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Enhanced pneumonia and disease in pigs vaccinated with an inactivated human-like (δ-cluster) H1N2 vaccine and challenged with pandemic 2009 H1N1 influenza virus

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ABSTRACT

Influenza is an economically important respiratory disease affecting swine world-wide with potential zoonotic implications. Genetic reassortment and drift has resulted in genetically and antigenically distinct swine influenza viruses (SIVs). Consequently, prevention of SIV infection is challenging due to the increased rate of genetic change and a potential lack of cross-protection between vaccine strains and circulating novel isolates. This report describes a vaccine-heterologous challenge model in which pigs were administered an inactivated H1N2 vaccine with a human-like (δ-cluster) H1 six and three weeks before challenge with H1 homosubtypic, heterologous 2009 pandemic H1N1. At necropsy, macroscopic and microscopic pneumonia scores were significantly higher in the vaccinated and challenged (Vx/Ch) group compared to non-vaccinated and challenged (NVx/Ch) pigs. The Vx/Ch group also demonstrated enhanced clinical disease and a significantly elevated pro-inflammatory cytokine profile in bronchoalveolar lavage fluid compared to the NVx/Ch group. In contrast, viral shedding and replication were significantly higher in NVx/Ch pigs although all challenged pigs, including Vx/Ch pigs, were shedding virus in nasal secretions. Hemagglutination inhibition (HI) and serum neutralizing (SN) antibodies were detected to the priming antigen in the Vx/Ch pigs but no measurable cross-reacting HI or SN antibodies were detected to pandemic H1N1 (pH1N1). Overall, these results suggest that inactivated SIV vaccines may potentiate clinical signs, inflammation and pneumonia following challenge with divergent homosubtypic viruses that do not share cross-reacting HI or SN antibodies.

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1. Introduction

Swine influenza is caused by influenza A virus of the *Orthomyxoviridae* family and the cause of an acute respiratory disease in swine. Orthomyxoviruses have a negative-sense, segmented RNA genome that allows for genetic reassortment and generation of novel viruses. Currently, three major influenza subtypes, H1N1, H3N2 and H1N2, co-circulate in the major swine producing regions of the United States (US) and Canada [1–4]. However, two additional subtypes, H3N1 and H2N3, have been identified in North American swine, and drift variants of the predominant subtypes are increasingly more common [4–11]. The increased rate of genetic change in North American swine influenza virus (SIV) H1 subtypes is attributed to the introduction of the human–avian–swine triple reassortant H3N2 subtype in 1998 and more importantly, to the acquisition of the triple reassortant internal gene (TRIG) cassette [10,12]. SIV subtypes include different combinations of the HA and neuraminidase (NA) genes, however, the TRIG cassette, which includes the NP, M, and NS, genes of classical swine lineage, PB2 and PA genes of avian lineage, and PB1 of human lineage, have been consistently identified among contemporary isolates circulating in the North American swine population [2]. The TRIG appears to have an enhanced ability to acquire a variety of surface glycoprotein gene segments generating novel isolates such as the H2N3 subtype identified in 2006, when the TRIG was shown to have acquired an avian H2 and N3, producing a novel triple reassortant SIV [10,13]. Antigenic drift resulted in the evolution of three distinct H1 phylogenetic clusters (α, β, and γ) from the classical swine lineage. The δ-cluster emerged in 2005 in the US and includes SIVs with the HA gene of human seasonal virus origin [10]. The hu-like H1 (δ) influenza viruses introduced a fourth cluster currently endemic in US swine [10,14].

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Table 1
Experimental design.

Group	Vaccine	Challenge	*N*	Weeks of age		
				Priming vaccine	Booster vaccine	Challenge
Vx/Ch[a]	MN08	pH1N1	10	4	7	10
NVx/Ch[b]	None	pH1N1	10	4	7	10
NVx/NCh[c]	None	None	5	4	7	10

[a] Vx/Ch: vaccinated/challenged.
[b] NVx/Ch: non-vaccinated/challenged.
[c] NVx/NCh: non-vaccinated/non-challenged.

Inactivated influenza A vaccines are approved for use in US swine in pigs 3 weeks of age or older and have played a significant role in preventing clinical disease [15,16]. However, inactivated vaccines have shown limited efficacy or cross-protective immunity against heterologous homosubtypic or heterosubtypic viruses [15,17–19]. Consequently, use of autogenous vaccines specific to the farm of origin has also increased in an attempt to control disease in the face of the escalating diversity within North American influenza A viruses [3]. In contrast, exposure to live H1N1 and H3N2 virus has demonstrated complete protection against an SIV with an unrelated HA protein in some studies, suggesting live exposure results in improved cross-protection between heterologous SIVs [20]. Collectively, genetically and antigenically diverse SIVs have made prevention more challenging due to the increasing lack of cross-protection among heterologous viruses and the inability to update vaccines as rapidly as viruses change [3].

A potential problem with vaccination was demonstrated when enhanced pneumonia in a subset of pigs administered an inactivated H1N1 vaccine followed by challenge with a heterologous virus was reported [19]. The viruses in that study were shown to have no cross-reactivity either as anti-sera or antigen in the hemagglutination inhibition (HI) assay. The inactivated α-cluster swine H1N1 vaccine failed to protect against challenge with a heterologous γ-cluster H1N2 SIV and resulted in enhanced pneumonic lesions in one-third of the pigs [19], and in all similarly treated pigs in a subsequent study [21]. In the study described here, pigs were administered an inactivated 2008 H1N2 hu-like δ-cluster SIV vaccine followed by challenge with 2009 pandemic H1N1 (pH1N1). We report the inactivated vaccine did not protect against challenge with pH1N1 virus. Furthermore, pigs in the vaccinated and challenged group (Vx/Ch) demonstrated enhanced macroscopic and microscopic pneumonia as well as an elevated inflammatory cytokine profile suggesting vaccination potentiated the clinical disease and pneumonia in the Vx/Ch group followed by heterologous challenge with pH1N1.

2. Materials and methods

2.1. Experimental design

Twenty-five, three-week-old cross-bred pigs were obtained from a herd free of SIV and porcine reproductive and respiratory syndrome virus (PRRSV) and treated with ceftiofur crystalline free acid (Pfizer Animal Health, New York, NY) and enrofloxacin injectable solution (Bayer Animal Health, Shawnee Mission, KS) according to label directions to reduce bacterial contaminants prior to the start of the study. Pigs were housed in biosafety level 2 (BSL2) containment during the vaccine phase of the study. Pigs were transferred to ABSL3 containment on the day of challenge for the remainder of the experiment. Pigs were cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center. The experimental design is described in Table 1.

SIV vaccine was prepared with A/Sw/MN/02011/08 H1N2 (MN08) at approximately 32 HA units and inactivated by ultraviolet irradiation with the addition of a commercial oil-in-water adjuvant (Emulsigen D, MVP Labs) at a v:v ratio of 4:1 virus to adjuvant. Pigs were vaccinated with 2 ml by the intramuscular route at approximately 4 weeks of age and boosted at 7 weeks of age (Table 1). Pigs challenged at 10 weeks of age were inoculated intratracheally with 2 ml of 1×10^5 50% tissue culture infectious dose ($TCID_{50}$) of A/CA/04/09 pH1N1 (pH1N1) propagated in Madin–Darby canine kidney (MDCK) cells, as previously described [14]. Pigs were observed daily for signs of clinical disease. Rectal temperatures were taken on −1, 0, 1, 2, 3, 4, and 5 days post-infection (dpi). Nasal swabs (Fisherbrand Dacron swabs, Fisher Scientific, Pittsburg, PA) were taken on 0, 3, and 5 dpi to evaluate nasal virus shedding by dipping the swab in minimal essential medium (MEM) and inserting the swab approximately 2.5 cm into each nares. Swabs were then placed into 2 ml MEM and stored at −80 °C until study completion. Pigs were humanely euthanized with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) on 5 dpi to evaluate lung lesions and viral load in the lungs. Postmortem samples included serum, bronchoalveolar lavage, lung and trachea.

2.2. Pathologic examination of lungs

At necropsy, lungs were removed and evaluated for the percentage of the lung affected with purple-red consolidation typical of SIV. The percent of the surface affected with pneumonia was visually estimated for each lung lobe, and then a total percentage for the entire lung was calculated based on weighted proportions of each lobe to the total lung volume [22]. Tissue samples from the trachea and right cardiac lung lobe were taken and fixed in 10% buffered formalin for histopathologic examination. Tissues were routinely processed and stained with hematoxylin and eosin. Lung sections were given a score from 0 to 3 and tracheal sections were given a score from 0 to 2.5 to reflect the severity of bronchial and tracheal epithelial changes based on previously described methods [9]. The lung sections were scored according to the following criteria: 0.0: no significant lesions; 1.0: a few airways affected with bronchiolar epithelial damage and light peribronchiolar lymphocytic cuffing often accompanied by mild focal interstitial pneumonia; 1.5: more than a few airways affected (up to 25%) often with mild focal interstitial pneumonia; 2.0: 26–50% airways affected often with moderate interstitial pneumonia; 2.5: approximately 51–75% airways affected, usually with significant interstitial pneumonia; 3.0: greater than 75% airways affected, usually with significant interstitial pneumonia. Trachea sections were scored according to the following criteria: 0.0: normal; 1.0: focal squamous metaplasia of the epithelial layer; 2.0: diffuse squamous metaplasia of much of the epithelial layer, cilia are focally evident; 2.5: diffuse squamous metaplasia with an absence of cilia. A single pathologist scored all slides and was blinded to the treatment groups.

2.3. Diagnostic microbiology

All pigs were screened for influenza A nucleoprotein antibody by ELISA (MultiS ELISA, IDEXX, Westbrook, Maine) prior to the start of the study to ensure absence of prior immunity. Bronchoalveolar lavage fluid (BALF) samples from 5 dpi were screened for aerobic bacterial growth on blood agar and Casmin (NAD enriched) plates. Diagnostic PCR for PCV2 [23] *Mycoplasma hyopneumoniae* [24] and an in-house RT-PCR for PRRSV were conducted on nucleic acid extracts from BALF.

2.4. Viral replication and shedding

Nasal swab samples were subsequently thawed and vortexed for 15 s, centrifuged for 10 min at 640 × *g* and the supernatant was passed through 0.45 μm filters to reduce bacterial contaminants. Ten-fold serial dilutions in serum-free MEM supplemented with TPCK trypsin and antibiotics were made with each BALF sample and nasal swab filtrate sample. Each dilution was plated in triplicate in 100 μl volumes onto PBS-washed confluent MDCK cells in 96-well plates. Plates were evaluated for CPE between 48 and 72 h post-infection. At 48 h, plates were fixed with 4% phosphate-buffered formalin and stained using immunocytochemistry with an anti-influenza A nucleoprotein monoclonal antibody as previously described [16]. A $TCID_{50}$ titer was calculated for each sample using the method of Reed and Muench [25].

2.5. Antibody detection assays

For use in the HI assay, sera were heat inactivated at 56 °C for 30 min, then treated to remove nonspecific hemagglutinin inhibitors and natural serum agglutinins by treatment with a 20% suspension of kaolin (Sigma–Aldrich, St. Louis, MO) and adsorption with 0.5% turkey red blood cells (RBCs). The HI assays were then performed with MN08 and pH1N1 viruses as antigens and turkey RBCs using standard techniques [26]. Reciprocal titers were divided by 10 and $\log_2$ transformed, analyzed, and reported as the geometric mean. Enzyme-linked immunosorbent assays (ELISAs) to detect total IgG and IgA antibodies against whole virus preparations of MN08 and pH1N1 present in serum and BALF were performed as previously described [19,27] with modifications. Concentrated MN08 or pH1N1 virus was resuspended in Tris–EDTA basic buffer, pH 7.8, and diluted to an HA concentration of 100 HA units/50 μl. Immulon-2HB 96-well plates (Dynex, Chantilly, VA) were coated with 100 μl of antigen solution and incubated at room temperature overnight. Serum and BALF were diluted in PBS and MEM, respectively followed by 2-fold serial dilutions. The assays were performed on each sample in duplicate. The mean of duplicate wells was calculated and antibody titers were designated as the highest dilution with an OD greater than 2 standard deviations above the mean of the NVx/NCh controls. Log_2 transformations of IgG reciprocal titers were analyzed and reported as geometric means. IgA reciprocal titers were divided by 32 to establish a scale before $\log_2$ transformation and analysis. IgA titers were reported as geometric means.

For the serum neutralization assay, sera were heat inactivated at 56 °C for 30 min, then two-fold serially diluted from 1:10 to 1:20,480 in 96-well plates, using 50 μl serum diluted in serum-free MEM supplemented with TPCK trypsin and antibiotics. Fifty microliters of SIV diluted to $10^{3.3}$ $TCID_{50}$/ml was added to each dilution and incubated at 37 °C for 1 h. Post-incubation, 100 μl of the serum and virus mixture was added to each well of confluent MDCK cells prepared in 96-well plates 48 h in advance and washed twice in sterile PBS. Plates were incubated for 48 h or until the presence of cytopathic effect (CPE). Presence of influenza virus was verified by immunocytochemistry for all plates, and titers were recorded as the highest dilution negative for virus or CPE. Reciprocal titers were divided by 10 and $\log_2$ transformed, analyzed, and reported as the geometric mean.

2.6. Cytokine assays

A 5 ml aliquot of BALF was centrifuged at 400 × *g* for 15 min at 4 °C to pellet cellular debris. The cell-free BALF was stored at −80 °C until assayed for cytokine levels. Levels of IL-8, IL-1β and IL-6 in BALF were determined by ELISA performed according to the manufacturer's recommendations (DuoSet ELISA, R&D Systems, Minneapolis, MN). Levels of IFN-α were determined by ELISA as previously described [28].

2.7. Statistical analysis

Macroscopic pneumonia scores, microscopic pneumonia scores, $\log_{10}$ transformed BALF and nasal swab virus titers, and $\log_2$ transformations of HI reciprocal titers, ELISA reciprocal titers and cytokine data were analyzed using analysis of variance (ANOVA) with a P-value ≤ 0.05 considered significant (JMP, SAS Institute, Cary, NC; GraphPad Prism Version 5.00, San Diego, CA). Response variables shown to have a significant effect by treatment group were subjected to pair-wise comparisons using the Tukey–Kramer test. Rectal temperature data were analyzed using a mixed linear model for repeated measures using SAS 9.1 for Windows (SAS Institute, Cary, NC, USA). Linear combinations of the least squares means estimates were used in a priori contrasts after testing for a significant ($P < 0.05$) treatment group effect of vaccination status. Comparisons were made between each group at each time-point using a 5% level of significance ($P < 0.05$) to assess statistical differences.

3. Results

3.1. Clinical disease

All pigs inoculated with pH1N1 developed clinical signs that included mild to moderate lethargy and inappetence. Some Vx/Ch pigs demonstrated coughing with an increased respiration rate and elevated respiratory effort whereas NVx/Ch pigs did not. Challenge with pH1N1 virus induced a significant ($P \leq 0.05$) febrile response in Vx/Ch and NVx/Ch groups at 1 dpi (Table 2). The initial febrile response in the NVx/Ch group began to subside by 2 dpi and was not different from NVx/NCh controls by 3 dpi. However, challenged pigs that were previously vaccinated with MN08 virus (Vx/Ch group) exhibited a sustained febrile response that was significantly higher than all other treatment groups from 48 through 96 h post infection.

3.2. Viral and microbiological assays

All pigs were free of influenza A virus antibodies prior to the start of the experiment. No extraneous viral or *M. hyopneumoniae* nucleic acids were detected in BALF collected at 5 dpi from any pigs. Routine aerobic bacterial cultures of BALF isolated *Bordetella bronchiseptica* from 3 Vx/Ch, 6 NVx/Ch and 2 NVx/NCh pigs.

3.3. Macroscopic and microscopic pneumonia scores

Pigs challenged with pH1N1 had purple-red colored cranioventral lung consolidation typical of influenza virus infection. The extent of lung consolidation ranged from 10.3% to 34.5% in Vx/Ch pigs and 3.9% to 13.4% in the NVx/Ch pigs. NVx/NCh lung consolidation scores ranged from 0% to 2.3%. Vx/Ch pigs that received the MN08 inactivated vaccine had significantly ($P \leq 0.0001$) greater percentages of pneumonia in the cranioventral and dorsocaudal

Table 2
Mean rectal temperatures.

Group	Vaccine virus	Challenge virus	Rectal temperature (°C)*				
			1 dpi	2 dpi	3 dpi	4 dpi	5 dpi
Vx/Ch	MN08	pH1N1	40.5[a]	40.1[a]	40.2[a]	39.9[a]	39.5[a]
NVx/Ch	None	pH1N1	40.4[a]	39.6[a]	39.3[b]	39.4[b]	39.2[a]
NVx/NCh	None	None	38.7[b]	38.4[b]	39.1[b]	39.3[b]	39.2[a]

* Values within a column not connected by the same letter are significantly different ($P \leq 0.05$).

Table 3
Mean macroscopic and microscopic pneumonia scores ± standard error of the mean (SEM) and mean $TCID_{50}$ log_{10} virus titers ± SEM in lung and nasal secretions*.

Group	Macroscopic pneumonia (%)	Microscopic pneumonia (0–3)	Log_{10} virus titers		
			BALF	NS 3 dpi	NS 5 dpi
Vx/Ch	20.5 ± 2.8[a]	2.5 ± 0.3[a]	2.1 ± 0.5[a]	1.4 ± 0.2[a]	1.7 ± 0.1[a]
NVx/Ch	8.9 ± 0.9[b]	1.7 ± 0.2[b]	3.4 ± 0.1[b]	1.1 ± 0.4[ab]	2.5 ± 0.2[b]
NVx/NCh	0.5 ± 0.5[c]	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.0 ± 0.0[b]	0.0 ± 0.0[c]

* Values within a column not connected by the same letter are significantly different ($P \leq 0.05$).

lung compared to the NVx/Ch pigs that had lesions primarily in the cranial and middle lung lobes (Fig. 1). Macroscopic lung lesions also included localized hemorrhagic, bullous emphysema in two pigs with enhanced pneumonia (figure not shown). Both challenge groups had significantly higher ($P \leq 0.0001$) pneumonia scores compared to the NVx/NCh pigs (Table 3).

Microscopic lesions of pneumonia were not observed in the NVx/NCh pigs; the scattered dark foci noted grossly in these pigs were atelectatic, unaffected lobules. All pigs challenged with pH1N1 demonstrated lung lesions consistent with SIV infection, and both inoculated groups had significantly greater ($P \leq 0.0001$) microscopic lesion scores compared to the NVx/NCh group. However, Vx/Ch pigs had significantly ($P \leq 0.05$) higher average microscopic lesion scores compared to NVx/Ch pigs (Table 3). Vx/Ch pigs also demonstrated more severe histopathological lesions than typically observed with uncomplicated SIV infection. These included severe necrotizing or proliferative bronchiolitis, prominent peribronchiolar lymphocytic cuffing and moderate lymphohistiocytic interstitial pneumonia (Fig. 2A). In addition, increased lymphocyte infiltration of the bronchiolar submucosa and suppurative bronchitis and bronchiolitis were observed in the Vx/Ch pigs (Fig. 3). NVx/Ch pigs that did not receive prior vaccination had moderate necrotizing bronchiolitis, subtle peribronchiolar lymphocytic cuffing and mild interstitial pneumonia. Microscopic tracheal lesion scores were also significantly ($P \leq 0.05$) higher in the Vx/Ch pigs compared to the NVx/Ch group and included epithelial attenuation and necrosis, regional loss of cilia and marked submucosal lymphocytic inflammation.

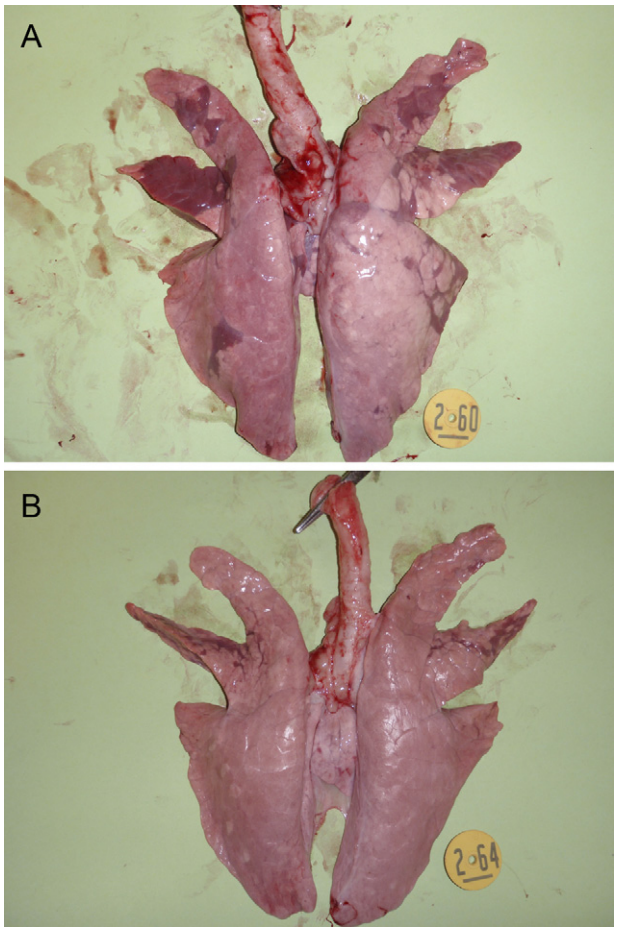

Fig. 1. Macroscopic lung lesions representing pigs in each challenge group. (A) Pigs in the Vx/Ch group had a greater percentage of lung involvement compared to the (B) NVx/Ch group.

3.4. Virus levels in lung and nasal secretions

Virus was not detected in NVx/NCh pigs at anytime throughout the study. Viral replication was detected in the lungs of all NVx/Ch pigs and in 8 of 10 Vx/Ch pigs at 5 dpi. Mean BALF log_{10} $TCID_{50}$ virus titers in the lung were significantly higher ($P \leq 0.05$) in the NVx/Ch pigs compared to Vx/Ch pigs (Table 3). Virus was not detected in nasal swabs from any group at 0 dpi. Nasal shedding was detected in 9 of 10 Vx/Ch pigs and in 5 of 10 NVx/Ch pigs at 3 dpi. At 5 dpi, 10 of 10 pigs in each challenge group had virus isolated from nasal secretions. No statistically significant difference was noted between the Vx/Ch and NVx/Ch pigs in virus titers in nasal secretions at 3 dpi although the mean log_{10} $TCID_{50}$ nasal swab titer was slightly higher in the Vx/Ch pigs. In contrast, the mean log_{10} $TCID_{50}$ nasal swab titer at 5 dpi in the NVx/Ch pigs was significantly higher ($P \leq 0.001$) than the Vx/Ch pigs in spite of the same number of pigs shedding virus.

3.5. Hemagglutination-inhibition and serum neutralization tests

Pigs in the NVx/NCh group remained seronegative throughout the study period. HI antibody titers in sera were observed only in MN08 vaccinated pigs prior to SIV challenge (0 dpi). No MN08 HI antibodies were detected at 0 dpi in the NVx/Ch group and no cross-reacting HI antibody response to the pH1N1 virus was detected in either the Vx/Ch or NVx/Ch group at 0 dpi. In addition, there was

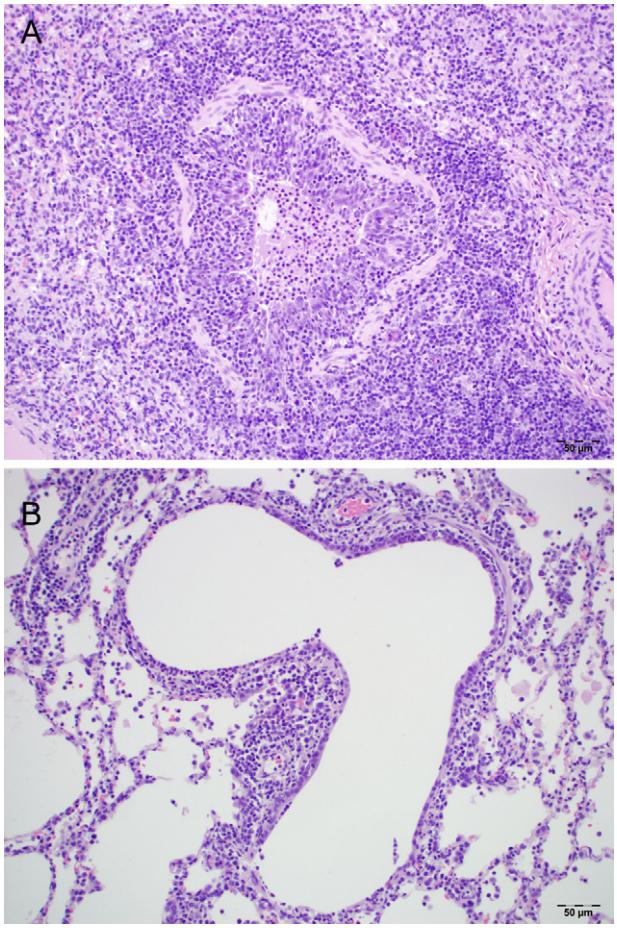

Fig. 2. Microscopic lung lesions representing pigs in each challenge group. (A) Vx/Ch pigs with enhanced pneumonia demonstrated necrotizing to proliferative bronchiolitis and marked peribronchiolar lymphocytic cuffing compared to (B) NVx/Ch pigs (200×).

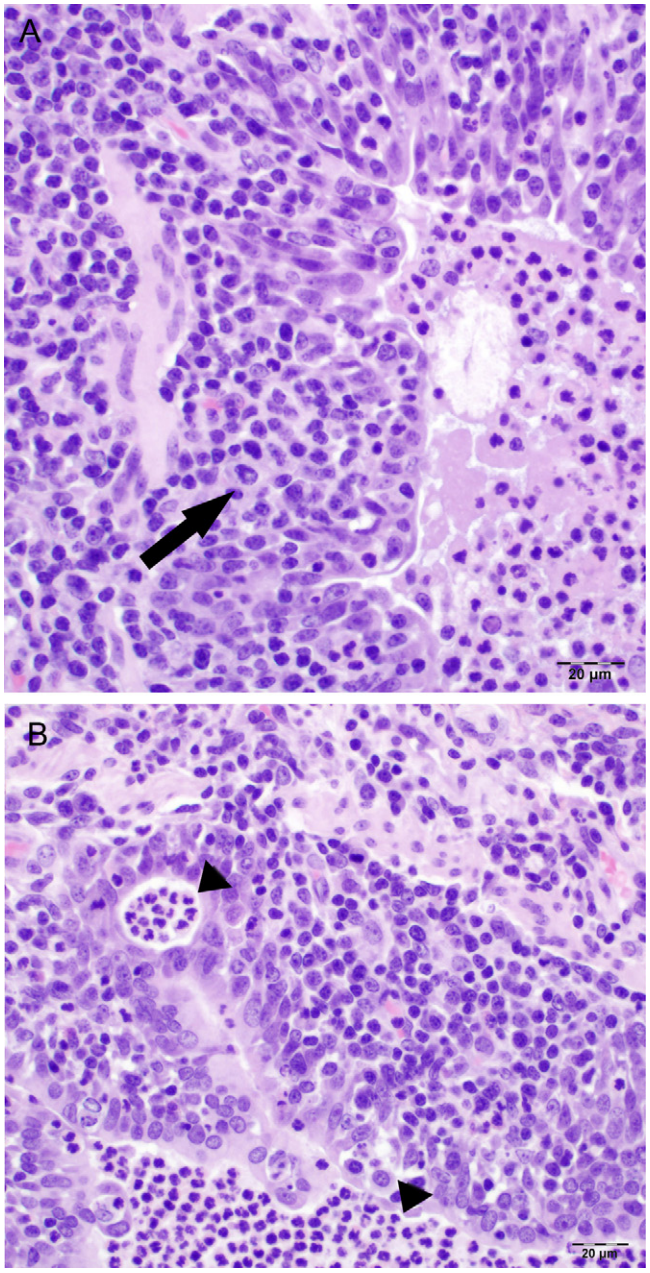

Fig. 3. Microscopic lung lesions demonstrated in Vx/Ch pigs with enhanced pneumonia includes subepithelial lymphocytic inflammation (arrow) and suppurative bronchiolitis (arrowheads) (400×).

no change in the level of MN08 HI antibodies in vaccinated pigs at 5 dpi compared to 0 dpi. HI and SN antibody titers are summarized in Table 4.

Serum neutralization titers against MN08 virus were only observed in the Vx/Ch pigs (Table 4). The geometric mean reciprocal titer was 2743.7 in the Vx/Ch group at 0 dpi and ranged between 640 and 10,240. In addition, serum neutralization titers against the pH1N1 virus were not observed in either the Vx/Ch or NVx/Ch groups at 0 dpi.

3.6. IgG and IgA antibody response to whole virus MN08 and pH1N1

Pigs vaccinated with MN08 and challenged with pH1N1 virus (Vx/Ch group) had anti-MN08 and cross-reacting anti-pH1N1 IgG in BALF. However, NVx/Ch pigs did not develop detectable anti-MN08 or anti-pH1N1 IgG antibodies in BALF that were different than the NVx/NCh group. Minimal anti-MN08 IgA antibody was detected in BALF in the Vx/Ch pigs that were not detected in the NVx/Ch pigs. However, the anti-pH1N1 IgA antibody response detected in the Vx/Ch pigs was not significantly different than the NVx/Ch or NVx/NCh pigs. Geometric mean IgG and IgA antibody titers are reported in Table 5.

Cross-reacting anti-MN08 and anti-pH1N1 IgG antibody responses were detected in serum of Vx/Ch pigs at 0 dpi. Pigs in the

Table 4
Hemagglutination inhibition and serum neutralization geometric mean reciprocal titers ± standard error of the mean (SEM).

Group	HI titers 0 dpi		SN titers 0 dpi	
	MN08	pH1N1	MN08	pH1N1
Vx/Ch	149.3 ± 12.7	0.0 ± 0.0	2743.7 ± 12.7	0.0 ± 0.0
NVx/Ch	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NVx/NCh	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Table 5
Isotype specific serum (0 dpi) and bronchoalveolar lavage fluid (5 dpi) geometric mean reciprocal antibody titers ± standard error of the mean*.

Group	Serum IgG		BALF IgG		BALF IgA	
	MN08 Ag	pH1N1 Ag	MN08 Ag	pH1N1 Ag	MN08 Ag	pH1N1 Ag
Vx/Ch	24,300 ± 1200[a]	18,400 ± 1200[a]	168.9 ± 1.3[a]	294.1 ± 1.2[a]	48.5 ± 35.8[a]	39.4 ± 35.6[a]
NVx/Ch[a]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[a]
NVx/NCh	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[b]	0.0 ± 0.0[a]

* Values within a column not connected by the same letter are significantly different ($P \leq 0.05$).
[a] NVx/Ch antibody responses were less than 2 standard deviations above the mean of NVx/NCh pigs.

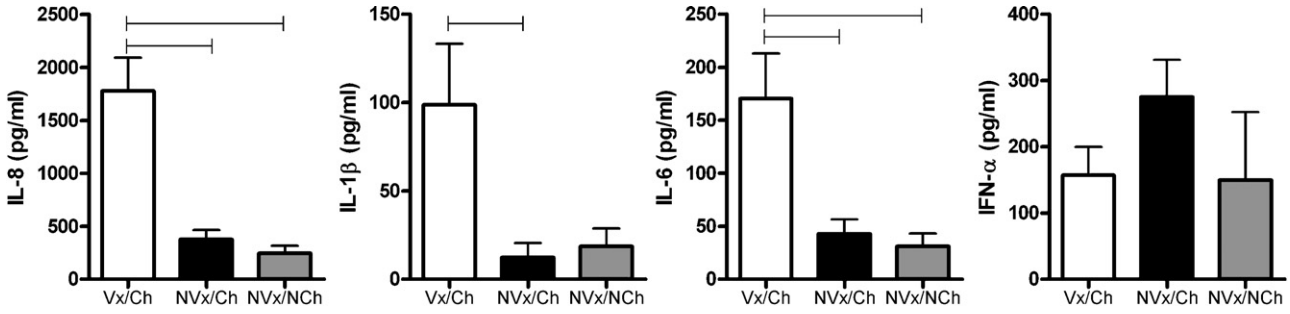

Fig. 4. Cytokine levels (pg/ml) in bronchoalveolar lavage fluid at 5 dpi. Error bars represent standard error of the mean. Bars connecting columns designate significant differences at $P < 0.05$.

NVx/Ch and NVx/NCh groups did not exhibit detectable IgG antibodies in serum at 0 dpi to either antigen. Geometric mean titers are reported in Table 5.

3.7. Cytokine analysis

Pigs in the Vx/Ch group had significantly ($P \leq 0.05$) higher concentrations of IL-8, IL-1β, and IL-6 in the lung on dpi 5 than NVx/ Ch pigs (Fig. 4). The Vx/Ch group averaged 1776 pg/ml of IL-8 compared to the NVx/Ch group that averaged 376.5 pg/ml of IL-8 on 5 dpi. IL-1β levels averaged 100 pg/ml in the Vx/Ch group compared to the NVx/Ch group that averaged 12 pg/ml. The same was observed for IL-6, with an average of 171 pg/ml versus 43 pg/ml in the Vx/Ch and NVx/Ch groups, respectively. Levels of IFN-α in the lung lavage were not significantly different between the challenge groups and the NVx/NCh group.

4. Discussion

This study demonstrates enhanced pneumonia in pigs administered an inactivated H1N2 SIV vaccine followed by challenge with a heterologous pH1N1 virus compared to non-vaccinated pigs challenged with the same virus. A/Sw/MN/02011/08 H1N2 (MN08) was chosen as vaccine virus to represent the δ-cluster of hu-like SIVs identified in 2005 and currently endemic in North American swine [10]. Presently, the δ-cluster SIVs are the most frequently isolated swine influenza virus from diagnostic submissions to the University of Minnesota Veterinary Diagnostic Laboratory (M. Gramer, University of Minnesota Veterinary Diagnostic Laboratory, personal communication). Pandemic A/CA/04/09 H1N1 (pH1N1) was chosen as the challenge virus to evaluate the risk pre-existing immunity against δ-cluster SIV vaccines might have on pH1N1 outbreaks in the pig population. In addition, previous serologic evaluation of swine influenza virus antiserum demonstrated a lack of cross-reactivity between the δ-cluster SIV anti-serum and pH1N1 in HI assays, fulfilling one criteria for duplication of the vaccine-heterologous challenge model used in the study where enhanced pneumonia was first observed [29]. Since the first US swine outbreak of pH1N1 in October 2009, a number of swine cases have been reported and pH1N1 may become endemic in the US swine population in addition to the previously circulating antigenic variants of swine-lineage H1N1 and H1N2.

Macroscopic and microscopic pneumonia typical of SIV infection was demonstrated at 5 dpi in both groups challenged with pH1N1. However, the Vx/Ch pigs had a significantly greater percentage of lung involvement compared to the NVx/Ch pigs in spite of previous vaccination. In addition, microscopic pneumonia and tracheal lesion scores were significantly higher in the Vx/Ch pigs compared to the NVx/Ch group. Experimental inoculation with SIV induces necrotizing bronchiolitis with peribronchiolar lymphocytic cuffing and interstitial pneumonia which can vary in extent and severity depending on the stage of infection and virulence of the virus [9,30]. However, in this study, marked differences in lung lesion profiles between the virus challenge groups were evident. In addition to the bronchiolar epithelial necrosis, pigs with enhanced lesions (Vx/Ch) had marked peribronchiolar lymphocytic cuffing, subepithelial bronchiolar lymphocytic inflammation and moderate interstitial pneumonia to an extent not observed in the NVx/Ch pigs. Interestingly, bronchi, bronchioles and alveoli also contained high numbers of neutrophils at 5 dpi atypical of an uncomplicated viral pneumonia. It is unlikely a secondary bacterial infection contributed to the suppurative inflammation considering *B. bronchiseptica* was isolated from a larger number of pigs in the NVx/Ch and NVx/NCh control group (8 pigs total) which did not have enhanced lung lesions or bronchopneumonia, compared to the Vx/Ch group with *B. bronchiseptica* (3 pigs). The increased pro-inflammatory cytokine response in the lungs of the Vx/Ch pigs, which included elevated IL-8 protein levels, may have contributed to the suppurative pneumonia in the Vx/Ch pigs due to the potent neutrophil chemotaxis property of IL-8 [31]. These data suggest specific differences in severity and distribution of microscopic lesions may provide distinguishing features between enhanced pneumonia and uncomplicated infection with SIV useful to identify such issues in field cases.

The mechanism of the enhanced pneumonia phenomenon has not been elucidated although consistent features among studies have been described previously and in this report [19,32]. These include (1) whole influenza virus antigen administered as a monovalent, inactivated vaccine combined with oil-in-water adjuvant; (2) challenge with a heterologous SIV with a homosubtypic HA three weeks post booster vaccination; (3) serum HI antibodies to

the priming antigen that do not cross-react with the challenge virus; and (4) whole virus, non-HI, non-neutralizing IgG antibodies detected by ELISA in serum and BALF cross-reacting with the challenge virus.

Inactivated whole-virus influenza vaccines are commonly used in the US swine industry and most are multivalent with H1 and H3 subtypes included. Inactivated SIV vaccines are efficacious against homologous challenge, although limited cross-protection is demonstrated against heterologous homosubtypic or heterosubtypic viruses [15,17–19]. Previous studies by our group have shown that the use of live attenuated virus may enhance the efficacy of vaccines against antigenically heterologous viruses of the same subtype, specifically through the development of cross-reactive antibodies at the mucosal level [19,33]. Cell-mediated immune responses, stimulated by live exposure, are also important for heterosubtypic immunity and recovery from infection. One study using immune pigs infected with heterosubtypic SIV demonstrated elevated CD8+ T cells in the lungs compared to pigs challenged with homologous virus indicating the importance of T cells in heterosubtypic immunity [34]. Collectively, these studies suggest live infection or vaccination with modified live vaccines may confer more efficient cross-protection than inactivated vaccines against divergent influenza viruses due to activation of both humoral and cell mediated immunity. Additional studies are needed to understand the differences in the immune response to inactivated vaccines versus live challenge and how this may affect cross-protective immune function and the aggravated lung pathology demonstrated in pigs in our model.

HI titers (1:40–1:320) and SN titers (1:640–1:10,240) to the MN08 antigen were demonstrated in vaccinated pigs at 0 dpi (day of challenge); however, antibody cross-reactive with the pH1N1 antigen was not detected by either HI or SN test. MN08 and pH1N1 are genetically related by H1 subtype, but have only 77% identity at the nucleotide level between the HA genes. The identity ranged from 91% to 94% for the polymerase genes and 95% for NP and NS. The NA genes are of different subtype and thus of low identity. The M genes also are derived from different source viruses (MN08 North American swine lineage; pH1N1 human influenza A virus with Eurasian swine-lineage) and thus of low identity as well. The MN08 virus is a contemporary member of the δ-cluster or hu-like SIVs. In the US, influenza viruses with hu-like HA were first identified in swine in 2005, all with triple reassortant internal genes similar to contemporary US swine influenza isolates. The HA and NA genes were related to recent human seasonal influenza virus lineages [10]. Influenza viruses related to earlier human seasonal H1N1 have been identified in pigs in China as well [35], but are distantly related to North American δ-cluster HA and were not shown to have reassorted with endemic Chinese swine viruses. In contrast, the pH1N1 virus, which has an HA similar to the γ-cluster H1 SIVs, contains genes from both North American and Eurasian swine influenza virus lineages with a constellation of the eight gene segments not known to circulate in swine prior to the emergence of the pandemic virus. Serologic cross-reactivity with pH1N1 has been demonstrated in HI tests with sera from pigs immunized with α-, β-, and γ-clusters of H1 SIV but not with δ-cluster antisera [29]. The lack of cross-reactive HI antibodies demonstrated in this study due to the divergent H1 cluster viruses used as the vaccine antigen and challenge virus suggests that HI antibodies may play an important role in preventing enhanced pneumonia.

In contrast to the absence of HI antibodies to pH1N1 in MN08-primed anti-sera, a whole-virus ELISA detected similar levels of anti-MN08 and anti-pH1N1 IgG antibodies in serum and BALF at 0 and 5 dpi, respectively. These data suggest the non-HI antibodies may have contributed to the enhanced macroscopic and microscopic lung lesions described in this report. Potential roles for vaccine-induced non-HI IgG antibody could include antibody dependent cell-mediated cytotoxicity (ADCC), activation of the classical complement cascade, or antibody dependent enhancement (ADE) through Fc-receptor mediated uptake of virus. Low avidity antibodies were recently shown to be associated with antigen–antibody complexes and complement fixation in lungs of fatal human cases of pH1N1 [36]. Inactivated influenza vaccines have been shown to induce antibodies reactive in ADCC [37] or to promote cell-mediated cytotoxicity and complement fixation [38,39]. Further studies are necessary to understand the role of non-HI antibodies and the mechanism for the development of enhanced pneumonia.

In contrast to the presence of whole virus IgG antibodies in BALF to both antigens, there were either minimal levels of IgA to the priming antigen or insignificant levels of IgA to the challenge virus in the respiratory mucosa at 5 dpi in the Vx/Ch pigs. Our results indicate the anti-MN08 IgA did not cross-react with the challenge virus. However, it is unknown if the presence of cross-reacting mucosal IgA, or a more robust response, would have prevented infection or the enhanced pneumonia in this swine study. A previous study by our group demonstrated a relative decrease in IgA and increase in IgG in 3 of 9 pigs with enhanced pneumonia compared to 6 pigs without enhanced lesions providing support for the potential cross-protective role of IgA antibody [19]. Antibody mediated immune reactions at the mucosal level, rather than systemic immunity, have been shown to be important for protecting the respiratory tract from infection with SIV [27]. Therefore, the potentiation of lung lesions described in this report may have been due to the insignificant levels of IgA in conjunction with higher levels of non-neutralizing IgG to the challenge virus.

The study described here suggests that cross-reactive, non-HI antibodies induced by inactivated vaccines may play a role in the enhanced pneumonia. However, it remains unclear if antibodies against specific epitopes on the HA protein are more involved in the immunopathology than others. Enhanced clinical disease implicating antibody responses to minor immunogenic proteins other than surface glycoproteins post-challenge with influenza virus has been reported in vaccinated pigs. A previous study used a DNA vaccine expressing an M2 and nucleoprotein (NP) fusion protein to induce anti-M2 antibodies and influenza-specific T-cell responses [40]. Interestingly, clinical signs and mortality were more severe upon challenge with SIV in the vaccinated pigs. The authors speculated that non-neutralizing antibodies to the M2 protein may have allowed increased viral uptake and expression of surface M2 protein, promoting cell death through ADCC or complement activation and T helper cells may have stimulated an exaggerated inflammatory response. Another recent study also suggested an association between prior human vaccination with the 2008–2009 trivalent inactivated influenza vaccine (TIV) and increased severity of clinical illness induced by infection with pandemic influenza A H1N1 [41]. Although cause and effect was not established between vaccination and illness, the authors concluded that prior vaccination with TIV may have increased the risk of medically attended pH1N1 illness in humans. This study demonstrated a potential vaccine-associated disease enhancement with human implications similar to the suggested outcome in this report. Clinical signs were correlated with one specific vaccine, implying the manufacturing process may have played a role in the disease enhancement. However, further studies are necessary to understand the role vaccine preparation may have on the clinical outcome to heterologous virus infection.

Swine with existing immunity to influenza virus through repeated exposure or vaccination with multiple strains may be partially protected against heterologous challenge, as was previously shown in vaccinated pigs challenged with pH1N1 virus [42]. However, the simultaneous increase in inactivated SIV vaccine use and the evolving antigenic diversity of influenza A viruses in swine creates a realistic potential for vaccine/challenge mismatch. Com-

mercial vaccines are typically multivalent to enable protection against exposure to multiple subtypes and antigenically diverse strains. Manufacturing regulations in the US limit the ability to alter fully licensed swine influenza vaccines as rapidly as the virus is changing. Future vaccines that provide adequate protection from infection and decrease the potential for vaccine-enhanced pneumonia will likely need to provide cross-protection at the respiratory mucosa and activate both the humoral and cell-mediated immune systems.

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