intraperitoneally seven weeks after the boost. They were challenged intranasally with either  $1 \times 10^3$  or  $1 \times 10^4$  TCID<sub>50</sub> of rWSN M2 avian (30  $\mu$ l). Animals were monitored daily for 14 days following the challenge for survival and weight loss. The percent weight loss was calculated for each individual animal per group by comparing the daily weight to the pre-challenge weight of the animal.

## 2.10. In vivo viral burden assay

To assess viral titer in vivo, five mice from each group were euthanized on day 5 post-challenge, and lungs were aseptically collected and homogenized to give a 10% (w/v) solution in PBS (pH=7.2). The homogenates were frozen at  $-70^{\circ}\text{C}$ . Solid debris was pelleted by centrifugation, and clear homogenates were serially diluted ten-fold in RPMI-1640 (Gibco, Grand Island, NY) and titrated on confluent MDCK cells. The limits of virus detection were  $10^{1.3}$  TCID<sub>50</sub>/ml and titers were calculated by the method described by Reed and Muench [45].

## 2.11. Statistical Analysis

Differences in antibody titers were determined using analysis of variance (ANOVA) and statistically different means ( $P < 0.05$ ) were further separated using Tukeys methods. Viral lung titers were analyzed using the Kruskal Wallis test and different means ( $P < 0.05$ ) were determined using Dunns test. Survival analysis was analyzed using the Kaplan-Meier method (GraphPad Prism; GraphPad Software).

## 3. Results

### 3.1 Construction of RASVs

The codon optimized WHc-M2e fusion construct (569 bp) was individually ligated into three vectors in order to determine optimal expression conditions. The gene encoding the fusion protein was cloned into the Asd<sup>+</sup> expression vectors pYA3341 (pUC *ori*), pYA3342 (pBR *ori*) and the lysis vector pYA3681 (pBR *ori*) to yield plasmids pYA4037, pYA4038 and pYA3664, respectively (Fig. 1). Plasmids were transformed into appropriate *S. Typhimurium* strains to yield  $\chi$ 8025(pYA4037),  $\chi$ 8025(pYA4038), and  $\chi$ 8888(pYA3664).

### 3.2 Synthesis of monomers and formation of virus-like particles

The relative amounts of fusion protein produced in *S. Typhimurium*  $\chi$ 8025(pYA4037),  $\chi$ 8025(pYA4038), and  $\chi$ 8888(pYA3664) were evaluated by immunoblot under both reducing (Fig. 2A) and non-reducing (Fig. 2B) conditions. Under reducing conditions cell lysates from the strains carrying the fusion protein reacted strongly and specifically with the anti-WHc antibody, as indicated by the presence of a single band. The same antibody reacted with two separate bands when the purified core particle HyW2 AM2A19 78 was run under the same conditions. One of the bands was of comparable size with the cell lysates and the smaller band probably indicates some proteolytic degradation during purification or

storage. Strains harboring the empty vectors pYA3341 and pYA3681 had no positive bands. The lysate from strain  $\chi$ 8025(pYA4037) containing a pUCori had a stronger positive band than either of the other lysates that have pBRori plasmid replicon, although equal amounts of protein were loaded into the gel.

Under native conditions immune-reactive protein bands were also detected only in lanes containing the purified core particle (served as control) or in lanes containing the lysates from strains  $\chi$ 8025(pYA4037),  $\chi$ 8025(pYA4038) or  $\chi$ 8888(pYA3664) (Fig. 2B), but not in lanes with lysates from the empty vector control  $\chi$ 8025(pYA3681) confirming that the staining was WHc-specific. Two distinct high molecular weight bands were observed probably representing the two different sized core particles, 180 and 240 subunits, commonly seen in hepatitis virus core particles [46] as well as two low molecular weight bands, probably representing monomers and dimers (Fig. 2B). Interestingly, these lower molecular weight bands were seen only in the *Salmonella* cell lysates, but not in the purified core particles suggesting that while core formation does occur in the *Salmonella* strain, only a portion of the monomers are incorporated into particles. Lysates were also examined for core particle formation via transmission electron microscopy (TEM). Particles of the expected size (~26 nm) were observed in all strains synthesizing the fusion protein (Fig. 2C).

As  $\chi$ 8025(pYA4037) synthesized more of the WHc-M2e fusion than  $\chi$ 8025(pYA4038) and as increased protein production generally correlates with increased immune response, the strain  $\chi$ 8025(4037) was chosen for in vivo testing. To test our hypothesis that a lysis phenotype could better deliver a core particle than one that did not lyse we chose to compare the constitutively attenuated strain  $\chi$ 8025(pYA4037) with the strain  $\chi$ 8888(pYA3664) displaying programmed delayed lysis.

### 3.3 Immune responses in orally immunized mice

To investigate the immunogenicity of the WHc-M2e fusions delivered by RASV, we compared the immunogenicity of strains  $\chi$ 8025(pYA4037) and  $\chi$ 8888(pYA3664) in mice orally immunized on days 1 and 21. Serum IgG responses to M2e, the woodchuck hepatitis virus core particle and purified *S. Typhimurium* LPS were measured by ELISA. All of the vaccinated groups had significantly ( $P < 0.01$ ) higher anti-M2e titers than mice immunized with the vector control strain  $\chi$ 8025(pYA3342) and BSG control mice. Mice immunized with strain  $\chi$ 8888(pYA3664) achieved significantly higher anti-M2e titers than those immunized with  $\chi$ 8025(pYA4037) at all three time points ( $P < 0.01$ ) (Fig. 3A).

Similar results were seen with regards to the antibody response to WHc particles (Fig. 3B). All of the vaccinated groups had significantly ( $P < 0.01$ ) higher titers than mice immunized with the vector control strain  $\chi$ 8025(pYA3342) and BSG control mice. Mice immunized with strain  $\chi$ 8888(pYA3664) achieved higher anti-WHc titers than those immunized with  $\chi$ 8025(pYA4037) at all three time points ( $P < 0.01$ ). In contrast, LPS titers did not significantly differ between any *Salmonella* vaccinated group (Fig. 3C) ( $P > 0.05$ ).

These results indicate that the WHc-M2e fusion protein delivered by strain  $\chi$ 8888 induced higher antibody titers in mice than when delivered by strain  $\chi$ 8025. Additionally, since the anti-LPS IgG responses in all groups, including the vector control, were not significantly different this difference is most likely due to better access to the antigen rather than a difference in fitness between the strains. This is reinforced by the fact that the superiority of  $\chi$ 8888(pYA3664) is observed even though it synthesized less WHcM2e fusion than strain  $\chi$ 8025(pYA4037) (Fig.2).

### 3.4. IgG isotype and mucosal IgA analysis

The immune responses to the M2e epitope were further examined by measuring the levels of IgG2a and IgG1 in the serum 2, 4 and 6 weeks post vaccination. The IgG2a titers to M2e in both vaccinated groups were higher than IgG1 and groups vaccinated with  $\chi$ 8888(pYA3664) induced higher titers of both isotypes than groups vaccinated with  $\chi$ 8025(pYA4037) (Fig. 4A). This indicates that all of the *Salmonella* vaccines induced a strong Th1 response against M2e regardless of whether the RASV strain was designed to lyse. Th1-type dominant responses are often observed after administration with attenuated *Salmonella* [47,48]

Both strains also induced secretion of mucosal IgA in vaginal fluids (Fig. 4B). As with IgG levels, groups vaccinated with  $\chi$ 8888(pYA3664) had higher levels than those vaccinated with  $\chi$ 8025(pYA4037).

### 3.5. Evaluation of protection from influenza challenge: Trial 1

To determine whether the RASV delivered WHc-M2e fusions provided protection against influenza, we challenged immunized mice with either  $1 \times 10^3$  or  $1 \times 10^4$  TCID<sub>50</sub> of rWSN M2 avian. At the low dose challenge we observed weight loss in all groups through day 8 post-infection. On days 8 through 12 the groups vaccinated with  $\chi$ 8888(pYA3664) had significantly higher ( $P > 0.05$ ) average weight than the other groups, signifying an earlier recovery from infection than the BSG control, vector control or  $\chi$ 8025(pYA4037) groups (Fig. 5A). At the high challenge dose we observed no difference in average weight throughout the 14 days period (Fig. 5B) and the survival rates were 70% and 60% for  $\chi$ 8025(pYA4037) and  $\chi$ 8888(pYA3664), respectively (Fig. 5C). The BSG and vector control groups had survival rates of 30% and 20% respectively (Fig. 5C). In summary, lower dose groups receiving  $\chi$ 8888(pYA3664) had a more rapid increase in body weight compared to both  $\chi$ 8025(pYA4037) and the controls, whereas at a higher dose groups receiving either vaccine had a relatively higher rate of survival as compared to the controls.

### 3.6. Comparison of administration routes

The data so far indicated that strain  $\chi$ 8888(pYA3664) was the most effective of all three in conferring protective immunity against virulent influenza challenge. Therefore we decided to compare the efficacy of this vaccine strain when administered to mice by alternate routes.  $\chi$ 8888(pYA3664) was given to mice orally ( $1 \times 10^9$  CFU) or intranasally ( $1 \times 10^7$  CFU) on days 1 and 21 and induction of protective immune response in each case were compared with that induced by purified HyW2AMA19 78 recombinant cores given subcutaneously. All vaccinated groups of mice had significantly higher anti-M2e titers than mice immunized with the vector control strain  $\chi$ 8025(pYA3342) ( $P < 0.01$ ) at all time points tested, with the only exception being the intranasal administration group at two-week time point (Fig. 6A). Both RASV administered groups exhibited relatively higher IgG2a as compared to IgG1 whereas the subcutaneous injection had approximately equivalent titers of both IgG1 and IgG2a (Fig. 6B).

### 3.7. Evaluation of protection from influenza challenge: Trial 2

To evaluate protection in immunized mice we challenged 15 mice per group with  $1 \times 10^4$  TCID<sub>50</sub> of rWSN M2 avian and monitored body weight and mortality in 10 of them. Although not significant, survival was lowest at 40% in the group receiving the RASV intranasally, followed by the vector control group at 60%, the purified antigen administered subcutaneously provided 70% protection, and the oral administration gave the best protection at 90% (Fig. 7A). There was no difference between groups with regard to average body weight through 14 days (Fig 7B). The differences in lung titers between the RASV immunized mice were marginal (Fig. 7C). Despite the lower titers in two of five mice the



geometric mean of the oral RASV group did not differ significantly from the vector control, however, the mean lung titers in s.c. group were significantly ( $P<0.05$ ) lower than the vector control group (Fig.7C) suggesting that the difference between the two routes may have influenced the degree of protection.

#### 4. Discussion

Vaccines based on the M2 protein of influenza may provide a novel and much needed alternative or addition to conventional influenza vaccines. Studies have defined the immunogenic epitope as being within the N-terminal 23 amino acids of the protein and determined that it is highly conserved among influenza serotypes. The differences that do exist, particularly between avian and humans strains, are located primarily between amino acids 6 and 13 [49]. Since this epitope is only 23 amino acids in length, two major factors in inducing a strong antibody response are associating it with an appropriate carrier and delivering it with an effective adjuvant. Three basic types of carriers have been used to deliver M2e to mammals; protein or peptide carrier conjugates [8,9,11–14,16], genetic or chemical fusions to VLPs [17–21] and fusion with the Toll-like receptor (TLR) ligand flagellin [15]. With the exception of the flagellin fused construct, all other fusions required adjuvants to achieve maximum protection. This protection includes a decrease in viral lung titers, mortality, and body weight loss [12,15,16,18,21].

Previously our group has demonstrated the presentation and delivery of both viral and protozoan epitopes on the HBc platform using *S. Typhimurium* [36]. In this study we demonstrate that a related platform, WHc, with the avian M2e epitope genetically fused into the immunodominant region can be expressed in *S. Typhimurium* as assembled VLPs (Fig. 2). As responses to *Salmonella* presented antigens are influenced by a number of factors we compared two strains, the constitutively attenuated strain  $\chi$ 8025 and a strain exhibiting delayed lysis  $\chi$ 8888. Differences in the responses to each of the strains are directly linked to these characteristics. Lysis presumably allows the immune system better accessibility to the core particle as opposed to its release after self-destruction of the bacteria and delaying this event allows *Salmonella* time to invade and express certain virulence factors prior to lysis, thus inducing a more robust immune response [26,30,50].

Humoral immune responses (IgG) to the M2e epitope and the VLP itself were higher when expressed in the strain exhibiting the delayed-lysis phenotype ( $\chi$ 8888) than in the one constitutively attenuated ( $\chi$ 8025), while the response to *Salmonella* LPS did not differ between the two (Fig. 3). Although antibody levels were generally as high as in previously reported studies using M2e, complete protection from weight loss or mortality was not achieved with either construct given orally or by the strain exhibiting delayed lysis when given intranasally. We did observe a reduction, although not significant, in lung viral titers in mice given the delayed lysis construct orally as compared to both the intranasal and vector control groups. Interestingly, injecting the purified VLP with an alum adjuvant increased this reduction with a geometric mean significantly different ( $P<0.05$ ) from that of the vector control. Given the observation that the injected particle with adjuvant induced a more balanced Th1/Th2 response as opposed to the *Salmonella* delivered particle (Fig. 6B), there is the possibility that the type of response induced may also be influencing the outcome (Fig. 7C).

Microbial pathogens and vaccines can drive CD4+ T cells to differentiate into Th1 or Th2 cells. Th1 helper cells direct cell-mediated immunity and promote IgG isotype switching to IgG2a while Th2 helper cells drive B-cell antibody production and promote isotype switching to IgG1[51,52]. *Salmonella* vectored vaccines generally induce a Th1 biased response, as is the case with the present constructs (Fig. 4A&C). When the same antigen as

presented by *Salmonella* was delivered s.c. with an alum adjuvant this response was more balanced with regards to the Th1–Th2 response (Fig. 6B). As most previous methods for delivering M2e have involved multiple injections using adjuvant, it may be assumed that they also induced either a balanced Th1–Th2 response or one more biased towards Th2. The single exception of adjuvant use also supports this as flagellin, when used as an adjuvant, has been reported to induce a Th2-biased response [53]. Therefore, despite the strong antibody response to M2e, one explanation for this delivery system's difference in protection as compared to previous studies may be that a Th1 biased response is less effective against influenza virus than a more balanced response. As the inclusion of a *sopB* mutation into *Salmonella* can shift it to a mixed Th1–Th2 response [54], this could easily be tested in future studies.

This explanation is supported by a recent trial involving *Salmonella* vectored delivery of M2e to poultry. Despite a strong antibody response, vaccination against M2e using *Salmonella* could only decrease morbidity and early viral shed against low pathogenic avian influenza (LPAI) viruses. There was no protection from mortality or viral shed when vaccinates were challenged with highly pathogenic avian influenza (HPAI) virus [55]. Despite the apparent lack of different IgG isotypes in chickens, both Th1 and Th2 dominant cytokines have been identified and biases towards one or the other have been associated with specific pathogens [56]. Therefore it is possible that failure against the HPAI challenge may be due more to the type of response induced than the inability of the antigen to induce protection, as M2e has been demonstrated to be able to protect against lethal challenges in mice.

In summary we have shown that the WHc fused to an epitope can be expressed in attenuated *Salmonella* vectors, form cores, and be delivered in an immunogenic manner to the host. Two strains were compared and the strain exhibiting a mechanism for release of cores by lysis induced higher antibody responses than one exhibiting constitutive attenuation. The type of response induced by the constructs was also discussed including possibilities for improvement by incorporation of the *sopB* mutation. Although the goal to completely eliminate symptoms was not met, this represents a significant step towards an affordable, universal vaccine against an important viral pathogen.

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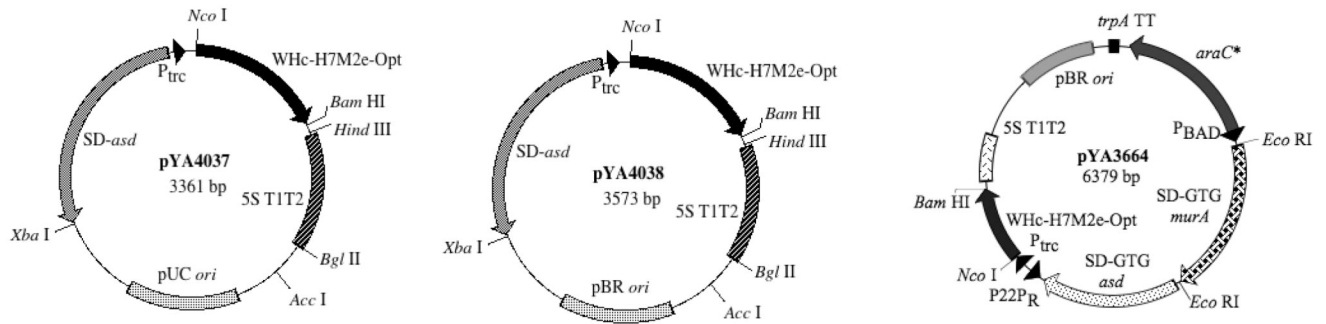
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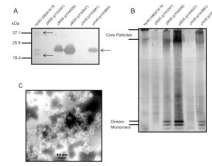
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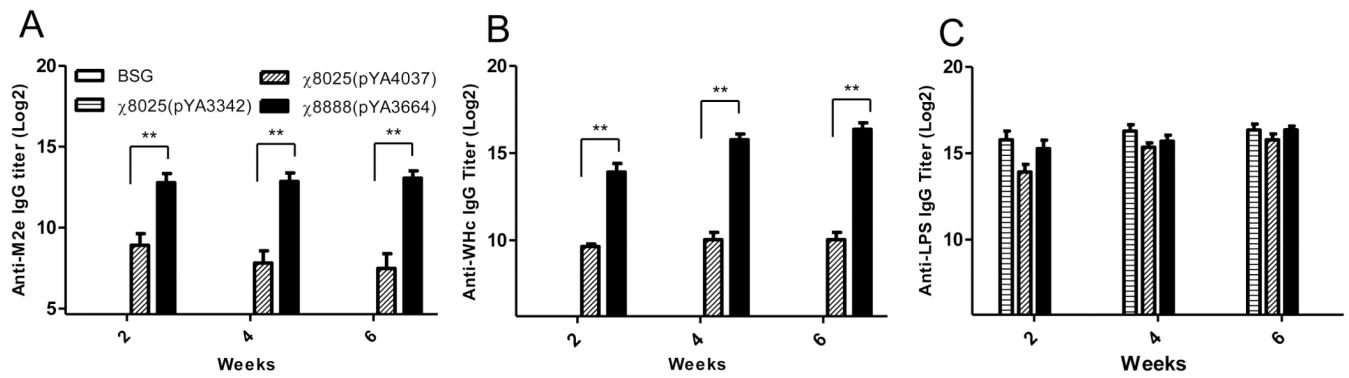


**Fig. 1.**

Plasmid maps of pYA4037, pYA4038, and pYA3664. The 569 bp WHc-H7M2e-opt fusion fragment flanked by *NcoI* and *BamHI* on either sides is in control of the *P<sub>trc</sub>-5S T1T2* promoter-terminator unit in *Asd*<sup>+</sup> vectors pYA4037 and pYA4038 and in control of *P<sub>22P<sub>R</sub>-5S T1T2</sub>* promoter-terminator unit in pYA3664 to finally yield a ~20 kDa fusion protein.

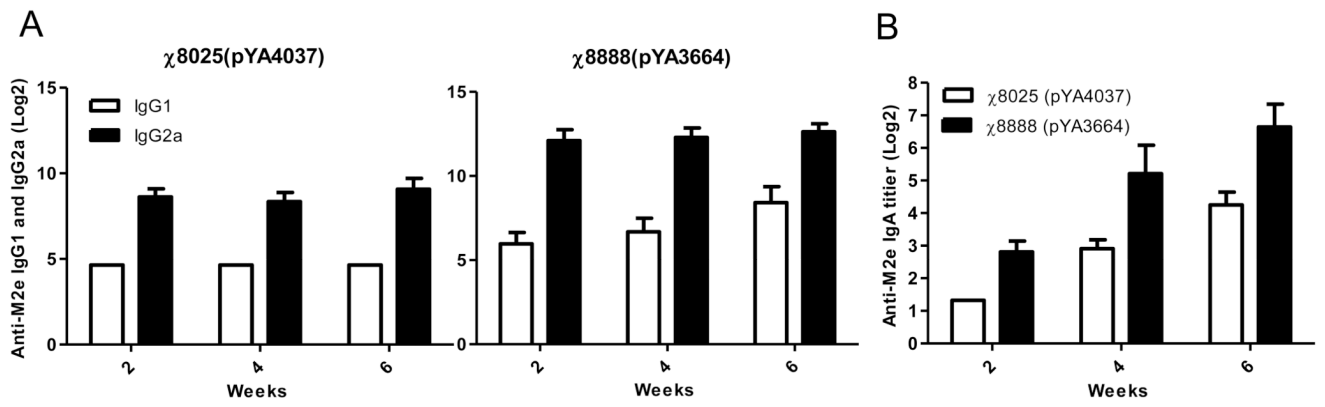


**Fig. 2.** Synthesis and visualization of WHc-M2e cores by staining with polyclonal rabbit anti-WHc antibody under (A) reducing or (B) native conditions and (C) electron micrograph of semi-purified WHc-M2e core particles from lysates of  $\chi$ 8025/pYA4037. Molecular mass (kDa) are given. Arrow indicates protein in each lane.

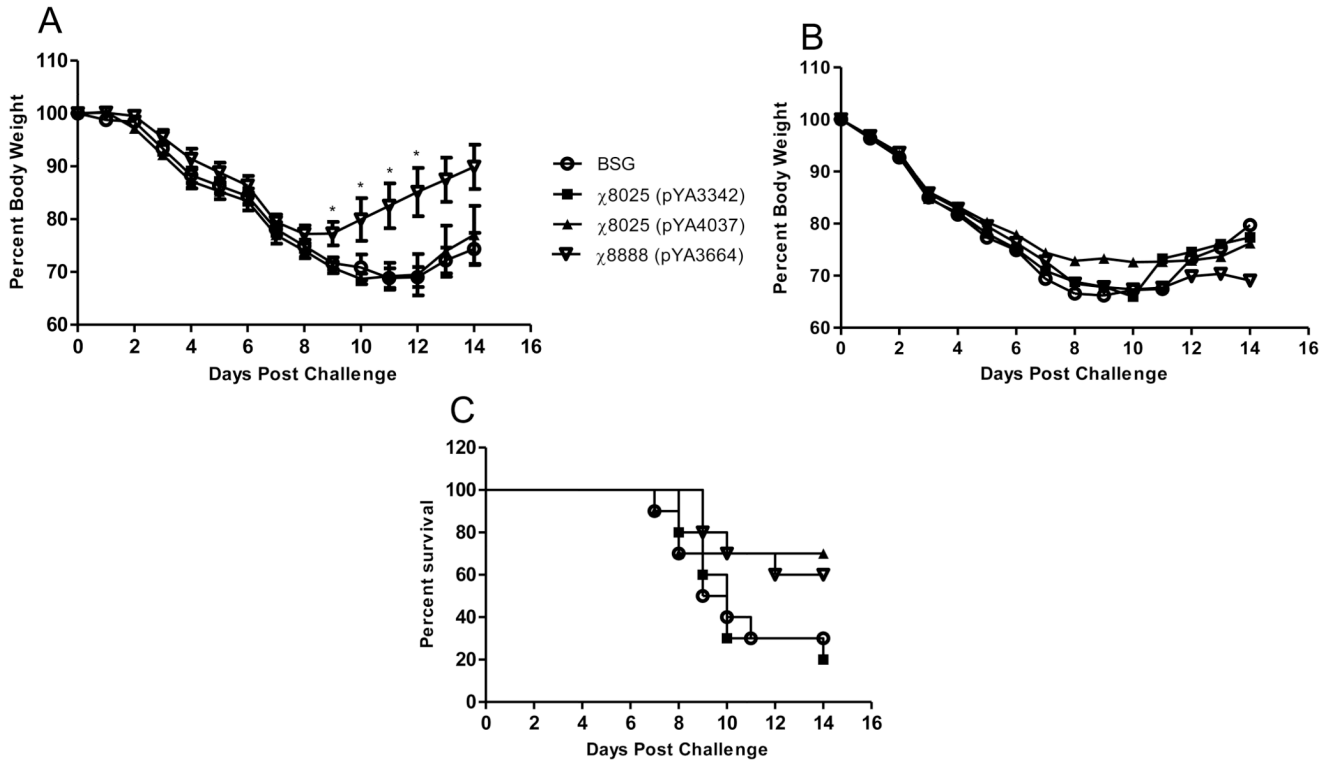


**Fig. 3.** Induction of IgG to (A) M2e peptide, (B) woodchuck hepatitis core and (C) purified *Salmonella* Typhimurium LPS after second oral immunization of mice with recombinant attenuated *Salmonella* expressing WHc-M2e cores. Data presented here are representative data of two independent experiments and are the geometric mean  $\pm$  SE of 10 mice. \*\*  $P < 0.01$

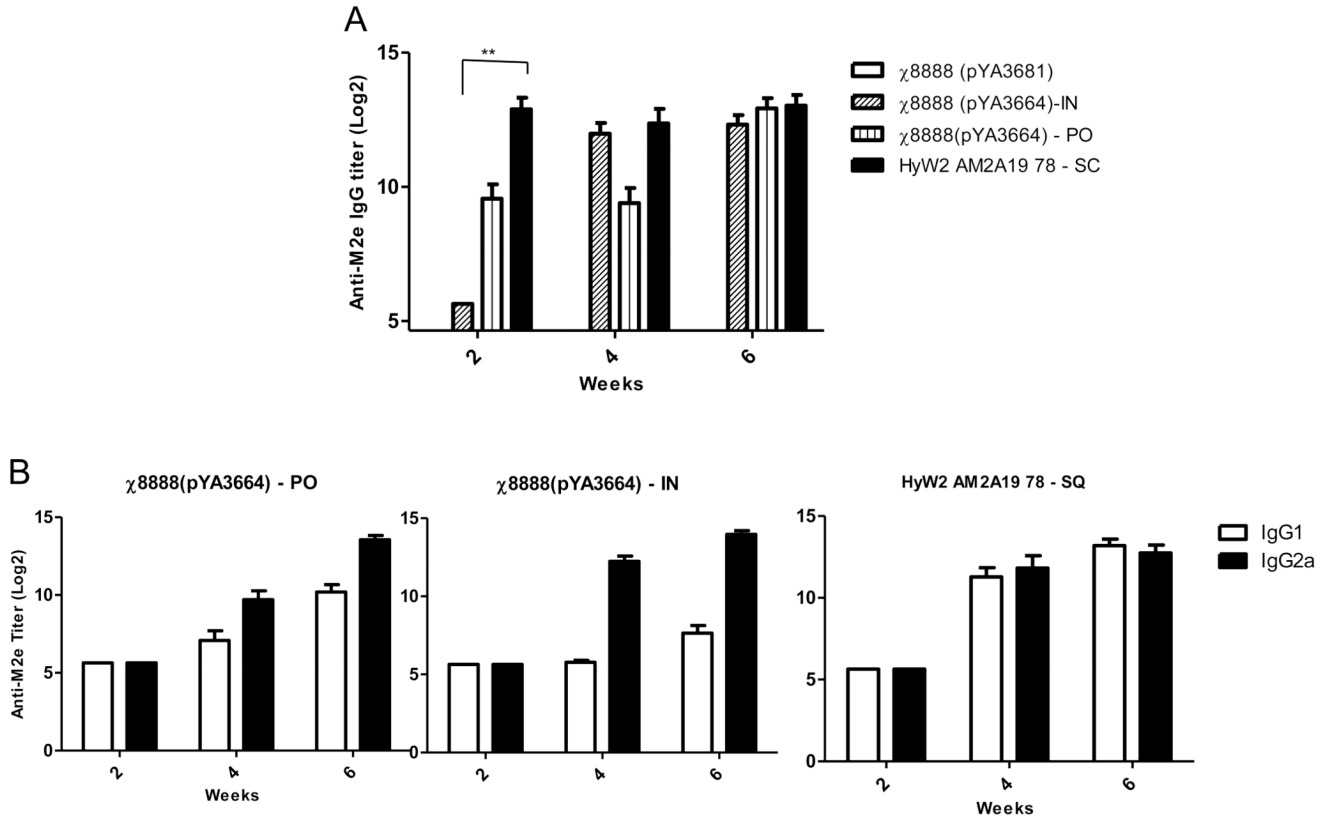




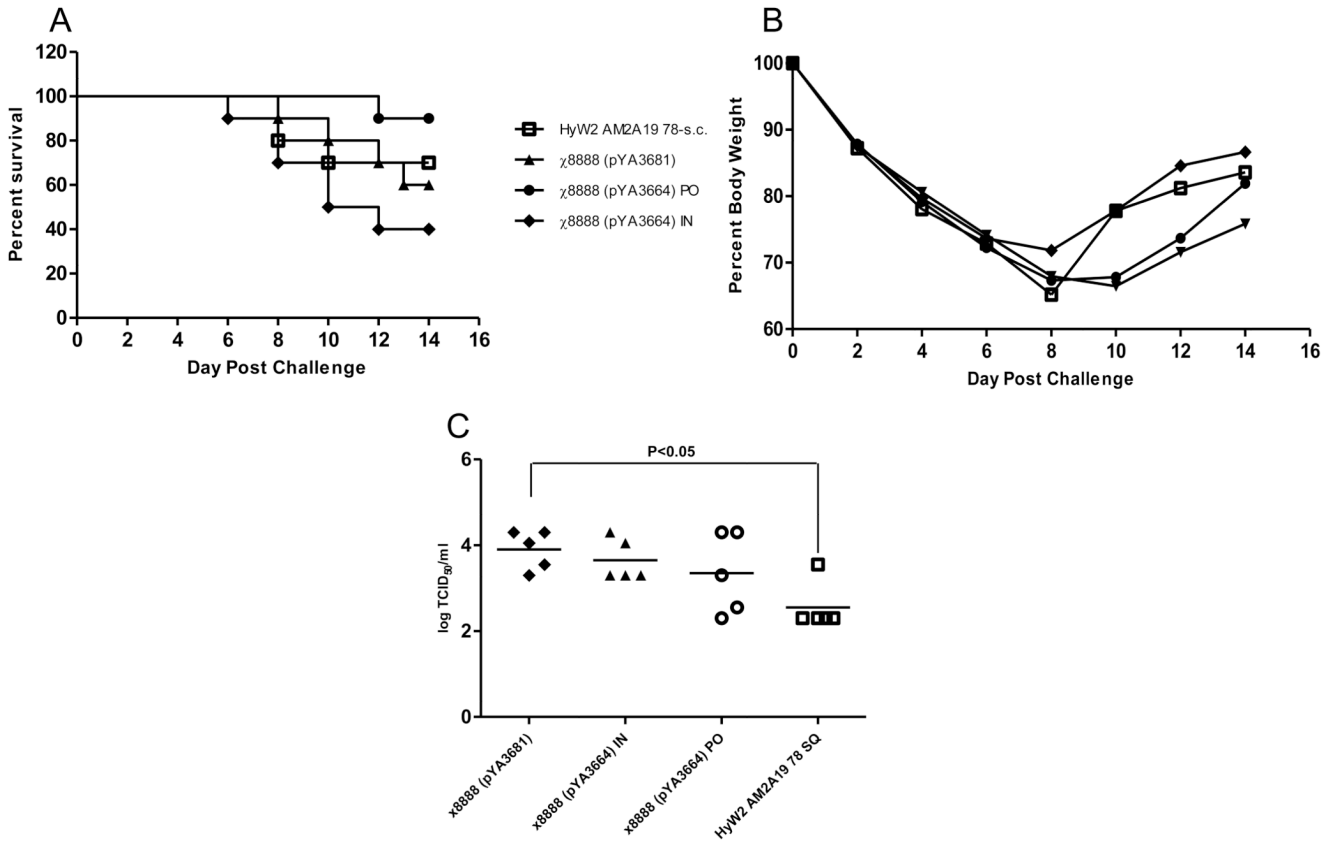
**Fig. 4.** Comparison of (A) serum IgG isotypes and (B) vaginal IgA levels for mice immunized twice with  $\chi$ 8025(pYA4037) and  $\chi$ 8888(pYA3664). Data presented here are representative data of two independent experiments and are the geometric mean  $\pm$  SE of 10 mice.



**Fig. 5.** Weight loss after challenge of immunized mice with (A)  $1 \times 10^3$  or (B)  $1 \times 10^4$  TCID50 of rWSN M2 avian virus. (C) Mortality curve after challenge with  $1 \times 10^4$  TCID50 rWSN M2 avian virus. \*  $P < 0.05$ . Data presented here are mean  $\pm$  SE of 10 mice.



**Fig. 6.** Induction of (A) IgG, and (B) IgG isotype antibodies to M2e peptide after oral or intranasal immunization of mice (n=10) with  $\chi$ 8888(pYA3664) as compared to subcutaneous injection of purified core particle. \* $P < 0.01$



**Fig. 7.** Mortality (A) and weight loss (B) after challenge of immunized mice with  $1 \times 10^4$  TCID<sub>50</sub> of rWSN-avian virus. (C) individual and geometric mean lung titers 5 days post challenge after immunization.

TABLE 1

## Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or Relevant Characteristics	Source or reference
Strains		
<i>E. coli</i> Top10	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74 nupG recA1 araD139</i> Δ( <i>ara-leu</i> )7697 <i>galE15 galK16 rpsL(Str<sup>R</sup>) endA1 λ<sup>-</sup></i>	Invitrogen
<i>S. enterica</i> serovar Typhimurium		
χ8025	Δ <i>cya</i> -27 Δ <i>crp</i> -27 Δ <i>asdA16</i>	Lab Collection
χ8888	Δ <i>araE25</i> Δ <i>araBAD1923</i> Δ <i>endA2311</i> Δ <i>asdA19::TTaraC</i> P <sub>BAD</sub> <i>c2 TT</i> ΔP <sub><i>murA7</i></sub> :: TT <i>araC</i> P <sub>BAD</sub> <i>murA</i> Δ( <i>gmd-fcl</i> )-26 Δ <i>relA1123</i>	Lab Collection
Plasmids		
pUC HyW2 AM2A19 78	Truncated woodchuck core with H7N7 derived M2e in immunodominant spike	Dr. Jean Noel Billaud
pYA4036	Codon optimized HyW2 AM1A19 78 in pUC19	This work
pYA3341	Asd <sup>+</sup> expression vector P <sub>trc</sub> promoter pUC <i>ori</i>	Lab collection
pYA3342	Asd <sup>+</sup> expression vector P <sub>trc</sub> promoter pBR <i>ori</i>	[31]
pYA3681	Asd <sup>+</sup> expression vector P <sub>trc</sub> promoter pBR <i>ori</i>	[32]
pYA4037	Codon optimized HyW2AM2A19 78 in pYA3341	This work
pYA4038	Codon optimized HyW2 AM2A19 78 in pYA3342	This work
pYA4151	HyW2 AM2A19 78 in pYA3681	This work
pYA3664	Codon optimized HyW2 AM2A19 78 in pYA3681	This work