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# Live attenuated influenza A virus vaccine protects against A(H1N1) pdm09 heterologous challenge without vaccine associated enhanced respiratory disease



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## ABSTRACT

Live-attenuated influenza virus (LAIV) vaccines may provide cross-protection against contemporary influenza A virus (IAV) in swine. Conversely, whole inactivated virus (WIV) vaccines have the potential risk of vaccine-associated enhanced respiratory disease (VAERD) when challenged with IAV of substantial antigenic drift. A temperature sensitive, intranasal H1N2 LAIV was compared to wild type exposure (WT) and an intramuscular WIV vaccine in a model shown to induce VAERD. WIV vaccinated swine challenged with pandemic A/H1N1 (H1N1pdm09) were not protected from infection and demonstrated severe respiratory disease consistent with VAERD. Lung lesions were mild and challenge virus was not detected in the respiratory tract of LAIV vaccinates. High levels of post-vaccination IgG serum antibodies targeting the H1N1pdm09 HA2 stalk domain were exclusively detected in the WIV group and associated with increased H1N1pdm09 virus infectivity in MDCK cells. In contrast, infection-enhancing antibodies were not detected in the serum of LAIV vaccinates and VAERD was not observed.

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## Introduction

Contemporary influenza A viruses (IAV) circulating in North American swine are characterized by extensive genetic and antigenic diversity that has increased since the introduction of the triple reassortant internal gene (TRIG) constellation in 1998 with surface proteins from human seasonal H3N2 (Zhou et al., 1999). The introduction of human seasonal influenza viruses again in 2005 (Vincent et al., 2009), human seasonal N2 in 2002 (Nelson et al., 2011, 2012b), and the spillover of the human 2009 pandemic H1N1 (H1N1pdm09) virus (Nelson et al., 2012a; Pasma and Joseph, 2010; Pereda et al., 2010) have continued to add to the complex IAV ecology in swine. As a result of these introductions, viral reassortment, and antigenic drift, novel IAV continue to emerge in

swine, including reassortant H3N2-TRIG/H1N1pdm09 viruses that recently caused multiple human infections (Ducatez et al., 2011; Lindstrom et al., 2012). Currently, the H1 $\alpha$ , H1 $\beta$ , H1 $\gamma$ , H1 $\delta$ 1, H1 $\delta$ 2, H1N1pdm09 and H3 cluster IV (CIV) represent seven antigenically distinct clusters co-circulating in U.S. swine (Lorusso et al., 2013; Vincent et al., 2009). Consistent with the increasing antigenic diversity recognized in swine-origin IAV is the lack of serological cross-reactivity demonstrated between phylogenetic clusters in hemagglutination inhibition (HI) tests (Lorusso et al., 2011).

Influenza vaccines are primarily used in adult female breeding swine and during the grow/finish phase of production to decrease IAV disease, lung lesions and transmission (Beaudoin et al., 2012; Chen et al., 2012; Ma and Richt, 2010; Vincent et al., 2008b). Protection against infection has relied on a systemic immune response induced by whole inactivated virus (WIV) products, the only preparation currently available for use in swine, in commercial or autogenous vaccines delivered by the intramuscular route (Van Reeth and Ma, 2013; Vincent et al., 2008b). The production of “off-the-shelf” commercially available efficacious WIV vaccines has proven difficult due to the number of valences required to

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minimize the antigenic distance between vaccine strains and circulating IAV in swine (Gasparini et al., 2011). Current WIV preparations used in swine include two or more representatives of H1 and H3 cluster-types mixed with oil-based adjuvants, however WIV vaccines have consistently been shown to provide only partial protection against heterologous IAV infection and shedding (Heinen et al., 2001; Kitikoon et al., 2006; Vincent et al., 2008a, 2010a). Formulating and updating effective WIV vaccines is additionally challenged by the difficulty in updating vaccine seed viruses faster than the rate of significant antigenic evolution, the time needed to approve and license WIV products, potential maternal antibody interference, and the lack of an adequate mucosal and cell-mediated immune response (Kappes et al., 2012; Kitikoon et al., 2006; Vincent et al., 2008b). Apart from sub-optimal protection provided by WIV adjuvanted preparations against mismatched circulating swine viruses in association with the absence of cross-neutralizing antibodies, these vaccines may be associated with vaccine-associated enhanced respiratory disease (VAERD) (Gauger et al., 2011, 2012; Kitikoon et al., 2006; Vincent et al., 2008a). We have recently described the presence of high titer cross-reacting antibodies targeting the conserved HA2 domain at a site adjacent to the fusion peptide (Khurana et al., 2013). In the absence of neutralizing antibodies against the HA1 globular head of H1N1pdm09, we observed the HA2 antibodies increased virus infection of MDCK cells in vitro and enhanced membrane fusion (Khurana et al., 2013).

Alternative IAV vaccine platforms and methods of delivery are needed to improve protection from heterologous infection and reduce the risk of VAERD. Live attenuated influenza virus (LAIV) vaccines administered by the mucosal route mimic natural infection and demonstrate the potential for broad cross-protective immunity (Masic et al., 2009, 2010; Pena et al., 2011; Richt et al., 2006; Vincent et al., 2007). In addition, LAIV vaccines may lack some of the potential concerns posed by WIV products. Pigs administered an intranasal H3N2 LAIV attenuated by truncation of the NS1 protein in the presence of matching maternally derived antibody (MDA) demonstrated better protection against challenge with a heterologous H3N2 compared to pigs that received an intramuscular WIV product (Vincent et al., 2012). Moreover, the WIV vaccinated pigs in the same study developed VAERD that was not observed in the LAIV group. This underscores another advantage of using modified live vaccines in the context of antigenically diverse IAV ecology observed in swine (Vincent et al., 2012). In addition to the NS-1 truncated attenuation, influenza viruses attenuated by modifications in the polymerase genes, rendering the virus sensitive to physiologic temperatures, have been shown to be efficacious in swine (Pena et al., 2011).

The objective of the study reported here was to determine if the use of a temperature sensitive LAIV would avoid or contribute to VAERD in an H1 model that previously demonstrated VAERD in pigs vaccinated with WIV (Gauger et al., 2011, 2012; Khurana et al., 2013). Here, pigs were vaccinated with  $\delta$ 1H1N2 LAIV or WIV and challenged with heterologous H1N1pdm09 virus. The two vaccine platforms were compared to wild type virus exposure to evaluate induction of humoral and cell-mediated immune responses in various compartments. In addition to the traditional HI assay, we measured antibody binding to the intact HA and to subdomains HA1 and HA2 of the challenge virus (H1N1pdm09). Additionally, a low challenge dose was administered to evaluate if VAERD was dependent on the relatively higher dose used previously (Gauger et al., 2011, 2012). These data demonstrate different outcomes of heterologous challenge after LAIV compared with WIV vaccination and suggest important differences in the type and specificity of antibodies elicited by the vaccine platforms that provides further insight into the conditions that lead to VAERD after heterologous challenge.

## Materials and methods

### Viruses and vaccine preparation

Antigen for both vaccine platforms was a  $\delta$ 1 cluster H1N2 A/Sw/MN/02011/2008 (MN08) propagated in Madin-Darby canine kidney (MDCK) cells. The LAIV vaccine was generated by introducing the MN08 HA and NA into the swine triple reassortant-lineage A/turkey/Ohio/313053/04 H3N2 (OH04) attenuated backbone using reverse genetics (Pena et al., 2011). The OH04 isolate was attenuated by modifying the polymerase genes creating a temperature-sensitive strain as previously described in detail (Pena et al., 2011). The LAIV was propagated at 35 °C on MDCK cells. The WIV was prepared using UV irradiated MN08 with the addition of a commercial oil-in-water adjuvant (Emulsigen D; MVP Laboratories, Inc., Ralston, NE) at a v-v ratio of 4:1 virus to adjuvant. Unaltered MN08 was used for the live virus exposure designated as wild-type (WT) immune group. A sham vaccine (SV) prepared using sterile MDCK cell culture supernatant diluted with PBS was added 4:1 with adjuvant, consistent with the WIV preparation. The challenge virus was prepared from A/California/04/2009 H1N1 (H1N1pdm09) propagated in MDCK cells.

### Experimental design

Ninety-six, three-week-old cross-bred pigs of mixed sex were obtained from a herd free of IAV and porcine reproductive and respiratory syndrome virus (PRRSV) and randomly divided into four groups consisting of twenty-four pigs. Upon arrival, pigs were housed in individual ABSL2 isolation rooms and treated prophylactically with ceftiofur (Pfizer Animal Health, New York, NY) according to label directions. Pigs were cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center.

Pigs received two doses of their respective vaccine at 4 and 7 weeks of age. Pigs in the LAIV- intranasal (LAIV-IN) group were vaccinated with 2 ml of MN08 temperature sensitive LAIV at  $1 \times 10^6$  50% tissue culture infective dose (TCID<sub>50</sub>) per ml by slowly dripping the vaccine alternately into both nostrils. Wild-type, unaltered MN08 was administered at the same dose and by the same method to pigs in the WT-IN immune group. The WIV-intramuscular (WIV-IM) vaccinated pigs were administered 128 HA units of inactivated, adjuvanted vaccine by the intramuscular route. One group of twenty-four pigs received 2 ml of a sham vaccine by the intramuscular route (SV-IM) and served as sham-vaccinated controls.

Eight pigs in each IAV MN08 immune group and 8 SV-IM pigs were euthanized at 0 dpi (pre-challenge) to collect bronchoalveolar lavage fluid (BALF) for antibody analysis in the lower respiratory tract. The remaining sixteen pigs in each IAV MN08 immune group and 8 SV-IM pigs were challenged with a low dose (2 ml,  $1 \times 10^3$  TCID<sub>50</sub>/ml) of H1N1pdm09 by the intratracheal route at 10 weeks of age to compare efficacies of the different vaccine platforms. Eight of the SV-IM pigs remained non-challenge controls (SV-NC) for the clinical evaluation and cytokine analysis. Pigs were observed daily for clinical disease and rectal temperatures were collected on - 1, 0, 1, 2, 3, 4 and 5 dpi. Nasal swabs (Fisherbrand Dacron swabs, Fisher Scientific, Pittsburg, PA) were taken from all pigs on 0, 3 and 5 dpi to evaluate nasal virus shedding as previously described (Vincent et al., 2012). Eight challenged pigs in each immune group and 8 challenged SV-IM pigs were humanely euthanized with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) at 5 dpi to evaluate lesions, viral replication and cytokine concentrations in the lungs. Postmortem samples included serum, lung, trachea and BALF. The

SV-NC pigs were euthanized on 21 dpi to evaluate cytokine concentrations in BALF representing IAV-naïve pigs.

#### Diagnostic microbiology

Prior to the start of the study, all pigs were screened for antibodies against influenza A nucleoprotein by ELISA (MultiS ELISA, IDEXX, Westbrook, ME) to confirm the absence of influenza exposure. BALF samples were cultured for aerobic bacteria on blood and Casmin (NAD enriched) agar plates and subjected to nucleic acid extraction using the MagMax™ Viral RNA/DNA Isolation Kit (Life Technologies, Carlsbad, CA) to detect potential confounding respiratory pathogens. A polymerase chain reaction (PCR) assay for PCV2 was conducted as previously described on nucleic acid extracts from BALF collected at each necropsy (Opriessnig et al., 2003). Commercial PCR assays for *Mycoplasma hyopneumoniae* and PRRSV were conducted with the Applied Biosystems® VetMax™ *M. hyopneumoniae* reagents or the NA and EU PRRSV-specific PCR assay (Life Technologies Carlsbad, CA), respectively, according to manufacturer's recommendations for serum.

#### Pathological examination of lungs

At 5 dpi, lungs were evaluated for macroscopic lesions consisting of purple or red consolidation typical of IAV in swine. The percent of the lung surface affected with consolidation was subjectively estimated for each lung lobe and the total percentage of pneumonia was calculated based on weighted proportions of each lobe relative to the total lung volume as previously described (Halbur et al., 1995). Microscopic lung and trachea lesions were evaluated by a veterinary pathologist blinded to the treatment groups after tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin. Individual scores were assigned for each of six parameters as previously described with modifications (Gauger et al., 2012). The percentage of intrapulmonary airway epithelial necrosis or proliferation was scored as follows: 0.0, no airway epithelial changes; 0.1–1.0, up to 25% of airways affected with bronchi or bronchiolar epithelial damage; 1.1–2.0, 26–50% of airways affected; 2.1–3.0, 51–75% of airways affected; 3.1–4.0, 76–100% of airways affected with intrapulmonary epithelial changes. The magnitude of peribronchiolar lymphocytic cuffing was evaluated regardless of the number of bronchi or bronchioles affected and were scored as follows: 0.0, no discernible peribronchiolar cuffing observed; 1.0, minimal, loosely formed cuffs; 2.0, mild, loosely to well-formed cuffs; 3.0, moderate, well-formed or prominent lymphocytic cuffs; 4.0, severe, thick or densely-formed peribronchiolar cuffing. The percentage of bronchi and bronchioles that demonstrated purulent exudate (suppurative bronchitis or bronchiolitis) and propria submucosa lymphohistiocytic inflammation were scored as follows: 0.0, no presence of neutrophils or inflammation; 0.1–1.0, up to 25% of airways affected with inflammation; 1.1–2.0, 26–50% of airways affected; 2.1–3.0, 51–75% of airways affected; 3.1–4.0, 76–100% of airways affected with neutrophils and marked propria submucosa lymphohistiocytic inflammation. The presence and severity of alveolar septal thickening with mononuclear inflammation (interstitial pneumonia) was scored as follows: 0.0, no interstitial pneumonia; 1.0, mild, focal to multifocal interstitial inflammation; 2.0, moderate, locally extensive to multifocal interstitial inflammation; 3.0, moderate, multifocal to coalescing interstitial inflammation and alveolar septal thickening; 4.0, severe, coalescing to diffuse interstitial thickening/inflammation. Two additional subjective scores were based on the overall severity of intrapulmonary epithelial exocytosis (microabscesses) in affected airways and magnitude of alveolar and/or interlobular edema.

Epithelial exocytosis was scored as follows: 0.0, none; 1.0, 1–3 microabscesses; 2.0, 4–6 microabscesses; 3.0, more than 6 microabscesses in the intrapulmonary epithelium. Edema was scored as follows: 0.0, none; 1.0, mild, focal; 2.0, moderate, locally extensive; 3.0, severe, diffuse. A composite score was computed using the sum of the 6 individual scores (range of 0–22). The average group composite score was used for statistical analysis.

The trachea was evaluated with two individual scores based on the magnitude of trachea epithelial attenuation or necrosis and the degree of inflammation (tracheitis). Trachea epithelial scores were defined as follows: 0.0, normal epithelium over the entire circumference; 1.0, mild epithelial changes (focal loss of cilia with epithelial cell degeneration or flattening); 2.0, mild epithelial attenuation of approximately 2–3 cell layers either segmental or multifocal (focal to multifocal loss of cilia and decreased number of goblet cells); 3.0, moderate epithelial attenuation of approximately 1–2 cell layers, either segmental or multifocal (multifocal loss of cilia and few goblet cells); 4.0, severe flattened epithelium with only a single layer of epithelium remaining, multifocal to extensive (almost complete loss of all cilia and no goblet cells remaining). Tracheitis scores were determined as follows: 0.0, no inflammation; 1.0, minimal amount of suppurative or subepithelial mononuclear inflammation; 2.0, mild, multifocal inflammation; 3.0, moderate, consistent inflammation affecting the trachea epithelium or subepithelial space; 4.0, severe, coalescing to diffuse inflammation of the epithelium or marked extension of inflammation into the subepithelial region. A composite score was computed using the sum of the two individual trachea lesion scores (range of 0–8). The average group composite score was used for statistical analysis.

#### Serologic and mucosal antibody assays

Hemagglutination inhibition assays were conducted on serum collected by anterior vena cava venipuncture from pigs in each group at 0 dpi (day of challenge) with MN08 or H1N1pdm09 virus as antigen and turkey red blood cells (RBC) as indicators using standard techniques as previously described (Vincent et al., 2008a). Reciprocal titers were divided by 10 and  $\log_2$  transformed, analyzed, and reported as the geometric mean.

Virus neutralization (VN) assays were conducted on BALF from the thirty-two pigs euthanized at 0 dpi and serum neutralization (SN) assays were conducted on serum from the thirty-two pigs challenged at 0 dpi as previously described (Gauger et al., 2011; Loving et al., 2012). Titers were recorded as the highest serum dilution negative for CPE and negative for virus as verified by immunocytochemistry for all plates. Reciprocal titers were divided by 10,  $\log_2$  transformed, analyzed, and reported as the geometric mean.

Serum from twenty-four LAIV-IN, WT-IN and WIV-IM pigs pre-challenge (0 dpi) were evaluated for binding to the H1N1pdm09 HA1 or HA2 domain in real-time kinetics using Surface Plasmon Resonance (SPR). A ProteOn SPR biosensor (Bio-Rad) was used to monitor steady-state equilibrium binding of individual pig sera (Khurana et al., 2011b, 2013). Antibody binding is expressed as maximum RU ( $\text{max RU}$ ) values.

Enzyme-linked immunosorbent assays (ELISA) to detect total IgG and IgA antibodies against whole virus preparations of MN08 and H1N1pdm09 present in serum and BALF from all pigs at 0 dpi were performed as previously described with modifications (Gauger et al., 2011). Sera were heat inactivated at 56 °C for 30 min and diluted in 5% Fraction V bovine serum albumin (BSA) (Life Technologies/Gibco, Grand Island, NY) in phosphate buffered saline (PBS) at 1:2000 and 1:4 for IgG and IgA assays, respectively, to adsorb non-specific binding. The BALF samples were incubated at 37 °C for 1 h with an equal volume of 10 mM dithiothreitol (DTT) to disrupt mucus prior to dilution with an equal volume of



10% BSA-PBS resulting in a final BALF dilution of 1:4. The BSA treated sera and BALF were incubated at 37 °C for 1 h immediately prior to testing.

Concentrated MN08 and H1N1pdm09 were 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 50 µl of either antigen and incubated at room temperature overnight. Plates were blocked for 1 h at room temperature with 150 µl of Starting Block (Thermo Fisher Scientific, Pittsburgh, PA) and washed 3 times with 200 µl of 0.05% Tween 20 in PBS (PBS-T). Independent assays were performed with each antigen using 50 µl of diluted sera or BALF in duplicate. Plates were incubated at room temperature for 1 h and washed 3 times with PBS-T. Fifty microliters of peroxidase-labeled goat anti-swine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD) or anti-swine IgA (Bethyl, Montgomery, TX) diluted 1:1500 in Starting Block was added to each well for 1 h at room temperature. Plates were washed 3 times with PBS-T prior to adding 50 µl of 2, 2'-azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS)-peroxide as the substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 8–10 minutes and the subsequent addition of 50 µl KPL Stop Solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The optical density (OD) was measured at 405 nm wavelength with an automated ELISA reader. Antibody levels were reported as the mean OD for each duplicate sample, and the mean OD of each treatment group was compared.

#### *Virus infection assay in MDCK cells*

Sera from individual LAIV-IN, WT-IN and WIV-IM vaccinated pigs pre-challenge (0 dpi) were analyzed in an H1N1pdm09 virus infection assay as previously described (Khurana et al., 2011a, 2013). A nucleoprotein-based ELISA was used to determine the virus titer expressed as a percent of control infection. Sera from H1N1pdm09 WIV-vaccinated animals were added as positive controls. The inhibition curve with these sera was not altered in the presence of pre-vaccination sera (Khurana et al., 2013).

#### *Lung cytokine assays*

BALF samples collected from twenty-four pre-challenge pigs at 0 dpi, twenty-four challenged pigs at 5 dpi and 8 SV-NC pigs at 21 dpi were centrifuged at 400 x g for 15 min at 4 °C to pellet cellular debris. Cytokine concentrations in cell-free BALF, which included IL-1β, IL-2, IL-6, IL-8, IL-12p70, IL-10, TNFα and IFNγ, were determined by multiplex ELISA performed according to the manufacturer's recommendations (SearchLight, Aushon Biosystems, Billerica, MA). Cytokine concentrations were reported as the mean of duplicate samples for statistical analysis.

#### *Statistical analysis*

Macroscopic and microscopic pneumonia scores, log<sub>2</sub> transformed HI and SN reciprocal titers, log<sub>10</sub> transformed nasal swab and BALF virus titers, mean OD ELISA IgG and IgA antibody levels, maximum RU HA1 and HA2 binding data, percent virus infection in the MDCK cell-based assay and cytokine concentrations 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 mean comparisons using the Tukey–Kramer test or a Dunnett's multiple comparison test for cytokine concentrations. Group means with statistical difference are indicated by connecting bars. 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.

## **Results**

### *Clinical disease*

Clinical signs consistent with IAV respiratory disease in swine were more severe over a prolonged period of time in the WIV-IM pigs compared to all other vaccinated and challenged immune groups and compatible with current descriptions of VAERD (Gauger et al., 2011, 2012). All WIV-IM pigs demonstrated coughing or respiratory distress at various times post-challenge and an average of 63% (10 of 16) of the WIV-IM pigs demonstrated IAV clinical signs from 1 to 5 dpi. In contrast, mild, transient lethargy and anorexia and mild coughing was occasionally observed in some of the pigs in the LAIV-IN or WT-IN vaccinated groups at 24 h post infection.

All MN08 vaccinated and sham-vaccinated immune groups challenged with H1N1pdm09 demonstrated mean febrile responses at 1 dpi defined as two standard deviations above the mean rectal temperature prior to challenge ( $\geq 39.9$  °C) that were significantly higher than the SV-NC group (Supplemental Fig. S1). However, the LAIV-IN and WT-IN mean rectal temperatures were significantly lower than the SV-IM challenge control group at 1 dpi. By 2 dpi, LAIV-IN, WT-IN and SV-IM mean rectal temperatures were not different than the non-challenged controls and significantly lower than the WIV-IM group. In addition, WIV-IM pigs demonstrated significantly elevated rectal temperatures at 3 and 4 dpi compared to all other immune groups and remained significantly higher at 5 dpi compared to the LAIV-IN, WT-IN, and SV-NC groups. These data are consistent with the prolonged elevated body temperatures described in VAERD-affected swine (Gauger et al., 2011).

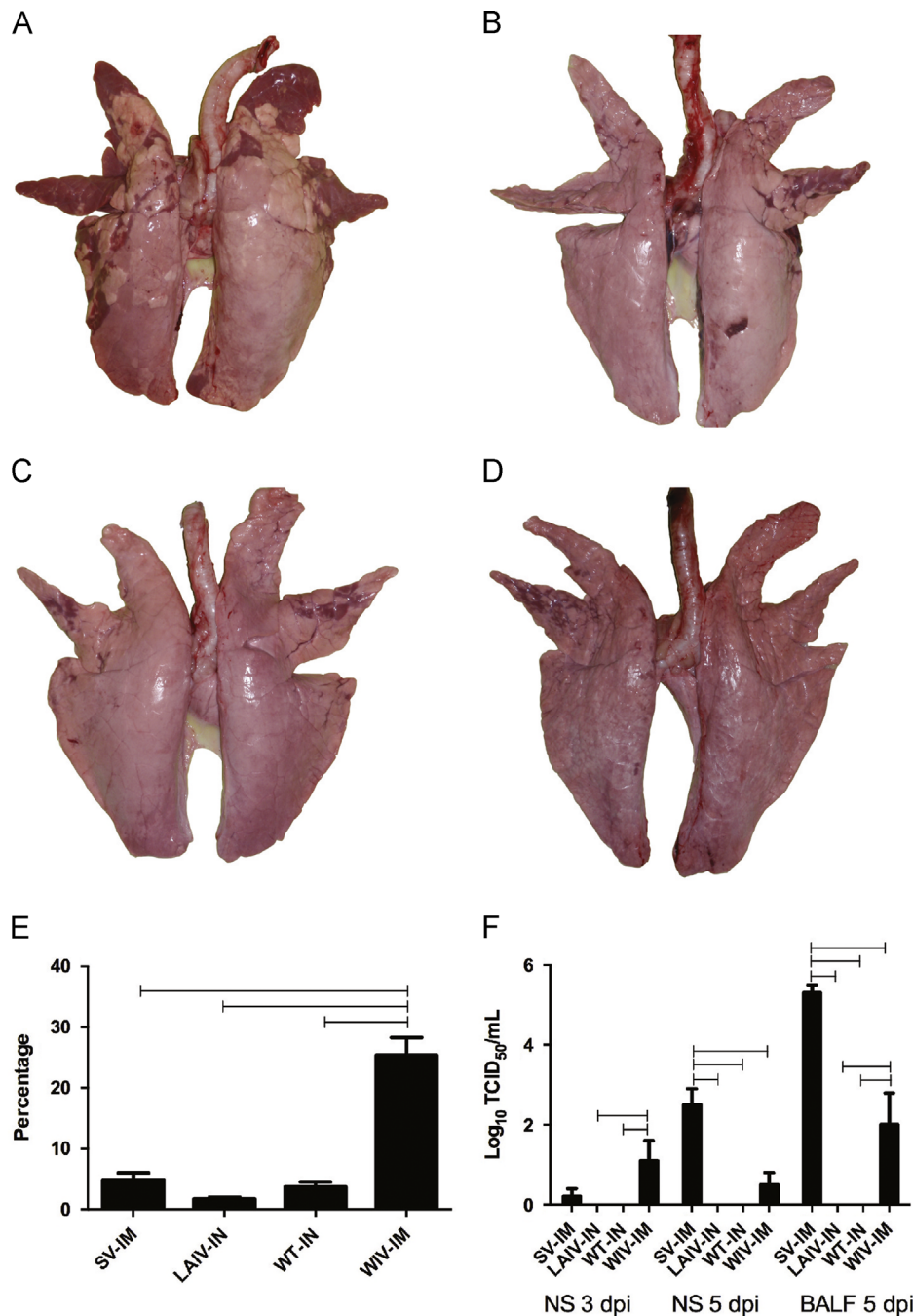
### *Viral and microbiological assays*

Influenza A virus antibodies were not detected in pigs prior to vaccination and virus was not detected pre-vaccination, one week post boost, or at 0 dpi. Extraneous viral or *M. hyopneumoniae* nucleic acids were not detected in BALF collected from pigs necropsied at 5 dpi. Aerobic bacterial cultures yielded no significant growth of respiratory pathogens, thus the severe lung pathology described in WIV-IM, VAERD-affected pigs was not associated with bacterial pneumonia.

### *Lung and trachea pathology and replication of challenge virus*

Pigs administered the WIV-IM vaccine and challenged with a low dose of H1N1pdm09 demonstrated enhanced macroscopic lung lesions (Fig. 1A) with an average of 25% macroscopic pneumonia, significantly higher compared to the mean lung consolidation demonstrated by the SV-IM pigs (Fig. 1B). Pigs that received the WT virus (Fig. 1C) or LAIV (Fig. 1D) intranasal vaccines demonstrated mild pneumonia, averaging 3.7 and 1.7 percent of the lung, respectively, that were similar to the percent pneumonia observed in the SV-IM pigs, and significantly lower compared to the WIV-IM immune group (Fig. 1E). Importantly, enhanced macroscopic lung pathology was not recognized in the LAIV vaccinated pigs in spite of the heterologous challenge.

Challenge virus was detected in nasal secretions in 1/8 SV-IM pigs and 4/8 pigs in the WIV-IM group at 3 dpi, likely influenced by the low challenge dose. However, by 5 dpi, virus was isolated from nasal



**Fig. 1.** Macroscopic lung lesions from representative pigs in H1N1pdm09 challenged immune groups. Pigs in the WIV-IM (A) group demonstrating VAERD had a significantly higher mean percentage of lung consolidation compared to all other groups. The SV-IM (B), WT-IN (C) and LAIV-IN (D) pigs demonstrated mild lung consolidation although group mean percentage of pneumonia was not statistically different from each other (E). H1N1pdm09 titers in nasal swab and bronchoalveolar lavage at 3 and 5 dpi (F). Data are expressed as the mean percent pneumonia or mean log<sub>10</sub> virus titer  $\pm$  standard error of the mean of each group. Connecting lines are significantly different at  $P < 0.05$ .

secretions of 8/8 pigs in the SV-IM group and 3/8 pigs in the WIV-IM group. Mean nasal virus titers at 3 dpi were significantly higher in the WIV-IM group compared to LAIV-IN and WT-IN pigs (Fig. 1F). In contrast, 5 dpi nasal virus titers were significantly higher in the SV-IM pigs compared to all other immune groups (Fig. 1F). Virus titers decreased between 3 and 5 dpi in the WIV-IM pigs in contrast to the SV-IM group that demonstrated higher titers by 5 dpi and higher numbers of individual pigs shedding virus. Importantly, no challenge virus was detected in nasal secretions from pigs immunized with LAIV-IN or WT-IN, suggesting adequate cross-protection against H1N1pdm09 by prior live or live-attenuated virus immunity (Fig. 1F). Virus was detected only in the SV-IM and WIV-IM BALF at

necropsy (5 dpi) (Fig. 1F). Although less than SV-IM challenge controls, pigs administered the WIV-IM vaccine had significantly higher virus titers compared to the LAIV-IN and WT-IN immune groups, which had no virus isolated from BALF. These findings suggest the VAERD-affected pigs demonstrated accelerated virus replication in the upper respiratory tract compared to the LAIV-IN and WT-IN groups. It is possible that by 5 dpi at the peak of airway epithelial damage, the infection was self-limiting due to lack of target cells compared to the SV-IM control group.

Microscopic lung lesion scores followed a similar trend as the magnitude of lung consolidation. The WIV-IM vaccine failed to protect pigs from infection or disease and resulted in lung lesions



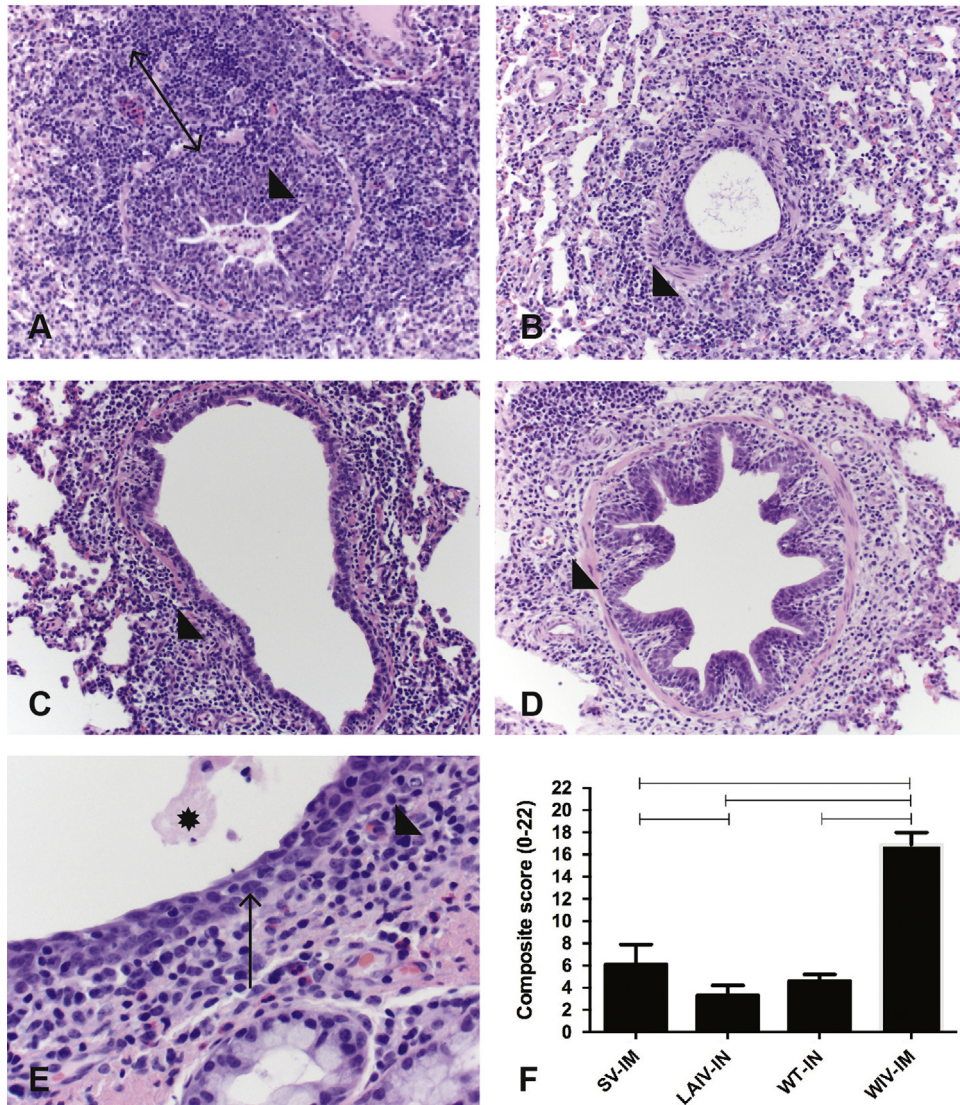
characterized as severe necrotizing bronchiolitis with marked peribronchiolar lymphocytic cuffing and interstitial pneumonia. Lesions were similar to what has been previously described for VAERD (Gauger et al., 2012) including a characteristic suppurative bronchiolitis and alveolitis with hemorrhage and edema and extensive mononuclear inflammation of the propria submucosa (Fig. 2A). Composite microscopic lung lesion scores were significantly higher in the WIV-IM pigs compared to all other immune groups (Fig. 2F). Significant differences were not recognized in microscopic lung lesion scores between the SV-IM (Fig. 2B) and WT-IN immune groups (Fig. 2C). However, the LAIV-IM (Fig. 2D) pigs demonstrated the least amount of microscopic lung inflammation compared to all immune/challenged groups and were significantly lower than the WIV-IM and SV-IM microscopic lung lesion scores. These data suggest the LAIV vaccine induced partial cross-protection that was associated with an absence of VAERD unlike the WIV vaccinated/challenged pigs.

In addition to pathologic changes in lungs, the trachea in VAERD-affected pigs (WIV-IM) was characterized by severe epithelial attenuation or necrosis (Fig. 2E) that occasionally resulted in a single layer of

epithelium. The submucosa was infiltrated with large numbers of lymphocytes, neutrophils and to a lesser extent with macrophages (Fig. 2E). Neutrophils had often transmigrated the tracheal epithelium or formed microabscesses that were rarely observed in LAIV-IN, WT-IN or non-vaccinated pigs. The WIV-IM trachea lesion composite scores were significantly higher compared to all other immune groups (data not shown). Trachea lesion scores were not statistically different between the SV-IM pigs and the LAIV-IN and WT-IN immune groups. However, a trend for lower trachea composite scores was observed in the pigs that were immunized with the intranasal wild type or attenuated viruses.

### Serology

Homologous HI titers detected the day of challenge (0 dpi) were significantly higher in the WIV vaccinated pigs compared to all other immune groups with a geometric mean reciprocal titer of 415 (Fig. 3A). The intranasally administered LAIV and WT MN08 virus induced lower geometric mean HI titers of 104 and 113, respectively, although all pigs in those immune groups had HI



**Fig. 2.** Microscopic lung lesions from representative pigs in H1N1pdm09 challenged immune groups. Pigs in the WIV-IM group demonstrating VAERD (A) had marked peribronchiolar cuffing (double arrow) and increased inflammation of the propria submucosa (arrowhead) with marked interstitial pneumonia. Pigs in the SV-IM (B), WT-IN (C) and LAIV-IN (D) groups demonstrated similar lung lesions consisting of mild peribronchiolar cuffing (arrowhead) and interstitial pneumonia. VAERD-affected pigs also demonstrate severe necrosis (arrow) and sloughing (asterisk) of the tracheal epithelium with marked submucosal inflammation (arrowhead) (E). (Lung, 200X; Trachea, 400X). WIV-IM mean microscopic pneumonia scores (F) were significantly higher compared to all other immune groups. Data are expressed as the mean composite score  $\pm$  standard error of the mean of each group. Connecting lines are significantly different at  $P < 0.05$ .

titers of 40 or higher. All immune groups had HI titers significantly higher compared to the SV-IM pigs. Cross-reactive heterologous HI titers to the H1N1pdm09 challenge virus were not detected at 0 dpi in any pigs regardless of HI titer to MN08.

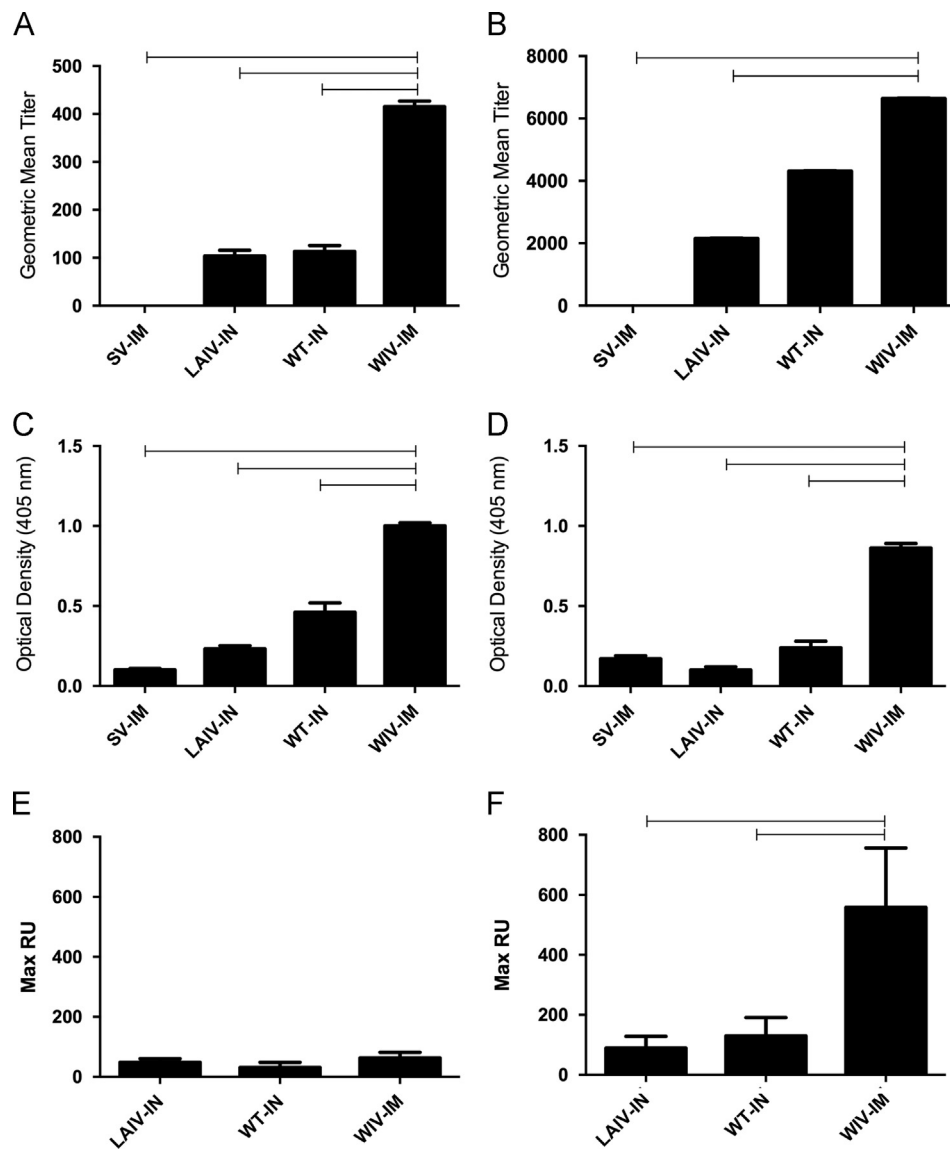
Homologous serum neutralizing (SN) antibody titers were detected at high levels in all MN08-immune pigs prior to challenge (Fig. 3B). Significant differences were not detected between WIV-IM and WT-IN immune group SN titers, and both were significantly higher than the LAIV-IN SN titer. Cross-reactive anti-H1N1pdm09 SN antibodies were not detected in any of the MN08 immune groups at 0 dpi. The SV-IM pigs were HI and SN antibody negative against both antigens prior to challenge.

To further explore the type of antibodies elicited by the different vaccine modalities we used whole-virus ELISA to measure binding of pre-challenge IgG antibodies in sera to the priming (MN08) and challenge (H1N1pdm09) virions, respectively (Fig. 3C and D). The highest homologous virus binding antibodies were detected in the WIV-IM group followed by the WT-IN and then the

LAIV-IN vaccinated pigs (Fig. 3C). Importantly, binding to the heterologous H1N1pdm09 challenge virus was prominent in the WIV-IM pigs, but minimal in the WT-IN or LAIV-IN immune groups that demonstrated binding levels similar to SV-IM (unvaccinated) control animals (Fig. 3D). This finding was very striking based on the low amino acid similarity of the HA and NA between MN08 and H1N1pdm09 (78% for HA and 43% for NA).

#### Serum antibody binding to HA1 and HA2 of H1N1pdm09

We further explored the specificity of the heterologous virus antibody binding using recombinant HA1 (aa 1–320) and HA2 proteins from the H1N1pdm09 that were expressed in a bacterial system and shown to be properly folded. These HA domains were coated onto SPR chips as previously described (Khurana et al., 2013). Sera from all immune groups displayed low and similar mean levels of anti-HA1 H1N1pdm09 antibodies ( $\text{max RU}$ ) (Fig. 3E). This low level of cross-reactive antibodies against the HA1 domain of H1N1pdm09 was



**Fig. 3.** Serum antibody responses in pigs vaccinated with LAIV, WT, WIV or SV prior to challenge (0 dpi). Hemagglutination inhibition (A) and serum neutralization (B) geometric mean reciprocal titers to homologous vaccine virus were significantly higher in the WIV-IM pigs compared to all immune groups. Cross-reactive HI or SN antibodies against H1N1pdm09 were not detected. Whole virus MN08 (C) and cross-reactive H1N1pdm09 (D) serum IgG levels detected in WIV-IM pigs were significantly higher than LAIV, WT or SV pigs. Anti-HA1 H1N1pdm09 serum antibody levels (E) were similar in LAIV, WT and WIV vaccinated pigs. Serum from WIV-IM vaccinates demonstrated preferential binding to the HA2 domain of H1N1pdm09 (F). Data are expressed as the geometric mean titer or mean optical density/max RU  $\pm$  the standard error of the mean of each group. Connecting lines are significantly different at  $P < 0.05$ .

consistent with the lack of detectable HI or SN serum antibodies against the challenge virus. However, WIV-IM vaccinated pig sera at 0 dpi demonstrated significantly higher cross-reactive binding against the H1N1pdm09 HA2 domain compared to sera from LAIV-IN or WT-IN vaccinated pigs (Fig. 3F). Elevated cross-reactive HA2-targeting antibodies were reported in our earlier paper describing the mechanism of VAERD (Khurana et al., 2013) and is consistent with the higher serum antibody binding observed in WIV-IM vaccinated animals to the H1N1pdm09 whole virus (Fig. 3F vs. 3D). The homology between the HA2 domains of MN08 and H1N1pdm09 is approximately 89% (significantly higher than for the entire HA), yet very minimal HA2-binding activity was found in the sera of animals primed with either WT-IN or LAIV-IN.

#### Serum H1N1pdm09 infectivity assay

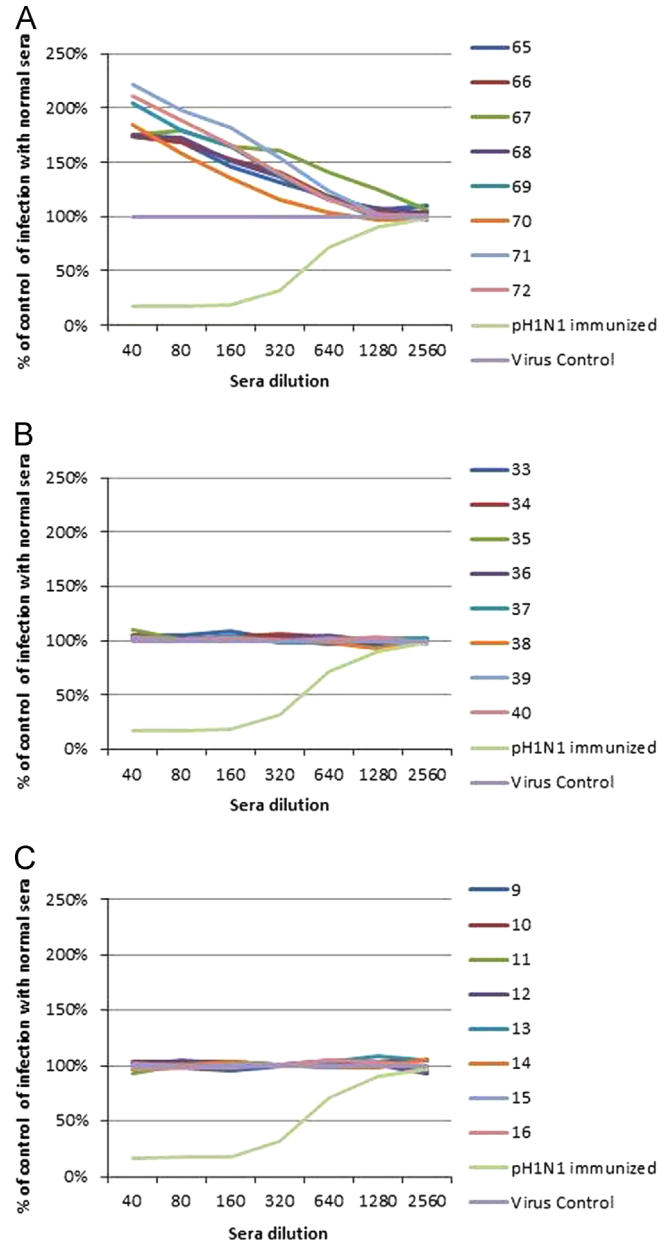
In order to link the observed H1N1pdm09 HA1/HA2 total binding with the function of the MN08 vaccine induced antibodies, we evaluated these sera in the MDCK-based microneutralization assay (based on the CDC recommended protocol) using H1N1pdm09 virus (Khurana et al., 2011a). Archived sera from H1N1pdm09 WIV-vaccinated animals demonstrated the expected efficient neutralization of H1N1pdm09 in the MDCK assay (Fig. 4, green curves). However, sera from the MN08 WIV-IM vaccinated animals from this study did not inhibit, but rather increased H1N1pdm09 infection in MDCK cells in a concentration-dependent manner (180–220% of virus-only control cultures at the lowest dilution) (Fig. 4A). Addition of pre-vaccination sera did not change the neutralization dilution curves for the H1N1pdm09 immune sera similar to what was described in the earlier study (Khurana et al., 2013). In contrast, sera from WT-IN (Fig. 4B) or LAIV-IN (Fig. 4C) vaccinated pigs did not enhance virus infection of MDCK cells compared to virus only control. Together these data demonstrated that vaccination with MN08 WIV-IM elicited high titer cross reactive antibodies preferentially targeting the HA2 stalk of the heterologous H1N1pdm09 challenge virus, resulting in fusion/infection enhancement in vitro and likely in vivo during early time points post-infection, in the absence of HA1-targeting antibodies (3 dpi, Fig. 1F). In contrast, the LAIV-IN vaccine modality did not induce infection-enhancing antibodies, had reduced virus titers in vivo at 3 dpi (and all time points), and did not lead to VAERD after heterologous challenge (Figs. 1–2).

#### Mucosal antibody responses

LAIV-IN and WT-IN pigs demonstrated VN antibodies against the homologous MN08 virus in the BALF prior to challenge (0 dpi), but not against the heterologous H1N1pdm09 challenge virus. MN08 or H1N1pdm09 VN antibodies were not detected in the BALF from WIV-IM pigs (data not shown). Anti-MN08 (Fig. 5A) and –H1N1pdm09 (Fig. 5B) IgG antibody responses in the lower respiratory tract were detected at 0 dpi in all MN08 immunized pigs regardless of the vaccine type or route of delivery. There were significant levels of anti-MN08 IgG in the BALF from LAIV and WT vaccinated pigs compared to the WIV-IM group; however, cross-reactive anti-H1N1pdm09 IgG levels were similar among all vaccine groups. Anti-MN08 (Fig. 5C) and –H1N1pdm09 (Fig. 5D) IgA antibodies in the BALF at 0 dpi were significantly higher in pigs that received intranasal immunization compared to the WIV-IM and SV-IM groups. Anti-MN08 and –H1N1pdm09 IgA antibody levels in WIV-IM vaccinates were detected at baseline levels similar to the non-vaccinated SV-IM group.

#### Cytokine concentrations in BALF

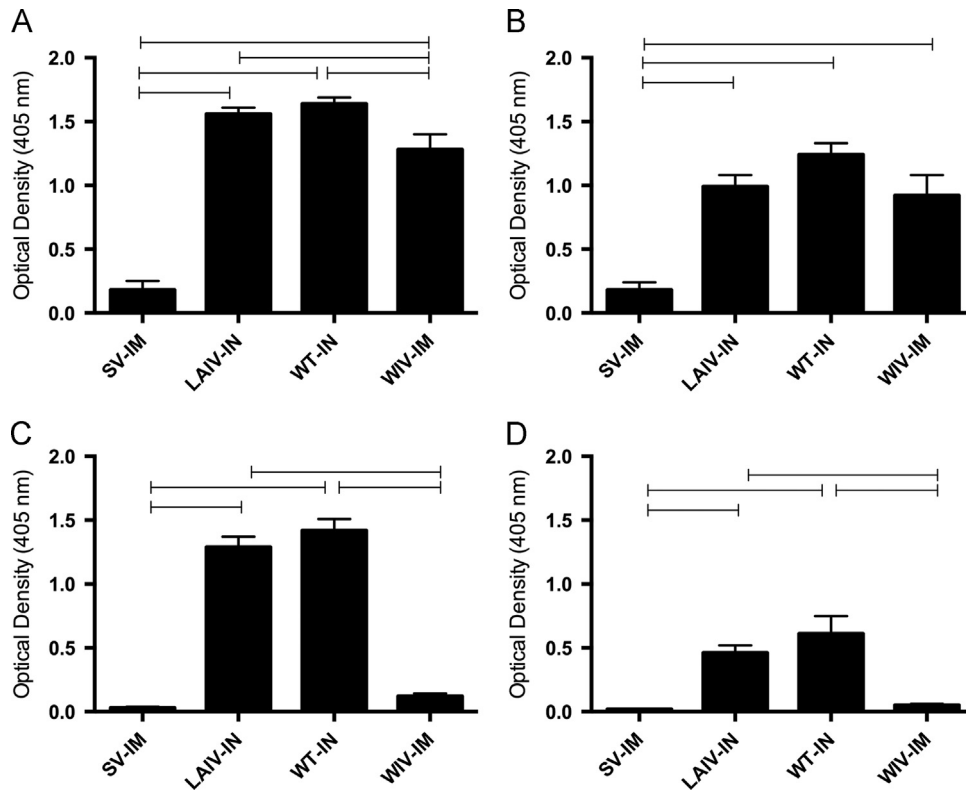
Three weeks after the booster dose of vaccines or WT exposure and just prior to challenge, pro-inflammatory cytokine levels in the



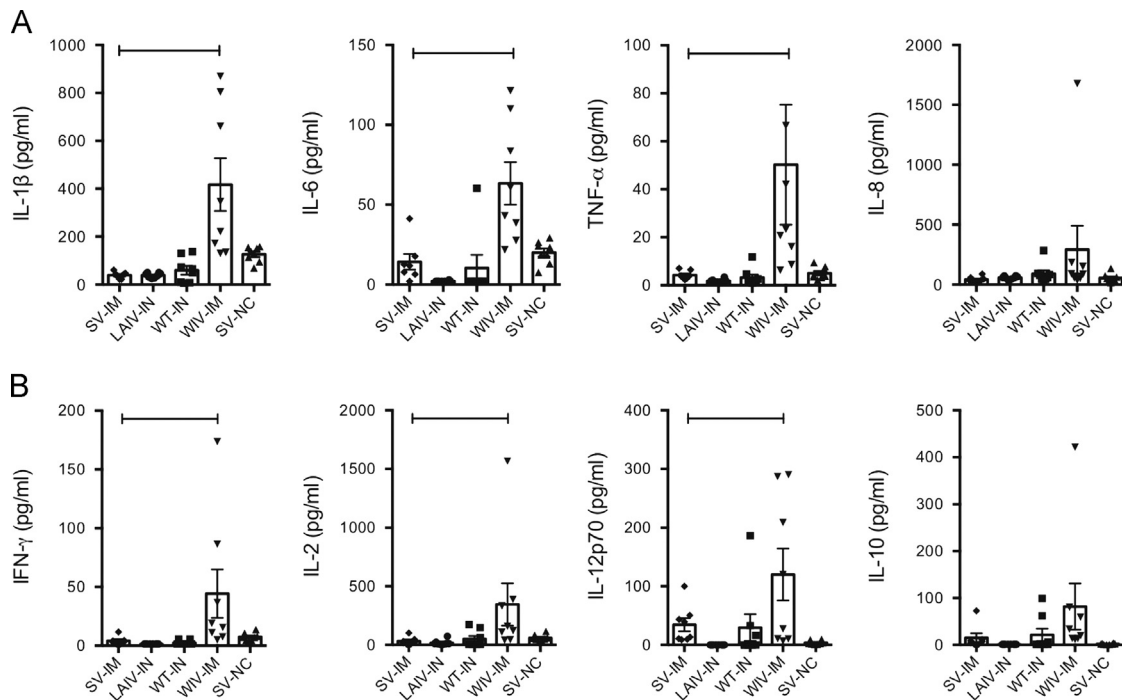
**Fig. 4.** Virus infection assay in Madin–Darby canine kidney cells detected by a nucleoprotein-based ELISA. WIV-IM vaccinated pig sera enhanced H1N1pdm09 infectivity in MDCK cells (A). Sera from WT-IN (B) and LAIV (C) vaccinated pigs did not demonstrate H1N1pdm09 virus infectivity that was different from virus control sera. Control sera from H1N1pdm09 immunized pigs demonstrate a dose depend neutralization of H1N1pdm09 virus. Data are expressed as a percent of control infection.

lung were at baseline levels in all vaccinated groups (data not shown). However, at 5 dpi, the WIV-IM vaccinated, VAERD-affected group demonstrated significantly elevated concentrations of IL-1 $\beta$ , TNF- $\alpha$  and IL-6 compared to pre-challenge baseline levels and compared to the SV-IM and intranasal vaccinated pigs as well as non-challenged pigs (SV-NC) (Fig. 6A). Levels of cytokines associated with adaptive immunity were evaluated as an indirect measure of T-cell activity. Concentrations of IFN- $\gamma$ , IL-2 and IL-12p70 were also higher in the WIV-IM group over all other immune groups and sham-vaccinated pigs (Fig. 6B). These increased levels of cytokines were concurrent with the influx of lymphocytes, macrophages, and neutrophils and the tissue damage in VAERD lungs, observed as early as 1 day (Gauger et al., 2012) and peaking at 5 days post H1N1pdm09 infection (Fig. 2).





**Fig. 5.** Antibody responses in bronchoalveolar lavage from pigs vaccinated with LAIV, WT, WIV or SV at 0 dpi. LAIV-IN and WT-IN homologous (A) IgG were significantly higher compared to WIV-IM vaccinated pigs. However, cross-reactive (B) IgG were detected at similar levels regardless of vaccine. Homologous (C) and cross-reactive (D) IgA antibodies were significantly higher in pigs that received intranasal vaccination compared to WIV-IM and SV vaccinates. Data are expressed as the mean optical density  $\pm$  standard error of the mean. Connecting lines are significantly different at  $P < 0.05$ .



**Fig. 6.** Lung pro-inflammatory cytokine concentrations from H1N1pdm09 challenged and non-challenged immune groups. Post-challenge TNF- $\alpha$ , IL-1 $\beta$  and IL-6 protein concentrations in bronchoalveolar lavage fluid were significantly higher in pigs administered the WIV-IM vaccine and demonstrating VAERD compared to other vaccinated/challenged groups (A). Significant differences between cytokine levels were not observed between SV-IM, WT-IN and LAIV-IN groups. Post-challenge IFN- $\gamma$ , IL-2 and IL-12p70 protein concentrations in bronchoalveolar lavage fluid were significantly higher in pigs administered the WIV-IM vaccine and demonstrating VAERD compared to other vaccinated/challenged groups (B). Significant differences between cytokine levels were not observed between SV-IM, WT-IN and LAIV-IN groups. A vaccinated/non-challenged (SV-NC) group euthanized at 21 dpi was included in the cytokine evaluation as a non-infected control. Data presented as box and dot plots with the mean cytokine concentration (pg/ml)  $\pm$  standard error of the mean. Significantly different cytokine concentrations between the WIV-IM and SV-IM group are identified by connecting lines at  $P < 0.05$  with some data not shown. SV-IM, sham vaccinated/intramuscular vaccine; LAIV-IN, live attenuated influenza virus/intranasal vaccine; WT-IN, wild type virus/intranasal exposure; WIV-IM, whole inactivated virus/intramuscular vaccine; SV/NC, non-vaccinated/non-challenged.

## Discussion

WIV influenza vaccines are used frequently in swine in the US and rely on the induction of a systemic immune response to protect the respiratory tract against influenza infection (Ma and Richt, 2010; Vincent et al., 2008b). WIV preparations described in previous reports have demonstrated adequate protection against homologous challenge or heterologous viruses with cross-reactive HI activity. However, efficacy against heterologous IAV is often deficient (Bikour et al., 1996; Lee et al., 2007; Van Reeth et al., 2004; Vincent et al., 2008a), similar to the situation with human seasonal vaccines. In addition, enhanced respiratory disease has been observed in several cases where heterologous infection occurred after vaccination with mismatched WIV vaccines (Gauger et al., 2011, 2012). Our recent publication suggested that the balance between protective antibodies primarily targeting the globular head domain and stalk-targeting antibodies against the conserved HA2 may play a role in infection outcome (Khurana et al., 2013). Among the cross-reactive HA2 antibodies, there may be some protective or possibly broadly neutralizing antibodies as recently described (Gocnik et al., 2008; Hashem et al., 2010). However, in the VAERD pig model, we have identified polyclonal cross-reactive HA2-targeting antibodies that may attach to virions and enter the endocytic compartment where they promote rather than block the fusion process (Gauger et al., 2011; Khurana et al., 2013).

It was not clear if these enhancing antibodies were more likely to be generated by WIV vaccines or irrespective of the vaccine platform. Furthermore, the increasing antigenic diversity of contemporary IAV circulating in North American swine increases the need for improved vaccines and methods of delivery that could provide sufficient cross-protection against heterologous infections. Improved vaccines could decrease viral transmission among pigs reducing the likelihood of reassortment that often results in newly emerging strains that may also jump to humans (Garten et al., 2009; Nelson et al., 2012b; Shu et al., 2012). Live attenuated influenza vaccines with genetic modifications through targeted mutations have demonstrated more consistent cross-protection and reduction in shedding compared to WIV products (Pena et al., 2011; Vincent et al., 2007).

The current study demonstrated that swine administered a temperature sensitive LAIV vaccine exhibited mitigated clinical signs, fewer macroscopic and microscopic lung lesions compared to the WIV-IM and unvaccinated (SV) animals and protection against infection and replication in the upper respiratory tract following challenge with a heterologous IAV (Figs. 1 and 2). Although the average lung consolidation described in the positive control SV-IM H1N1pdm09-challenged pigs was lower than what was previously reported (Vincent et al., 2010b), there was a trend for reduced macroscopic pneumonia in the LAIV-IN and WT-IN pigs. The lower challenge dose of virus in this study likely contributed to the reduced or delayed magnitude of lung lesions observed in pigs infected with H1N1pdm09. Most importantly, enhanced respiratory disease was only observed in WIV-IM vaccinated animals but not in pigs that received the intranasal LAIV vaccine, suggesting a decreased risk of VAERD after mismatched vaccination. The magnitude of protection described in the LAIV group was similar to pigs administered the wild type MN08 (WT-IN), indicating the attenuated virus evaluated here paralleled the cross-protection by natural infection in this study and as previously described (Van Reeth et al., 2003). Since no cross-reactive H1N1pdm09 serum neutralizing antibodies were measured in any vaccine group, other mechanisms of protection could be postulated, including cell mediated immunity against conserved internal genes.

Whole virus, cross-reactive IgG antibodies were only detected in sera from WIV-IM vaccinated animals. Further analysis revealed

these WIV-IM serum antibodies preferentially bound to the conserved HA2 domain of H1N1pdm09 and increased *in vitro* infectivity of MDCK cells as previously reported (Khurana et al., 2013). In both studies, the HA2 targeting antibodies and antibody-mediated enhanced MDCK cell-infectivity correlated with increased lung pathology. In addition, the enhanced MDCK cell-infectivity demonstrated in the WIV-IM pig sera (Fig. 4A) is consistent with the elevated virus titers detected at 3 dpi in nasal swabs in the VAERD-affected pigs. In contrast, cross-reactive anti-HA1 or -HA2 antibodies were detected at minimal levels in serum from LAIV-IN or WT-IN, with no evidence of preferential binding to HA2. Therefore, when challenged with mismatched virus, pigs lacking protective antibodies in the presence of high titer anti-HA2 antibodies that target a critical site on the HA2 stem (close to the fusion peptide) may have an increased risk of VAERD. Future studies should evaluate potential differences in the type of antibodies elicited by inactivated vaccines with different adjuvants and replicating virus vectors.

Intranasal LAIV vaccines have the advantage over inactivated products of inducing local IgA that has been correlated with cross-protection against heterologous challenge (Loving et al., 2013; Vincent et al., 2007, 2012). The temperature sensitive LAIV vaccine used in this study demonstrated a cross-reactive H1N1pdm09 specific IgG antibody response to whole virus in the BALF at 0 dpi that was similar to levels demonstrated by the WIV pigs (Fig. 5B). However, significantly higher levels of homologous and cross-reactive (anti-H1N1pdm09), whole virus IgA were detected in the lower respiratory tract of LAIV-IN and WT-IN vaccinates compared to the WIV-IM, VAERD-affected group prior to challenge (Fig. 5C and D). The mild post-challenge clinical signs and lung lesions demonstrated by the LAIV immune group in the absence of anti-H1N1pdm09 serum neutralizing antibodies suggests a role for cross-reactive mucosal IgA in protection from infection and clinical disease. Although 0 dpi BALF neutralizing antibodies were not detected against heterologous virus in the VN assay, the cross-reactive IgA detected in BALF from the LAIV-IN group still may have been associated with the absence of VAERD and reduced infection. This effect could be through differential processing of antigen bound by IgA versus IgG or through interference with virus infection since polymeric IgA is more cross-reactive than IgG (Asahi et al., 2002; Tamura et al., 1991). On the other hand, the level of BALF IgG antibodies was not significantly different between the three vaccine groups. Therefore, any protective role associated with IgA antibodies may be offset by IgG antibodies with no or opposing biological activities. Due to limited volumes, it was not possible to determine the antigen's epitopes targeted by the BALF antibodies. Regardless of the underlying mechanism (antibody or CMI), mucosal vaccination and local IgA may be crucial to the reduction in viral replication and nasal shedding in the upper and lower respiratory tract.

Elevated pulmonary pro-inflammatory cytokines are a consistent feature of the VAERD phenomenon in swine and may contribute to excessive inflammation or lung damage (Gauger et al., 2011; Gauger et al., 2012; Van Reeth et al., 2002). Post-challenge cytokine concentrations in the BALF were highly variable in all experimental groups in the present study. Still, significantly higher group mean cytokine levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were only observed in the WIV-IM, VAERD-affected pigs compared to the sham-vaccinated and LAIV (or WT) vaccinated groups (Fig. 6) that also demonstrated minimal lung lesions (Figs. 1E & 2F), further suggesting an association of aberrant cytokine responses with the incidence of VAERD. The proinflammatory cytokines measured in the BALF of animals with VAERD at day 5 post-challenge may have been produced by an influx of inflammatory "activated" macrophages, dendritic cells, and neutrophils responding to the initial tissue damage by enhanced fusion/virus replication in the epithelial cells. This may have ultimately

limited the level of viral replication observed at 5 dpi in lung and the upper respiratory tract. MDCK cells are epithelial cells and do not express Fc receptors. Therefore, Fc-mediated antibody enhancement is not implicated by increased virus infectivity *in vitro*, whereas increased virus fusion in the presence of antibody from WIV vaccinated pigs in epithelial cells is a potential mechanism as previously demonstrated (Khurana et al., 2013). However, a possible role for Fc receptor mediated uptake of antibody bound virus and VAERD-associated aberrant lung cytokine responses *in vivo* requires further investigation.

The results of this study demonstrate critical advantages to the use of LAIV vaccines in swine populations where a diverse IAV ecology has been established. Intranasal LAIV vaccines have the ability to induce a broad, cross-protective immune response similar to natural infection that may include priming of cell-mediated immunity and protective local immunity without the risk of VAERD. A source of resistance to the use of LAIV in swine is a potential reversion to virulence and reassortment between wild-type and vaccine strains. Reversion to virulence has never been confirmed in human LAIV products and the temperature sensitive platform used in this report has multiple attenuating mutations, reducing the opportunity for reversion. Additionally, the LAIV used here is from the North American swine-lineage, contributing no extraneous genetic material if reassortment occurred. LAIV vaccines have consistently been shown to significantly reduce viral replication and shedding which, if performance were mimicked in the field, LAIV would greatly decrease virus transmission, reduce the emergence of divergent IAV, and potentially reduce zoonotic events such as those that recently occurred in the human population (Bowman et al., 2012; Killian et al., 2013; Wong et al., 2012). These benefits outweigh concerns delaying the progress of licensing LAIV for use in the swine population.

## Conclusions

Inactivated influenza vaccines are commonly used in United States swine increasing the opportunity of mismatched vaccination against antigenically diverse viruses circulating in swine. This study demonstrates that vaccination with WIV, but not LAIV or WT, elicited cross-reactive HA-2 targeting antibodies that in the absence of anti-HA1 neutralizing antibodies may have been associated with enhanced respiratory disease after challenge with the mismatched H1N1 virus in the pig model. These data suggest live attenuated vaccines administered in the upper respiratory tract may induce broad cross-protection, reduce the risk of enhanced disease, and could serve as an alternative to whole inactivated virus vaccines in swine.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.virol.2014.10.003>.

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