



Swine influenza matrix 2 (M2) protein contributes to protection against infection with different H1 swine influenza virus (SIV) isolates

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ABSTRACT

A swine influenza virus (SIV) vaccine-challenge pig model was used to study the potential of a conserved matrix 2 (M2) protein vaccine alone or in combination with an inactivated H1N1-vaccine to protect against H1N1 and H1N2 viruses. The H1N1-vaccine and heterologous H1N2-challenge virus model has previously been shown to prolong fever and increase SIV-associated pneumonic lesions. The M2 vaccine in combination with the H1N1-vaccine reduced the H1N2 induced fever but not virus shedding. The M2 vaccine alone reduced respiratory signs and pneumonic lesions to levels similar to the negative control pigs following H1N2 infection. This study found that the M2 protein has potential as a vaccine for SIV-associated disease prevention. However, development of an immune response towards the major envelope HA protein was required to reduce SIV shedding.

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1. Introduction

Influenza A virus causes respiratory disease in avian and mammalian species [1]. Immunization with inactivated influenza virus vaccines is currently the most common method used to prevent influenza-associated disease in the human, equine and swine populations. Protection induced by these vaccines has been associated with the induction of antibodies to the two major viral envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [2]. Little to no heterologous cross-reactive immunity (Het-I) between influenza subtypes or genetically diverse viruses within subtype is induced by these vaccines [3]. As a result, influenza A vaccines are often manufactured as a bi- or trivalent vaccines containing genetically different types and/or subtypes of the viruses circulating in each host population. Vaccine efficacy is directly correlated with how closely the vaccine virus matches the field viruses within each subtype [3,4]. Mismatch of the vaccine and field viruses is a frequent cause for vaccine failure in the field. Because influenza type A viruses undergo antigenic drift, human influenza vaccines are re-evaluated yearly. The viruses used in human vaccines are determined by intensive field surveys with laboratory evaluations involving the collaboration of many authorities and organizations around the world.

The emergence of a new swine influenza viruses (SIV) in the United States swine population in the late 1990s has complicated the development of efficacious vaccines for pigs [5]. The entrance of the H3N2 virus into the US swine population has resulted in the generation of new genetically diverse viruses due to viral reassortment. The presence of multiple genetically diverse viruses within each subtype has reduced the success of vaccine efficacy in swine. The importance of matching virus strains within subtypes was recently demonstrated in Europe where a study found that a bivalent H1N1/H3N2 vaccine provided suboptimal protection in pigs against a H1N2 challenge [6]. Interestingly, a separate study observed disease enhancement in pigs that were vaccinated with an inactivated H1N1 virus followed by infection with a genetically different H1N2 virus [7]. Unlike human influenza vaccines, SIV vaccines are not revised annually. Maintaining up to date SIV vaccines would require the same active surveillance and networks of laboratories requiring extensive funding similar to required for human vaccines. Similar laboratory networks and funding is not currently available to the swine industry, reducing the response time to newly emerging viruses.

The concept of a “universal influenza vaccine” based on the conserved matrix 2 (M2) minor envelope protein of the influenza A virus has been a focal point for influenza-associated-disease prevention research [8,9]. The M2 protein forms a proton channel, which is essential in uncoating the virus during the initial stage of infection [10,11]. It is a transmembrane protein composed of a non-glycosylated ectodomain (M2e) made up of 24 amino acids at

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the N-terminus, 19 amino acids spanning the lipid bilayer and 54 amino acids make up the C-terminus cytoplasmic tail [12]. Studies with influenza A and B viruses have shown that antibodies against the extracellular domain of the M2 protein inhibit virus replication in MDCK cells [13] and M2e-specific antibodies decrease the viral load in mouse lungs [14]. Passively transferred antibodies to the M2e protein fused with hepatitis B virus core (HBc) protein demonstrated 90–100% protection against lethal challenge with influenza virus in mice [15].

M2 vaccine-challenge studies conducted in animal models that are not natural hosts for influenza A infection were promising. Several studies in mice found that M2e is capable of inducing antibodies that reduce clinical disease and prevent lethal challenge [14,16,17]. Another vaccine study using a M2 peptide conjugate conducted in mice, ferrets and rhesus monkeys found that the vaccinated animals shed reduced levels of virus in nasal secretions and had reduced amounts of viral antigen in the lungs [18]. An early study in pigs, a natural host of influenza A viruses, suggested that M2e may play a role in heterosubtypic immunity as low levels of M2e-antibodies were detected in pig's sera following a primary H3N2 influenza infection and much higher levels were observed subsequently with infection with a H1N1 virus [19]. In contrast, one experiment investigated if the induction of the cell mediated immune (CMI) response based on two conserved influenza A proteins, M2 and nucleoprotein (NP) provided protection against SIV infection [20]. This study found that pigs vaccinated with a DNA-construct expressing the M2e–NP fusion protein and infected with SIV lacked antibodies to the HA and NA protein and had enhanced disease. These results suggest that the presence of a CMI induced response to the M2e and NP proteins alone without the presence of antibodies against the two major envelope glycoproteins (HA and NA proteins) is insufficient to provide protection against SIV infection and may even result in a negative outcome.

The purpose of this study was to investigate the efficacy of a M2 protein vaccine in combination with an inactivated H1N1 virus in preventing SIV vaccine-associated disease enhancement in pigs using a previously described vaccine-heterologous virus challenge model. This model demonstrated SIV vaccine-associated disease enhancement in an earlier study [7]. This model was used in this study as SIV vaccine-induced disease enhancement can potentially occur in the field from mismatched vaccine and field viruses. Our primary objective was to study the efficacy of the M2 protein when used in combination with a monovalent H1N1 inactivated vaccine in protecting against a heterologous subtype (H1N2) SIV infection compared to the protection induced by the M2 protein alone.

2. Materials and methods

2.1. Experimental design

2.1.1. Virus and vaccine preparation

The virus used to prepare the inactivated SIV vaccine was A/Sw/IA/15/1930 H1N1 (IA30). Viruses used as challenge inocula were the homologous virus (IA30) and a heterologous virus, A/Sw/MN/00194/2003 H1N2 (MN03). Both viruses had been used in a previously described experimental vaccine-challenge model [7]. All viruses were propagated in Madin-Darby canine kidney (MDCK) cells. The virus was harvested and cell debris clarified by centrifugation prior to inoculating pigs. Pigs were inoculated intratracheally with a dose of 5 ml of 1×10^7 50% tissue culture infective dose (TCID₅₀)/milliliter (ml).

The IA30 inactivated vaccine was prepared as described previously [7]. Briefly, IA30 virus with a hemagglutinin unit of 400 per ml was UV-inactivated and tested for viability by inoculating MDCK cells. A commercial adjuvant was added to the inactivated virus at a ratio of 1:1 (Emulsigen, MVP Laboratories, Inc., Ralston, NE).

The recombinant M2 (rM2) protein was produced using a baculovirus expression system and isolated as described previously [21]. A concentration of 50 µg/ml of rM2 protein was combined with the same adjuvant used with the IA30 inactivated vaccine.

2.1.2. Animals

All study procedures and animal care activities were conducted in accordance with the guidelines and under the approval of the Iowa State University (ISU) Institutional Committee on Animal Care and Use.

Forty-eight twelve-day-old pigs obtained from a conventional herd serologically free of SIV, porcine reproductive and respiratory syndrome virus (PRRSV) and *Mycoplasma hyopneumoniae* were allotted randomly to 9 treatment groups. The experimental design is described in Table 1. Throughout the study, pigs were housed in identical isolation rooms based on their challenge status. Pigs were provided feed and water ad libitum.

Pigs in the appropriate groups received an inactivated SIV vaccine described earlier and/or a recombinant M2 protein vaccine at a different site at 3 and 5 weeks of age. The IA30 vaccine and/or rM2 vaccine were administered intramuscularly (IM) to pigs in groups 2–4 and 6–8 at a dose of 2 ml per pigs. Groups assigned to receive both the inactivated SIV vaccine and rM2 vaccine were injected IM at different sites. Pigs were inoculated intratracheally with either the homologous virus (vaccine strain) or a heterologous virus at 7 weeks of age (Trial day 0).

Nasal swabs were collected at –1, 2, 3, and 5 days post infection (DPI). Blood was collected at –29, –22, –15, –8, –1 and 5 DPI. Sera was stored at –20 °C and assayed simultaneously at the end of the trial.

2.2. Clinical evaluation

Pigs were evaluated 2 days prior to infection (–2 DPI) and daily for 5 days after SIV infection for respiratory disease. Pigs were observed for signs of respiratory disease including labored and/or abdominal breathing and coughing both at rest and after obtaining the rectal temperature measurement. Rectal temperatures were obtained from all pigs at 2 days prior to infection (Day –2) and daily for 5 days after SIV infection.

2.3. Necropsy

Pigs were euthanized using a pentobarbital-based euthanasia solution (Beuthanasia, Schering-Plough, Kenilworth, NJ, USA) followed by exsanguination. The lungs were removed and evaluated for pneumonia. Macroscopic lesions associated with SIV pneumonia, consisting of well demarcated dark-purplish areas of lung consolidation [22], were sketched onto a standard lung diagram. The proportion of lung surface with lesions was determined from the diagram using a Zeiss SEM-IPS image analyzing system as previously described [23]. Bronchial swabs were obtained from each pig and cultured for swine respiratory bacteria using standard microbi-

Table 1
Experimental design, vaccination status, SIV infection status and number of pigs.

Group	Vaccination	SIV infection	Total number of pigs
1	No	No	6
2	IA30	IA30	6
3	IA30 + rM2 protein	IA30	6
4	rM2 protein	IA30	3
5	No	IA30	6
6	IA30	MN03	6
7	IA30 + rM2 protein	MN03	6
8	rM2 protein	MN03	3
9	No	MN03	6

ological procedures. Bronchoalveolar lavage (BAL) fluid collection was performed by using 25 ml of phosphate buffer saline (PBS) with 100 U/ml penicillin and 100 mg/ml streptomycin as previously described [24]. A portion of lung tissue was collected from all lung lobes, fixed in 10% neutral buffered formalin processed and embedded in paraffin using an automated tissue processor. Lung sections were scored for microscopic lung lesions consistent with SIV (necrotic bronchiolitis) as described previously [23]. The evaluation primarily focused on airway damage but also took into consideration the degree of inflammation surrounding airways and involving alveoli.

The presence of SIV-specific antigen was assessed in the formalin-fixed lung tissues using a previously described immunohistochemistry (IHC) staining method [25]. IHC was performed on sections cut from one paraffin-embedded lung tissue block and included two pieces (1–2 cm) of lung collected at necropsy.

2.4. Virus isolation

Following sample collection at –1, 2, 3, and 5 DPI, nasal swabs were immediately placed in infecting medium (minimum essential medium with 7% BSA, 300 U/ml penicillin, 300 mg/ml streptomycin and 1 mg/ml trypsin). Viruses isolated from nasal swabs were titrated using ten-fold serial dilution in cell culture media and inoculated onto MDCK cells followed by incubation at 37 °C with 5% CO₂ as described previously [26]. Virus was identified by staining with anti-influenza A nucleoprotein monoclonal antibody (clone HB-65, ATCC, Rockville, Maryland) followed by rabbit anti-mouse IgG conjugated horseradish peroxidase (Dako Cytomation, Carpinteria, California) [26]. The color was developed using a chromogen aminoethyl carbazole substrate (Sigma, St. Louis, Missouri). Each assay contained mock-infected negative control cells and positive control cells infected at a known virus titer. The titer of the virus in each nasal swab was expressed as log₁₀ TCID₅₀ per milliliter calculated by the method of Reed and Muench [27].

2.5. Hemagglutination-inhibition (HI) assay

The HI assays were performed according to a standard protocol routinely used at ISU-Veterinary Diagnostic Laboratory using 0.5% rooster erythrocytes for hemagglutination [28]. Virus antigens utilized in the HI assays included both the challenge viruses, A/Swine/Iowa/15/1930 H1N1 (IA30) and A/Swine/Minnesota/00194/2003 H1N2 (MN03).

2.6. ELISA for mucosal SIV-specific antibody production and serum SIV M2e-specific antibody production

2.6.1. ELISA for mucosal SIV-specific antibody

BAL fluids were incubated at 37 °C for 1 h with an equal amount of 10 mM dithiothreitol (DTT; Sigma–Aldrich, St. Louis, MO) to disrupt mucus present in the fluids. ELISA assays for SIV antibodies in the respiratory tract were performed as previously described [29]. Briefly, inactivated challenge virus was diluted to a hemagglutination (HA) concentration of 100 HA units/50 µl. Immulon-2HB 96-well plates (Dynex, Chantilly, VA) were coated with 100 µl of SIV antigen and incubated at room temperature overnight. Plates were blocked for 1 h with 100 ml of 10% BSA in PBS and washed 3 times with PBST washing buffer (0.1 M phosphate buffer saline pH 7.2 with 0.02% Tween 20). The assay was performed on each BAL sample in duplicate. Negative controls (DTT with equal amount of PBS solution) were included on each plate. Plates were incubated at room temperature for 1 h and incubated with peroxidase-labeled goat anti-swine IgG (Kirkegaard and Perry, Gaithersburg, MD) or peroxidase-labeled goat anti-swine IgA (Bethyl, TX) at 37 °C for 1 h. The ABTS/peroxidase was added as the substrate (Kirkegaard

and Perry, Gaithersburg, MD). Antibody levels were reported as the mean optical density (OD) and the mean OD of each treatment group was compared.

2.6.2. ELISA for serum SIV M2e-specific antibody

Serum samples collected at –29, –1 and 5 DPI were assayed for SIV M2e-specific antibodies as described previously [21]. In brief, M2e coated plates were blocked with 100 µl of 5% milk diluent (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD) for 1 h at room temperature and washed 3 times with PBST washing buffer. Samples were diluted 1:50 using 5% milk diluent. Each diluted sample was run in duplicate using 100 µl of sample per well and incubated for 1 h at 37 °C. Excess antibodies were removed by washing 3 times with PBST washing buffer. To detect the presence of SIV M2e-specific antibodies, peroxidase-labeled goat anti-swine immunoglobulin G (Sigma–Aldrich, St. Louis, MO) was used and color was developed with a pre-warmed 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS) substrate freshly prepared according to the manufacturer's protocol (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). The enzyme–substrate reaction was stopped by adding 100 µl of 1% sodium dodecyl sulfate (Sigma–Aldrich, St. Louis, MO) in PBS into each well and the OD was measured at 405 nm. Positive samples were run in duplicates on each plate and the sample diluent was included in the blank wells as controls. The M2e-specific antibody levels were reported as the mean OD of the duplicates.

2.7. Flow cytometry analysis

2.7.1. Culture procedures

Peripheral blood mononuclear cells (PBMC) were collected in heparinized blood collection tubes (Becton, Dickinson and Company, Franklin Les, NJ) and isolated by differential centrifugation. PBMCs were collected 1 day prior to challenge and at necropsy. The PBMCs were counted prior to staining with carboxyfluorescein succinimidyl ester (CFSE) green fluorescent dye (Sigma–Aldrich, St. Louis, MO) following a procedure previously described [30]. Briefly, 2×10^7 PBMCs were centrifuged ($400 \times g$) for 10 min, supernatants were aspirated, and cells were stained with $1 \times$ PBS pH 7.2 containing 5 µM CFSE. Cells were gently vortex for 5 min. The staining was quenched by adding 2 ml of fetal bovine serum and incubated for 2 min to adsorb the dye. Cells were then washed three times with RPMI 1640 (Mediatech, Huntingford, VA). Once stained, cells were recounted and added to 96-well U-bottomed microtiter plates (Costar, Corning, NY) at a density of 5×10^5 cells per well in 100 µl medium (RPMI containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin). PBMCs were cultured for 4 days with inactivated IA30 and MN03 viruses (100 HA units/100 µl) and M2e antigen (10 µg/100 µl) in duplicate. Positive control samples were cultured with 5 mg/ml ConA in duplicate and the culture media was used as negative control.

2.7.2. Cell surface marker staining

Stained PBMCs were centrifuged ($400 \times g$) for 10 min and the supernatant was discarded. Primary antibodies to swine leukocyte surface antigens in PBS containing 1% BSA and 0.1% sodium azide (FACS buffer) was added to wells containing cells. Primary antibodies, including phycoerythrin (PE)-conjugated anti-CD4 and biotinylated anti-CD8 were added to the appropriate wells. After incubating for 20 min, the cells were washed with FACS buffer and resuspended in 50 µl of secondary antibody streptavidin-conjugated cyochrome dye secondary antibody (Pharmingen, BD Bioscience, CA). Cells were incubated, washed, resuspended and fixed with 2% formalin in PBS before flow cytometric analysis.

The program Modfit Proliferation Wizard (Verity Software House Inc., Topsham, Maine) was used to analyze cell prolifera-

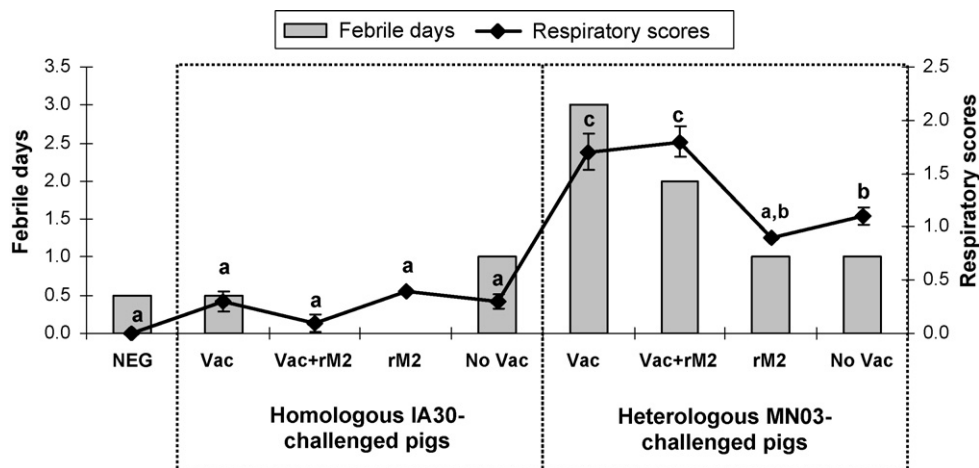


Fig. 1. Mean rectal temperature ($\geq 40^{\circ}\text{C}$) and clinical scores (0–3) for respiratory distress associated with SIV. Scoring: 0 = normal; 1 = mild dyspnea at rest; 2 = moderate dyspnea and/or tachypnea at rest; 3 = severe dyspnea and tachypnea with distinct abdominal breathing. ^{a,b,c}Values not sharing the same letters are significantly different ($P \leq 0.05$).

tion. The results are presented as the mean number of proliferating cells \pm standard error mean per 10,000 PBMCs. The number of cells proliferating was calculated by the following formula: (% proliferation to specific antigen \times number of cells in the R1 gate) – (% proliferation with no stimulation \times number of cells in the R1 gate) [31]. R1 is the region containing live lymphocytes based on forward and side light scatter properties of porcine lymphocytes.

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed to determine group differences for each measured parameter. Significant differences between treatment groups were evaluated using the Tukey-Kramer Honestly Significant Difference multiple comparison test when $P \leq 0.05$. All data analyses were performed using JMP[®] statistical software (SAS Institute, Cary, NC).

3. Results

3.1. Clinical evaluation

Respiratory signs were mild in all pigs challenged with the homologous IA30 virus (groups 2–5), independent of vaccination status. Only the non-vaccinated, IA30-challenged pigs (group 5) were febrile with rectal temperatures $\geq 40^{\circ}\text{C}$ at 1 DPI (Fig. 1). Pigs challenged with the heterologous MN03 virus (groups 6–9) demonstrated significantly increased respiratory disease levels compared to the negative control pigs and the pigs challenged with IA30. Respiratory signs including cough and elevated respiratory rates were

significantly increased in the IA30 vaccinated pigs challenged with MN03 with or without rM2 vaccination (groups 6 and 7) compared to the rM2 vaccinated or non-vaccinated MN03-challenged pigs (groups 8 and 9). Pigs vaccinated with the IA30 vaccine without rM2 vaccine followed by challenge with MN03 virus (group 6) were febrile for an average of 3 days, which was one day longer than pigs vaccinated with only rM2 protein (group 7), although no statistical difference was observed.

One pig that received only the rM2 vaccine prior to infection with IA30 virus died one day after SIV infection of unknown causes; however, typical SIV-associated lung lesions were observed and SIV-specific antigen was detected by immunohistochemistry staining. No significant bacteria including *M. hyopneumoniae* were cultured from the respiratory tract. The pig had no HI antibodies to either viral antigens, but M2-specific IgG antibodies were detected by the M2e-ELISA (data not shown).

3.2. Macroscopic and microscopic lesion scores and viral antigen in lungs

As shown in Table 2, the percentage of macroscopic lesions consistent with SIV in the lungs of pigs that were vaccinated and challenged with IA30 (groups 2–4) was significantly decreased compared to the non-vaccinated group (group 5). The macroscopic lesions in all vaccinated groups challenged with IA30 (groups 2–4) did not differ significantly from the non-vaccinated, non-challenged pigs in group 1. Microscopic pneumonia scores were minimal in the pigs challenged with IA30. Only the non-vaccinated, IA30-challenged group was positive for SIV antigen.

Table 2
Percentage of macroscopic lesions and microscopic lesion scores \pm standard error of the mean (S.E.M.) and swine influenza virus (SIV) antigen detection at 5 days post infection (DPI). Means with different letters within columns are statistically different ($P \leq 0.05$).

Group	Vaccination	SIV infection	% Macroscopic lesions [†]	Microscopic lesion scores [‡]	SIV antigen
1	No	No	0.2 \pm 0.1 ^a	0.0 \pm 0.0 ^a	–
2	IA30	IA30	2.1 \pm 1.1 ^a	0.0 \pm 0.0 ^a	–
3	IA30 + rM2	IA30	0.8 \pm 0.3 ^a	0.0 \pm 0.0 ^a	–
4	rM2	IA30	4.7 \pm 0.9 ^{a,b}	2.5 \pm 0.6 ^{b,c}	–
5	No	IA30	10.0 \pm 2.5 ^b	1.3 \pm 0.6 ^{a,b}	+
6	IA30	MN03	26.8 \pm 3.3 ^d	3.3 \pm 0.4 ^c	+
7	IA30 + rM2	MN03	20.0 \pm 2.1 ^{c,d}	3.2 \pm 0.2 ^c	+
8	rM2	MN03	8.9 \pm 0.7 ^{a,b}	2.7 \pm 0.6 ^c	+
9	No	MN03	16.9 \pm 2.3 ^{b,c}	2.8 \pm 0.4 ^c	+

[†] As determined by lesion sketches and image analysis.

[‡] SIV microscopic lesion scores are based on the severity of bronchiolar epithelial damage (necrotic bronchiolitis).

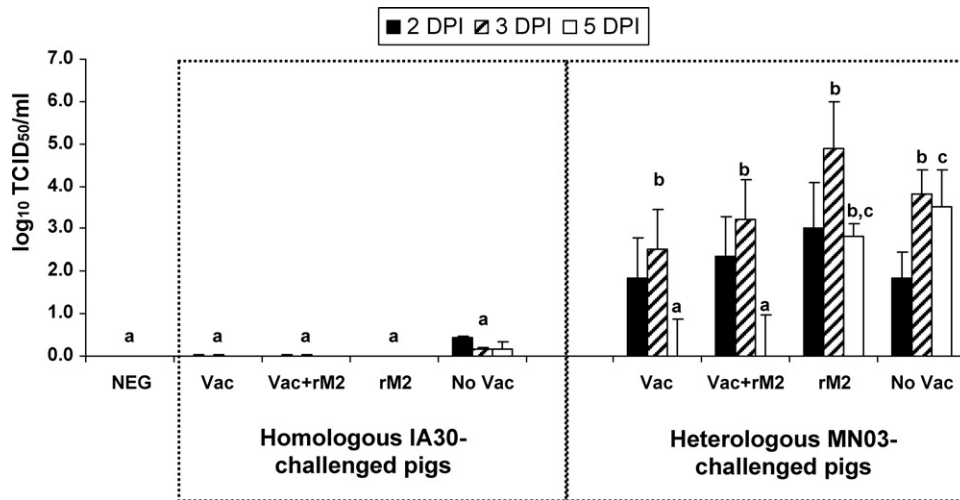


Fig. 2. Virus titers in nasal swabs collected from pigs at 2, 3 and 5 DPI. Results are represented as mean log₁₀ TCID₅₀/ml ± S.E.M. Different letters within the same DPI are significantly different ($P \leq 0.05$).

In contrast to the IA30 challenge groups, increased macroscopic lung lesions were observed in pigs inoculated with the IA30-vaccine and challenged with the heterologous MN03 virus (group 6). Pigs in group 6 had significantly greater percentages of lung lesions compared to the non-vaccinated pigs (group 9) and rM2 vaccinated pigs (group 8). The enhanced disease observed in this study were consistent with previous results observed with pigs immunized with the IA30-vaccine and challenged with the MN03 virus [7]. Although not statistically significant when compared to groups 6 and 9, the addition of rM2 vaccine with the IA30 vaccine (group 7) appeared to have resulted in a slight reduction in the severity of lung lesions, and pigs in group 8 that received only the rM2 vaccine had no evidence of vaccine enhancement of pneumonia. There were no significant differences in the microscopic pneumonia scores between groups challenged with the MN03-virus and SIV antigen was detected in the lungs of all the MN03-challenged pigs at 5 DPI (Table 2). Interestingly, SIV antigen detected in the lungs of IA30-vaccinated pigs (group 6) and non-vaccinated pigs (group 9) challenged with MN03 was not confined to the epithelial lining of the large airways but were scattered in alveoli that were also heavily infiltrated with mononuclear cells. In contrast, SIV antigen detected in MN03-challenged pigs that received the rM2 vaccine with IA30-vaccine (group 7) or rM2 vaccine alone (group 8) was confined to the epithelial linings of large airways.

3.3. Virus levels in nasal secretions

No virus was isolated from the negative control pigs at any time point in the study. Pigs challenged with IA30 virus shed low levels of virus, with non-vaccinated pigs shedding the greatest amount, although the levels were not significantly different between groups. In contrast, pigs in all groups challenged with MN03 virus (groups 6–9) shed equivalent levels of virus that were significantly greater than the levels shed by the IA30-challenged pigs at 2 and 3 DPI (Fig. 2). At 5 DPI no virus was detected in nasal secretions of MN03-challenged pigs in the vaccinated groups 6 and 7. Virus was still detected in nasal secretions from pigs that had received the rM2 vaccine or were not vaccinated (groups 8 and 9).

3.4. Hemagglutination-inhibition test

No pigs were positive for SIV antibodies as measured by HI assay prior to vaccination, confirming that pigs were negative for SIV viruses at the beginning of the study. Pigs in the negative control

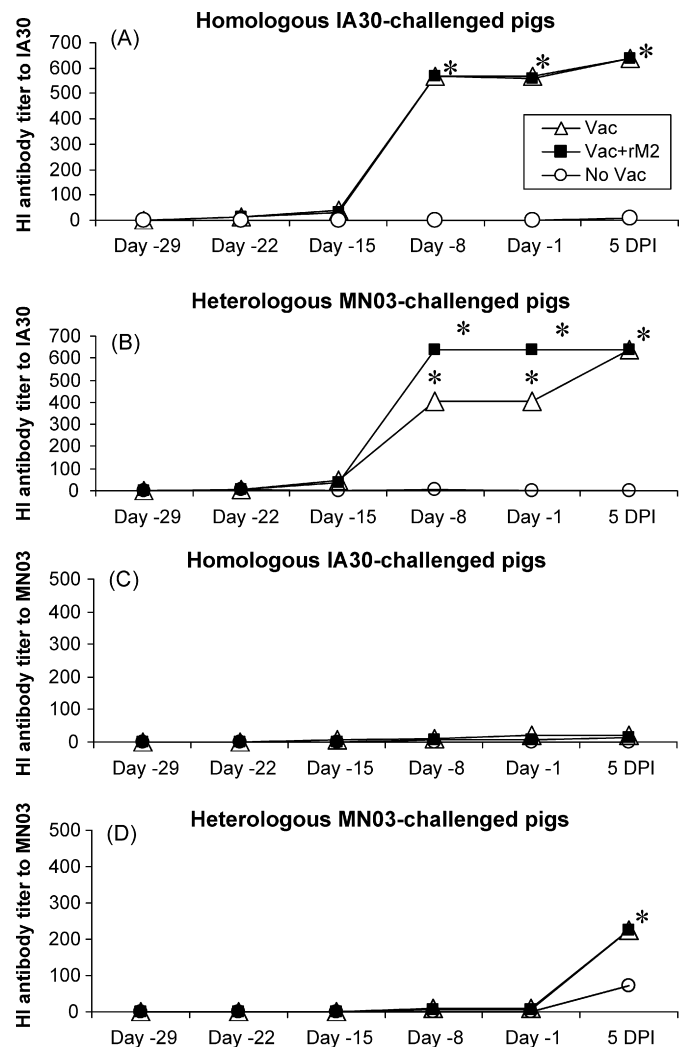


Fig. 3. Mean hemagglutination-inhibition (HI) antibody titers from IA30 challenged pigs and -MN03-challenged pigs against the IA30 antigen (A) and (B) and the MN03 antigen (C) and (D). There were no positive HI titers against either virus antigen in negative control pigs and pigs prior to vaccination. * Significantly higher values observed at the same DPI ($P \leq 0.05$).

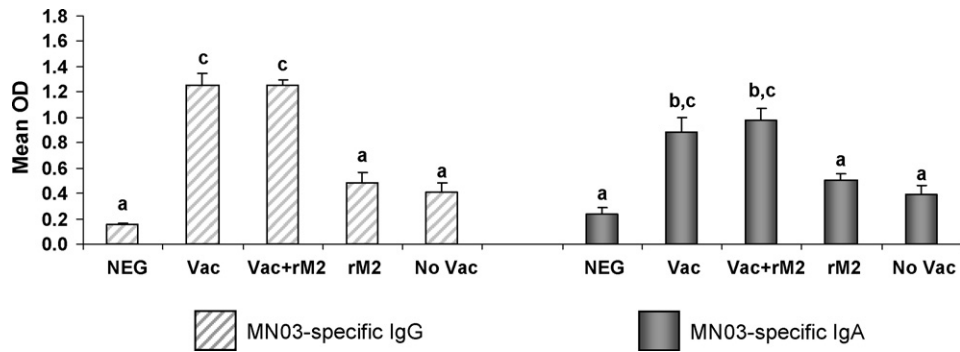


Fig. 4. Mean O.D. ± S.E.M. of SIV MN03-specific IgG (▨) and SIV MN03-specific IgA (■) from lower airways of negative control pigs (group 1) and pigs challenged with IA30 virus (groups 2–5) measured by ELISA. Means with different letters within a column are statistically different ($P \leq 0.05$).

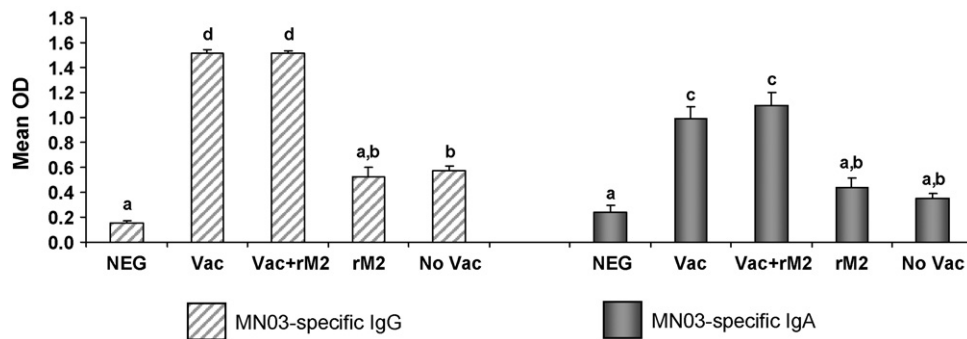


Fig. 5. Mean O.D. ± S.E.M. of SIV MN03-specific IgG (▨) and SIV MN03-specific IgA (■) from lower airways of negative control pigs (group 1) and pigs challenged with MN03 virus (groups 6–9) measured by ELISA. Means with different letters are statistically different ($P \leq 0.05$).

group remained seronegative throughout the study period (Fig. 3). Prior to SIV challenge, HI antibody titers to the vaccine antigen (IA30) were observed only in vaccinated pigs (groups 2, 3, 6 and 7). No cross-reactive HI antibody response to the MN03 antigen was observed in sera from vaccinated pigs or IA30 challenged pigs. In addition, no HI antibodies cross reacted with IA30 in the non-vaccinated pigs challenged with the MN03 virus. However, the level of HI antibodies to the MN03 antigen in IA30 vaccinated pigs that had been challenged with the MN03 virus (groups 6 and 7) were significantly higher compared to the non-vaccinated, MN03-challenged pigs (group 9) at 5 DPI.

3.5. ELISA

3.5.1. Local SIV-specific antibody response

While IgG antibodies that recognized the IA30 antigen in the sera of the pigs challenged with MN03 were barely detectable by the HI test at 5 DPI, antibodies that recognized both the MN03 and the IA30 viruses were detected in BAL fluid collected from pigs that were only vaccinated with the IA30 antigen. Vaccinated pigs that were infected with the IA30 virus had similar amounts of cross-reacting IgG- and IgA-anti-MN03 antibodies in the BAL fluids (Fig. 4). In contrast, pigs that were vaccinated and challenged with MN03 virus had levels of cross-reacting IgG-anti MN03 antibodies that were significantly higher than the cross-reacting IgA-anti MN03 antibodies (Fig. 5).

3.5.2. Serum M2e-specific antibody response

Serum M2e-specific antibody responses were evaluated at 3 time points; prior to rM2 vaccine administration (–29 DPI), after receiving 2 vaccinations (prior to SIV-challenge; –1 DPI) and 5 DPI. No M2e-antibody levels were detected between the groups at –29 (data not shown). However, prior to SIV-challenge, the level of M2e-antibodies detected in pigs that received only the rM2 vaccine was

significantly higher compared to all other groups (Table 3). Serum M2e-specific antibody responses were not significantly different between the groups at 5 DPI.

3.6. Flow cytometry and cell surface marker analysis

Proliferation of different T cell populations in peripheral blood to the recall antigens, IA30, MN03 and rM2 proteins, were studied at two time points; one day (–1 DPI) prior to SIV infection, and at 5 DPI. Since the treatment status prior to SIV infection between IA30-vaccinated pigs from groups 2 and 6, IA30 and rM2 protein-vaccinated pigs from groups 3 and 7 and rM2-vaccinated pigs from groups 4 and 8 were considered the same, –1 DPI data retrieved from these groups of pigs were combined for statistical analysis. Significant differences in the number of T cells proliferating in response to the recall antigens between the treatment groups were detected only at the time point prior to SIV infection (Fig. 6). Pigs that received the IA30-vaccine with or without rM2 vaccine had significantly higher numbers of CD8+ T cells that proliferated

Table 3

SIV-M2e IgG specific serum antibodies ± standard error of the mean (S.E.M.). Means with different letters within columns are statistically different ($P \leq 0.05$).

Groups	Vaccination	SIV infection	Serum IgG-M2e-specific antibody	
			–1 DPI	5 DPI
1	No	Neg	0.077 ± 0.016 ^a	0.123 ± 0.022 ^a
2	IA30	IA30	0.122 ± 0.011 ^{a,b}	0.145 ± 0.029 ^a
3	IA30 + rM2	IA30	0.262 ± 0.093 ^{a,b}	0.189 ± 0.060 ^{a,b}
4	rM2	IA30	0.449 ± 0.063 ^{b,c}	0.207 ± 0.025 ^{a,b}
5	No	IA30	0.086 ± 0.010 ^{a,b}	0.140 ± 0.018 ^a
6	IA30	MN03	0.156 ± 0.044 ^{a,b}	0.133 ± 0.020 ^{a,b}
7	IA30 + rM2	MN03	0.265 ± 0.127 ^{a,b}	0.325 ± 0.073 ^b
8	rM2	MN03	0.652 ± 0.292 ^c	0.125 ± 0.015 ^{a,b}
9	No	MN03	0.088 ± 0.008 ^a	0.162 ± 0.022 ^{a,b}

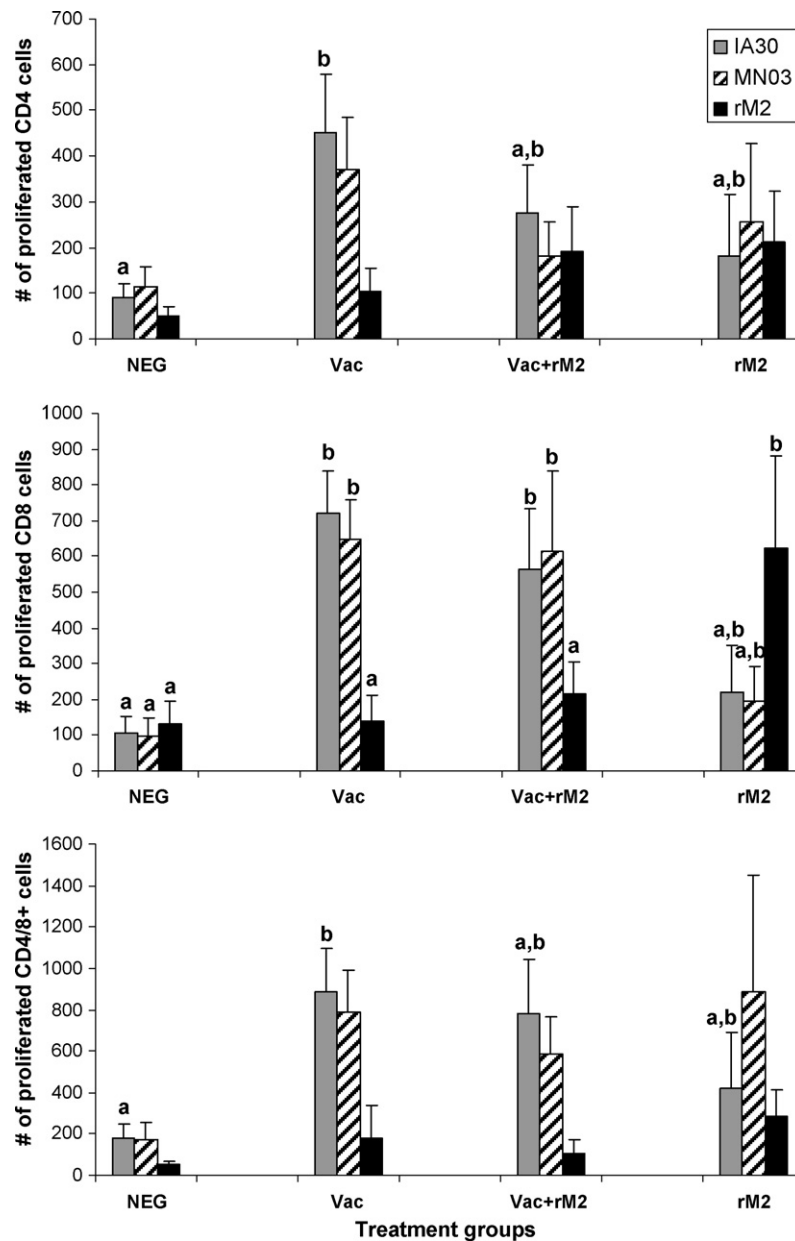


Fig. 6. Mean numbers of CD4+, CD8+ and CD4/8+ T cells \pm S.E.M. that proliferated to the IA30 (■), MN03 (▨) and rM2 (■) antigen as determined by flow cytometry and cell surface marker staining. Peripheral blood mononuclear cells were collected from pigs two weeks after the second vaccination prior to SIV infection (-1 DPI). Means with different letters compared between treatment groups within the same antigen are statistically different ($P \leq 0.05$).

in response to both IA30 and MN03 antigen stimulation. Pigs that received only the rM2 vaccine had significantly higher numbers of CD8+ T cells that proliferated in response to the rM2 protein compared to the negative control pigs or pigs vaccinated with only IA30-vaccine. No significant difference in the numbers of the CD4+ and CD4/8+ T cells was detected between all of the groups. No significant difference in the T cell populations was detected between the groups at 5 DPI.

4. Discussion

The goal of this study was to investigate the potential for the M2 protein to protect against a H1N2 subtype SIV infection when used in combination with an inactivated H1N1 SIV vaccine. This study was performed using conventional pigs and a previously described experimental H1N1-vaccine and heterologous H1N2 SIV-challenge model [7]. Pigs in this model have demonstrated enhanced disease

when vaccinated with an inactivated H1N1 (IA30) virus prior to infection with the heterologous H1N2 (MN03) virus. In this study, groups of pigs that received the rM2 protein were included to evaluate the efficacy of the rM2 protein alone to provide protection against SIV infection.

Similar to the previous findings by Vincent et al. [7], increased pneumonic lesions and SIV vaccine-associated disease were observed in this study in pigs vaccinated and then challenged with a heterologous SIV. The findings of this study further confirmed that the immune response induced by an inactivated swine influenza virus (H1N1) vaccine can have a negative impact when pigs are infected with a heterologous swine influenza virus (H1N2). These results underscore the possibility of similar scenario under field conditions since the current commercial swine influenza vaccines typically include older H1N1 and H3N2 viruses that may differ genetically from the viruses that circulate in the swine population. In this study, the addition of rM2 vaccine with the IA30-vaccine

appeared to have helped reduce the number of febrile days by one. In addition, the rM2 vaccine reduced macroscopic lung lesions (8.9%) compared to the lesions in the IA30-vaccinated group (26.8%) and the non-vaccinated, MN03-challenged group (16.9%). However, the rM2 vaccine administered with the IA30-vaccine did not reduce virus shedding significantly.

This study had two groups of pigs that were vaccinated with only the rM2 vaccine and were then challenged with either the IA30 or the MN03 viruses. Pigs vaccinated with rM2 and infected with MN03 virus did not demonstrate the disease enhancement observed in the IA30 vaccinated group. In fact, compared to the IA30-vaccinated pigs, the rM2-vaccinated, MN03-challenged pigs were febrile one day less, had significantly reduced respiratory signs and the percentage of pneumonic lesions were not statistically different from the negative control pigs. One rM2-vaccinated pig was found dead a day after infection with the H1N1 virus. Immunohistochemistry tests confirmed the presence of SIV antigen in epithelial linings of the large airways with no significant microscopic lesions. While the cause of death of this pig was unknown, an earlier study in pigs, demonstrated SIV vaccine-associated disease enhancement and death in pigs challenged with a H1N1 virus following vaccination with nucleoprotein and M2 DNA-vaccine [20]. That study noted that the presence of a strong cell mediated immune response towards NP and M2 protein alone without anti-HA neutralizing antibodies may have been damaging to the host. Pigs vaccinated with a purified M2 protein fused with hepatitis B core fusion protein (M2eHbc) prior to challenge with H1N1 had M2-specific antibodies may have demonstrated SIV vaccine-associated disease enhancement. Earlier findings indicate that M2-specific antibodies are non-neutralizing but appear to contribute to virus reduction through antibody-dependent natural killer cell activity (ADCC) especially during the initial stage of infection when the amount of virus is low [32]. The expression of M2 protein on the surface of SIV-infected cells in the presence of non-neutralizing anti-M2 antibodies, but in the absence of anti-HA neutralizing antibodies may induce cell membrane damage via ADCC and/or complement fixation. The cause of death of the pig that was rM2-vaccinated and H1N1-challenged in this study was not determined. So it is unknown if this enhanced immune response is a possible cause of death of this single pig. However, no pigs in any other group were impacted. Prior to SIV infection, pigs receiving only the rM2 vaccine had significantly higher numbers of M2-specific CD8+ T cell in their PBMCs compared to IA30-vaccinated pigs with or without rM2 vaccine. The rM2-vaccinated, MN03-challenged pigs had reduced SIV-associated clinical signs and pneumonia, suggesting a beneficial role of the M2 specific CD8+ T cells under these conditions. The unexpected difference between these two groups requires further examination in future studies to look at the mechanism of both the disease enhancement and the role an immune response to the rM2 may play in reducing disease severity.

Of the antibodies produced in response to influenza infection, HA antibodies are considered the most important as they are primarily responsible for virus neutralization [33]. Protective antibodies against infection with SIV are based on both quality and quantity of the HA antibodies produced. Studies in humans, mice and ferrets suggests that serum anti-HA IgG antibodies transudate into the bronchoalveolar passages and prevents the lower respiratory tract from being infected [34,35]. Clinical protection against experimentally challenged SIV induced disease appears correlated with the serum HI titer of the individual pig due to priming of the immune system to the HA protein and the production of HI antibodies that are antigenically similar to the infecting strain [36,37]. In addition, locally produced SIV-specific IgA antibodies found in the mucosal lung areas are equally important for protecting the local airways from influenza virus infection [29]. These mucosal SIV-specific antibodies in the nasal wash fluids and BAL fluids can

be detected as early as 4–5 days post primary SIV exposure [29,38] and the predominant SIV-specific antibody isotype found in both mucosal areas following primary infection is IgA, as opposed to IgG, which is the highest isotype detected in serum [29]. Studies suggest that local IgA plays a significant role in defending against SIV-induced disease as it provides heterosubtypic cross-protection [3,39].

Analysis based on the HA1 region of the HA nucleotide sequence revealed up to 21% difference between the H1N1 (IA30) and H1N2 (MN03) viruses used in this vaccine-challenge model [40]. In this study, the serum IgG antibody response to the IA30 vaccine was present at high levels in the vaccinated pigs prior to SIV challenge. Serum cross-reactivity to the MN03 antigen was not detected by the HI assay prior to infection. Yet after infection, MN03-virus specific IgG levels were significantly higher in the vaccinated pigs compared to the non-vaccinated pigs. This indicates that the IA30 vaccine primed an antibody response that was cross-reactive to the MN03 virus. The mucosal antibody response at 5 DPI from IA30-vaccinated pigs contained high levels of IgG that were cross-reactive with the MN03 antigen using a whole virus-based ELISA. It is possible that the MN03-specific IgG antibodies detected in the BAL fluids of the IA30-vaccinated, MN03-challenged pigs were directed against a conserved area of the HA protein such as the HA2 region and were of non-neutralizing nature. These findings are supported by the presence of mucosal MN03 cross-reactive IgG antibodies that did not reduce the level of virus shed in the early phases of infection. In fact, the presence of the non-neutralizing antibodies may have contributed to the potentiated lung lesions found in the IA30-vaccinated, MN03-challenged pigs. The mucosal MN03-IgA cross-reactive antibodies were also present with MN03-IgG cross-reactive antibodies at 5 DPI indicating that the IA30 vaccine had primed the local IgA antibody response. Recent data suggests specific anti-SIV-IgA antibodies have a broad spectrum activity towards differing SIV subtypes compared to IgG antibodies, which may contribute to increased protection against heterologous infections [3,39]. In this study, the IA30-vaccinated pigs challenged with MN03 virus had significantly lower levels of anti-MN03 IgA antibodies than IgG antibodies. It is possible that the level of pre-existing IgA antibodies prior to MN03 infection were at an insufficient level to overcome the disease enhancement caused by the non-neutralizing IgG antibodies.

The administration of the rM2 vaccine in addition to the IA30 vaccine did not appear to reduce the HI antibody production to the IA30 antigen. In contrast, the combined vaccination strategy appeared to suppress the production of M2 specific antibodies as the level was significantly lower in pigs receiving both vaccines compared to pigs that received only the rM2 vaccine. Previous data has shown that the influenza A proteins, HA and NA, when presented as antigens on the same viral particle have an “intravirionic antigenic competition” and the antibody production to the HA protein out competes the NA protein [41]. This phenomenon disappears when the HA and NA proteins are administered separately [42]. This “antigenic competition” resulting in a reduced or biased antibody response has been reported in combination vaccines studies [43–45]. Furthermore, it has been determined that the M2 protein is poorly immunogenic in nature [46]. Thus, it is possible that the concentration of the rM2 protein administered alone as a vaccine, or when used in combination with the IA30 vaccine was suboptimal for providing complete protection against SIV infection. Future M2 vaccine studies are needed to determine if optimizing the rM2 and HA protein concentration and ratio would enhance protection against disease.

Other M2 protein-based vaccine trials have been conducted in mice, monkeys and ferrets, which are not natural hosts for influenza, found reduction in both clinical signs and virus excretion. The findings in this study conducted in pigs, a natural host

of influenza A viruses, indicates a potentially positive outcome in using rM2 protein as a vaccine for SIV-associated disease prevention. However, the results of this study underscore the importance of inducing an immune response against the major envelope protein, HA to reduce SIV excretion. New innovative intervention strategies, including vaccines consisting of various influenza A protein combinations, may be useful for the control of influenza A viruses in the future.

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