Divergent Immune Responses and Disease Outcomes in Piglets Immunized with Inactivated and Attenuated H3N2 Swine Influenza Vaccines in the Presence of Maternally-Derived Antibodies

Matthew Robert Sandbulte
Iowa State University

Ratree Platt
Iowa State University, rplatt@iastate.edu

James A. Roth
Iowa State University, jaroth@iastate.edu

Jamie N. Henningson
University of Kansas Main Campus

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Abstract
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Keywords
Influenza A, Maternally derived antibodies, Live attenuated influenza virus, Whole inactivated virus, Heterologous immunity

Disciplines
Large or Food Animal and Equine Medicine | Veterinary Microbiology and Immunobiology | Veterinary Preventive Medicine, Epidemiology, and Public Health

Comments
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Authors
Matthew Robert Sandbulte, Ratree Platt, James A. Roth, Jamie N. Henningson, Kathleen A. Gibson, Daniela S. Rajão, Crystal Lynn Loving, and Amy L. Vincent

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Divergent immune responses and disease outcomes in piglets immunized with inactivated and attenuated H3N2 swine influenza vaccines in the presence of maternally-derived antibodies

Matthew R. Sandbultea, Ratree Platta, James A. Rotha, Jamie N. Henningsonb, Kathleen A. Gibsont, Daniela S. Rajãoc, Crystal L. Lovingc, Amy L. Vincentc,*

a Department of Veterinary Microbiology & Preventive Medicine, Iowa State University, Ames, IA, USA
b Department of Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS, USA
c Virus and Prion Research Unit, National Animal Disease Center, USDA-ARS, 1920 Dayton Avenue, Ames, IA 50010, USA

corresponding author. Tel.: +1 515 337 7557.
E-mail address: amy.vincent@ars.usda.gov (A.L. Vincent).

Abstract

Live-attenuated influenza virus (LAIV) prime-boost vaccination previously conferred protection against heterologous H3N2 swine influenza challenge, including in piglets with maternally derived antibodies (MDA). Conversely, a whole-inactivated virus (WIV) vaccine was associated with enhanced disease. This study was aimed at identifying immune correlates of cross-protection. Piglets with and without MDA received intramuscular adjuvanted WIV or intranasal LAIV, and were challenged with heterologous H3N2. WIV induced cross-reactive IgG, inhibited by MDA, and a moderate T cell response. LAIV elicited mucosal antibodies and T cells cross-reactive to the heterologous challenge strain. The presence of MDA at LAIV vaccination blocked lung and nasal antibody production, but did not interfere with T cell priming. Even without mucosal antibodies, MDA-positive LAIV vaccinates were protected, indicating a likely role for T cells. Based on the data, one LAIV dose can induce cell-mediated immunity against antigenically divergent H3N2 influenza virus despite passive antibody interference with humoral immune responses.

Introduction

Swine influenza A virus (IAV) vaccines are commonly administered in commercial herds, but multiple factors hinder their success in controlling influenza outbreaks. Vaccines containing whole-inactivated virus (WIV) antigens elicit neutralizing antibodies to antigenically similar strains and confer protection against homologous challenge (Bikour et al., 1996; Van Reeth et al., 2001). However, IAV strains with even a few mutations in the viral surface proteins can evade neutralization (Jin et al., 2005). Antigenic evolution frequently leads to evasion of pigs’ WIV-induced immunity (Lee et al., 2007; Van Reeth et al., 2001; Vincent et al., 2010). Furthermore, both HA subtypes of swine-lineage IAV (H1 and H3) include multiple co-circulating phylogenetic clusters, which are antigenically distinct (Lorusso et al., 2010; Loving et al., 2013; Nelson et al., 2012). Therefore, it is difficult to maintain well-matched IAV vaccines for swine (Ma and Richt, 2010). Immunizing sows to stimulate colostrum-mediated passive transfer of antibodies to their piglets is a common practice in the swine industry. Passively acquired antibodies reduce infection and disease with similar strains until antibody titers wane below a protective threshold (Loeffen et al., 2003). However, maternally-derived antibodies (MDA) can interfere with immune responses to vaccines (Kitikoon et al., 2006).

Live-attenuated influenza virus (LAIV) vaccines delivered intranasally have shown promise as a means to induce T cells and mucosal immunity that cross-react with antigenically drifted and heterosubtypic strains, both in pigs and humans (Hoft et al., 2011; Masic et al., 2010). We previously reported protective immunity against heterologous H3N2 and heterosubtypic H1N1 infections in young pigs immunized with an experimental LAIV engineered to express a truncated NS1 protein (Kappes et al., 2012; Vincent et al., 2007). This LAIV vaccine, administered intranasally in two doses, provided cross-protection even when administered to piglets with high titers of homologous MDA (Vincent et al., 2012). This was in marked contrast to a WIV vaccine that failed, and in fact exacerbated heterologous infection severity, when given to piglets with maternal immunity. H3N2 LAIV induced humoral immunity such that bronchoalveolar lavage (BAL) specimens contained significant levels of IgA and IgG with reactivity to the challenge strain, although not in MDA-positive piglets (Vincent et al., 2012). Determining correlates of cross-protective immunity could facilitate improved regulatory evaluation of commercial LAIV vaccines since traditional serological parameters are not induced reliably by...
LAIV in swine or humans (Belshé et al., 2000; Loving et al., 2013). No LAIV vaccines for swine influenza are currently licensed. General requirements for approval of live vaccines, by regulatory agencies such as the USDA Center for Veterinary Biologics, include demonstrating efficacy, safety in target and non-target host species, stability of the attenuated phenotype, and an environmental risk assessment (Rippke et al., 2012).

In the previous study that compared H3N2 WIV and LAIV vaccines, MDA(+) piglets vaccinated with the adjuvanted WIV and challenged with an antigenically divergent H3N2 IAV strain developed more severe respiratory tract lesions than non-vaccinated controls (Vincent et al., 2012). The adverse outcome in WIV vaccinated was similar to the virus-associated enhanced respiratory disease (VAERD) phenomenon described in pigs that were vaccinated with an H1N2 WIV and challenged with heterologous H1N1 (Gauger et al., 2012, 2011), except that MDA were not involved in the H1-subtype VAERD pathogenesis. Both the H3 and H1 models of VAERD are characterized by increased percentages of macroscopic pneumonia with severe bronchiointerstitial pneumonia, necrotizing bronchiolitis, and interlobular and alveolar edema and hemorrhage (Gauger et al., 2012; Vincent et al., 2012). IAV-specific IgG antibodies that fail to neutralize the challenge virus are likely involved in VAERD pathogenesis (Khurana et al., 2013). High levels of virus-binding IgG were detected in lungs of pigs affected by H3N2 VAERD, 5 days post-infection. The mechanism by which non-neutralizing antibodies might promote inflammation and the possible involvement of vaccine-primed B and T cells has not been resolved.

Objectives of this study included testing the efficacy of a single dose LAIV regimen in piglets with and without MDA; determining the effects of MDA on cellular and mucosal immune responses; and identifying immune responses correlated with VAERD versus cross-protection upon heterologous challenge.

## Results

### Serological response to vaccines

Immunization of sows with multiple doses of TX98 WIV vaccine elicited homologous serum HI antibody titers of 160 or greater. Non-vaccinated sows had HI titers < 20, and were also seronegative by nucleoprotein-specific commercial ELISA (Ciacci-Zanella et al., 2010). Neonatal piglets from the vaccinated sows acquired passive HI antibody at similar high titers, which waned steadily (Fig. 1A). There was very limited HI cross-reactivity to the challenge strain, CO99 (Fig. 1B), consistent with previous studies (Richt et al., 2003; Vincent et al., 2012). Piglets were vaccinated with WIV or LAIV at weaning. Serum HI antibody responses to both vaccines were low or below detection limits prior to challenge (Fig. 1A). The WIV vaccine elicited IgG antibodies detectable by ELISA, including moderate cross-reactivity with the heterologous challenge strain (Fig. 1C and D).

### Mucosal antibody response to vaccines

Subgroups of pigs were sacrificed at 0 dpi to analyze respiratory tract local immunity prior to infection. There was no evidence of maternally derived IAV-specific IgA in BAL samples of non-vaccinated (NV) piglets 7–8 weeks of age (Fig. 2A and B). In MDA(−) piglets, a single LAIV dose elicited a strong IgA response, and these antibodies cross-reacted with CO99. In contrast, no BAL IgA response was detected in animals given two doses of WIV. In MDA(+) piglets, the BAL IgA response to LAIV was inhibited. With regard to IAV-specific IgG, passive maternal antibodies remained in lungs of NV pigs at moderate levels, 7–8 weeks after piglets had ingested colostrum (Fig. 2C and D). LAIV induced the highest BAL

![Fig. 1](image-url)  
**Fig. 1.** Serum antibody levels due to maternally derived antibodies and responses to vaccination. Reciprocal geometric mean HI titers against TX98 H3N2 antigen (A) and CO99 antigen (B) are shown for time points prior to and after challenge. MDA(+) piglets were from sows immunized with the TX98 WIV vaccine. Treatment groups were non-vaccinated (NV), vaccinated 1X at 0 dpv and 14 dpv with TX98 WIV, vaccinated 1X with TX98 LAIV at 0 dpv only (LAIVx1), or vaccinated 1X with LAIV at 0 dpv and 14 dpv (LAIVx2). IgG antibodies binding to TX98 (C) and CO99 (D) whole viral antigens were measured by ELISA in 34 dpv sera. Open bars designate groups without MDA, and solid bars designate groups with MDA. Statistically significant differences between MDA statuses within a vaccine group are marked with asterisks, and differences between vaccine treatment groups with matched MDA statuses are identified by connecting lines (P < 0.05).
IAV-specific IgG response in MDA(−) groups but, like the IgA isotype, this was inhibited by MDA. WIV immunization induced moderate IAV-specific BAL IgG; this was also reduced in MDA(+) piglets, although the difference was not statistically significant from MDA(−) pigs.

IAV-specific IgA levels were also measured in nasal wash (NW) specimens collected at −1 dpi from the same piglets. Although the magnitude of IgA signal was lower in NW, the pattern of results approximately mirrored results from 0 dpi BAL samples. Among MDA-negative piglets, LAIV vaccines had significantly more TX98-binding IgA than WIV vaccines (Fig. 2E and F). The presence of MDA at vaccination blocked NW IgA responses to LAIV. Overall, results from pre-challenge samples indicate that LAIV vaccination induced a robust local antibody response in the respiratory tract, while WIV vaccinees developed moderate quantities of lung IgG only, but the presence of MDA at vaccination suppressed humoral responses to both vaccines.

Cellular immune response to vaccines

T cell priming was analyzed by testing ex vivo recall responses to homologous and heterologous H3N2 strains. Based on expression of CD25, IFN-γ, and IL-10, peripheral blood CD4+CD8+ T cells were primed by WIV and LAIV vaccines (Fig. 3). In terms of surface CD25 expression after homologous TX98 stimulation, the CD4+CD8+ cells showed extensive group-to-group variation (Fig. 3A). The two treatment groups for which the CD25 marker was significantly upregulated compared to NV controls were MDA(−)
WIV and MDA(+) LAIVx2. Although CD25 recall responses varied in the MDA(+) LAIVx2 group, the mean was significantly higher than in either the MDA(-) group receiving a single dose or the MDA(-) group with two doses. In contrast, WIV vaccination only primed CD4+CD8+ cells for significant CD25 responses if administered to MDA(-) pigs. Similar patterns were present in CD25 data from cells stimulated with heterologous CO99 virus, including significant recall responses in MDA(+) LAIVx2 piglets (Fig. 3B).

Like the CD25 marker, statistically significant IFN-γ recall responses were detectable in CD4+CD8+ cells from MDA(+) LAIVx2 vaccinees, after TX98 or CO99 stimulation (Fig. 3C and D). There was evidence of IFN-γ recall responses in WIV-vaccinated groups, although not statistically different from non-vaccinates.

The third recall marker, IL-10, indicated statistically significant responses for the MDA(-) WIV and MDA(+) LAIVx2 groups, to both strains (Fig. 3E and F), which closely mirrored the CD25 responses. The same recall response markers were simultaneously analyzed in CD4+CD8-, CD4-CD8+, and γδ TCR+ peripheral blood lymphocyte populations. In these populations, none of the vaccine treatment groups showed statistically significant responses to the homologous TX98 strain (Supplemental Fig. S1) or heterologous CO99 (data not shown).

ELISPot results from virus-stimulated PBMC (Fig. 4A and B) were consistent with the flow cytometry-based intracellular IFN-γ data described above. Notably, IAV-specific IFN-γ SCs were detected with the highest frequencies in the MDA(+) LAIVx2
were similar until 5 dpi, when mean temperatures of the MDA(−) groups had mild clinical disease, while in the LAIV-vaccinated groups most severe in WIV-vaccinated groups (Fig. 5C). The NV groups had no clinical signs. Mean rectal temperatures of the MDA(−) and MDA(+) WIV groups surpassed 40.5 °C, whereas equivalent MDA(−) groups approached the normal baseline near 39.5 °C. WIV vaccination exacerbated the clinical severity of heterologous infection, while LAIV was cross-protective.

VWV-vaccinated piglets had higher macroscopic lung lesion scores than NV counterparts of matching MDA status (Fig. 5E). The mean percentage of lung area affected with pneumonia was 26.6% in the MDA(−) WIV group and 17.1% in the MDA(+) WIV group as compared to the MDA(−) NV control group at 10.5%. Piglets in LAIV-vaccinated groups showed no evidence of macroscopic pneumonia, regardless of MDA status or the number of LAIV doses received. Microscopic lesions of lung tissues were most pronounced in the WIV-vaccinated groups, whereas LAIV-vaccinated groups had the lowest scores (Fig. 5F and G). Tracheal microscopic lesion scores were significantly greater in WIV-vaccinated groups than LAIV groups, with trends similar to microscopic lung injury. Overall, analysis of respiratory tract pathological lesions shows a similar pattern to clinical disease scores, pointing to exacerbation by WIV, cross-protection by LAIV, and no major impact by the presence of MDA at vaccination.

Fig. 4. Vaccine priming of IAV-specific T cells in lung-draining lymph nodes. TBLN cells were harvested from non-infected pigs that were sacrificed 35 dpi, and tested by IFN-γ ELISpot for responses to TX98 and CO99. The mean number of spot-forming cells in duplicate wells is plotted for each pig, and the treatment group means and standard errors of the means are denoted with bars. Statistically significant differences between vaccine treatment groups with matched MDA status are identified by connecting lines (P < 0.05).

Vaccine efficacy against heterologous challenge

Virus shedding from the nasal cavity was limited during the challenge experiment, as virus was detected in 3 of 8 MDA(−) NV pigs, 8 of 8 MDA(+) NV pigs, 4 of 8 MDA(−) WIV pigs, 7 of 8 MDA(+) WIV pigs, and 0 of 8 MDA(+) LAIVx1 pigs, 5 of 8 MDA(+) LAIVx1 pigs, 0 of 8 MDA(−) LAIVx2 pigs, and 2 of 8 MDA(+) LAIVx2 pigs (data not shown). All positive nasal swabs had titers below 103 TCID50/ml. In lungs, the challenge virus replicated more extensively, based on 5 dpi BAL infectivity titers (Fig. 5A) and viral antigen detection in lung tissue (Fig. 5B). None of the LAIV-vaccinated pigs had detectable viable virus in BAL or IAV antigen in lung tissue. Among MDA(−) WIV-vaccinated pigs there was very little infectious virus in 5 dpi BAL, yet the amount of viral antigen detected in the lung tissues by IHC was as high as in NV animals. WIV vaccination had little effect in reducing lung viral load when administered to MDA(+) animals (Fig. 5A).

Composite clinical scores peaked about 3–4 dpi, with disease most severe in WIV-vaccinated groups (Fig. 5C). The NV groups both had mild clinical disease, while in the LAIV-vaccinated groups almost no clinical signs were observed. Mean rectal temperatures were similar until 5 dpi, when mean temperatures of the MDA(+) NV and MDA(+) WIV groups surpassed 40.5 °C, whereas equivalent MDA(−) groups approached the normal baseline near 39.5 °C. WIV vaccination exacerbated the clinical severity of heterologous infection, while LAIV was cross-protective.

Post-challenge lung immune responses

At necropsy (5 dpi), post-challenge BAL TX98- and CO99-binding IgG were present at higher levels (Fig. 6) compared to the pre-challenge levels (Fig. 2), indicating a local response induced by infection. Similarly, IAV-specific IgA reached higher levels in 5 dpi BAL than in equivalent 0 dpi samples. MDA(−) LAIV vaccinees continued to have the highest levels of IAV-specific IgA at 5 dpi. In all cases, the presence of MDA at the time of vaccination diminished the local antibody response to challenge.
H3N2 strains of several antigenically distinct lineages circulate in swine (Vincent et al., 2014). The diversity and rapid evolution of these viruses impairs efficacy of commercial vaccines containing WIV antigens (Ma and Richt, 2010). Sows are commonly immunized with WIV vaccines to enable colostrum-mediated protection of young piglets, although MDA can interfere with immunization of the young pigs (Kitikoon et al., 2006; Loeffen et al., 2003). Our previous study tested immune responses and cross-protective efficacy of H3N2 WIV versus LAIV (Vincent et al., 2012). Both vaccines conferred protection against homologous challenge, but in MDA(+) animals the outcomes of heterologous H3N2 challenge were markedly different. LAIV provided moderate cross-protection, even in MDA(+) pigs. WIV vaccination in MDA(+) animals failed to limit viral replication and it led to more severe respiratory tract lesions, i.e. VAERD. The adverse outcome was associated with the presence of virus-binding IgG that lacked HI cross-reactivity, which was also observed in an H1N1 model of VAERD (Gauger et al., 2011). The present study was aimed at identifying immune parameters associated with cross-protection versus disease enhancement following heterologous challenge.

LAIV vaccination conferred significant cross-protection against the heterologous CO99 virus challenge. Regardless of the dose

Fig. 5. Viral replication, disease, and respiratory tract tissue damage after heterologous H3N2 challenge. Groups of pigs, with and without MDA, received no vaccine (NV), two I.M. doses of TX98 WIV, one I.N. dose of TX98 LAIV, or two I.N. doses of LAIV. 35 days after the initial vaccine dose, piglets were challenged by I.T. inoculation with CO99. (A) BAL specimens were collected at necropsy (5 dpi) and titrated by TCID50 assay on MDCK cells. (B) Lung tissue sections were stained for IAV antigen by IHC. Clinical disease scores (C) and rectal temperatures (D) were monitored daily. Lungs were evaluated for macroscopic pneumonia (E) and microscopic pneumonia (F). Tracheal tissue was evaluated for microscopic lesions (G). Open bars and symbols represent MDA(-) groups, and filled bars and symbols represent MDA(+) groups. Error bars indicate standard errors of the means.

Discussion

H3N2 strains of several antigenically distinct lineages circulate in swine (Vincent et al., 2014). The diversity and rapid evolution of these viruses impairs efficacy of commercial vaccines containing WIV antigens (Ma and Richt, 2010). Sows are commonly immunized with WIV vaccines to enable colostrum-mediated protection of young piglets, although MDA can interfere with immunization of the young pigs (Kitikoon et al., 2006; Loeffen et al., 2003). Our previous study tested immune responses and cross-protective efficacy of H3N2 WIV versus LAIV (Vincent et al., 2012). Both vaccines conferred protection against homologous challenge, but
again emphasize the marked difference in efficacy between LAIV and WIV vaccines and dispel virus after its placement in the trachea. These results extended to non-anesthetized pigs, potentially allowing pigs to cough associated with a lower rate of culture-con

LAIV vaccines for swine IAV have been shown, in seronegative pigs, to elicit lung mucosal antibodies (Masic et al., 2010; Vincent et al., 2012). In the prior study, VAERD occurred only in pigs that had received WIV vaccination in the face of MDA. In the current study, we observed no increased propensity for VAERD in the MDA(+) pigs. This variation might be explained by considering the levels of functional cross-reactive antibodies to the CO99 challenge strain remaining at challenge. In the previous study, WIV vaccination induced robust HI titers in MDA(−) pigs with low cross-reactive HI activity against CO99 challenge virus, likely reducing disease severity. In the current study, MDA(−) WIV-vaccinated pigs had lower homologous HI titers and developed no detectable serum cross-reactive HI activity against CO99, likely leaving them susceptible to VAERD pathogenesis. Another difference between the two studies was in the delivery and dose of challenge virus. The CO99 inoculum titer was 20-fold less in the current study (10^{1.7} TCID_{50}/ml compared with 10^{5} TCID_{50}/ml) and was administered to non-anesthetized pigs, potentially allowing pigs to cough and dispel virus after its placement in the trachea. These results again emphasize the marked difference in efficacy between LAIV and WIV vaccines and the complexity of vaccination of pigs with variable immune statuses.

LAIV vaccines for swine IAV have been shown, in seronegative pigs, to elicit lung mucosal antibodies (Masic et al., 2010; Vincent et al., 2012). Similarly, naïve infants vaccinated with trivalent LAIV for seasonal influenza developed increased IgA levels, which were associated with a lower rate of culture-confirmed influenza illness (Ambrose et al., 2012). In the present study, vaccine-induced mucosal immunity was analyzed by measuring virus-binding IgA and IgG in upper and lower respiratory tract samples collected before challenge. Most LAIV vaccinated pigs developed mucosal IgA in both locations; however, IAV-specific IgG was only detected in the lower respiratory tract. In comparison, the WIV vaccine elicited moderate lung IgG responses before challenge but no mucosal IgA. Overall, local IgA and IgG cross-reacted with CO99 challenge virus. These findings agree with the concept that intranasally administered LAIV vaccines elicit superior mucosal antibody responses (Cheng et al., 2013). Cross-reactive mucosal antibodies in MDA(−) LAIV vaccinated pigs may have been sufficient to block infection without requiring full recruitment and activation of memory T cells. We hypothesized that intranasal LAIV would induce a mucosal antibody response even in pigs with circulating MDA. In fact, MDA(+) pigs had almost no local IgA and IgG responses to either vaccine. This suggests it could be difficult to use nasal IgA as a correlate of LAIV vaccine-induced immunity in field studies where many young pigs would have MDA from their vaccinated or exposed mothers.

Lower respiratory tract samples from infected pigs (5 dpi) were also assayed for IAV-specific antibodies, which transudated into the lungs or were produced by local B cells during infection. Vaccinated groups, particularly LAIV vaccinates, had increased BAL IgA and IgG levels after infection. Groups that were vaccinated in the face of MDA continued to have reduced levels of IAV-specific lung antibodies after challenge. Based on mucosal antibody levels only, one might predict MDA(+) LAIV vaccinates to be vulnerable to infection; however they were protected against heterologous challenge. This suggests that B cells were able, possibly with the aid of memory T helper cells, to mount an anamnestic antibody response early in infection (note the rapid rise in antibodies (Fig. 1B)), and/or that T cells provided robust protection.

Previous studies showed LAIV induction of T cells in MDA(−) pigs and in humans (Chirikova et al., 2011; He et al., 2006; Hoff et al., 2011; Lanthier et al., 2011; Loving et al., 2012; Masic et al., 2010). Porcine T cells can be differentiated into four major subsets based on surface phenotype markers: CD4+CD8−, CD4−CD8+, CD4+CD8+ (expressing CD8α chain only), and γδ TCR+. In the present study, cellular immune responses were analyzed by ex vivo

Fig. 6. Antibody levels in BAL at 5 days post-infection with heterologous CO99. Mean O.D. values in whole-virus ELISAs are shown for IgA antibodies against TX98 (A) and CO99 (B) and for IgG antibodies against TX98 (C) and CO99 (D). Open bars designate groups without MDA, and solid bars designate groups with MDA. Statistically significant differences between MDA statuses within a vaccine group are marked with asterisks, and differences between vaccine treatment groups with matched MDA statuses are identified by connecting lines (P < 0.05).
antigenic stimulation of T cells from the peripheral blood and draining lymph nodes (TBLN). T cell responses from PBMC and TBLN were generally equivalent to heterologous CO99 virus and homologous TX98 virus (Figs. 3 and S1), which supports the concept that the cellular immune response is more broadly reactive than serum HI antibodies. The T cell subset with the most significant responses to viral stimulation was the CD4+CD8+ population, which existed outside the thymus in pigs (Saalmuller et al., 1987). Porcine CD4+CD8+ cells have been characterized as memory cells with T helper function (reviewed by Gerner et al. (2009)), which also can express perforin and mediate cytolytic activity against virally infected cells (De Bruin et al., 2000; Denyer et al., 2006). The porcine CD4+CD8+ population was recently differentiated into CD27+ and CD27− subpopulations, with phenotypes corresponding to central and effector memory cells, respectively (Reuter et al., 2013). In the current study, priming of CD4+CD8+ T cells was evident in both WIV and LAIV vaccinated pigs, in terms of response markers CD25, IFN-γ, and IL-10 (Fig. 3). The presence of MDA at vaccination had distinct effects on induction of CD4+CD8+ T cell responses, depending on whether the vaccine was WIV or LAIV, as there was evidence of a dampening effect on WIV and an enhancing effect on LAIV. This could reflect differences in antigen processing for the inactivated versus replicating antigens. The two vaccines differ greatly in formulation and route of delivery, and previous studies showed that modulatory effects of preexisting antibodies can be either positive or negative, depending on factors such as antigen concentration and engagement of alternate Fc receptors (Crowe, 2001).

Between the treatment groups that received 1 or 2 doses of LAIV, higher recall responses were observed in the peripheral T cells of the groups receiving 2 doses. Presumably, the first dose primed a small population of cells, which expanded after the second dose. Even so, IFN-γ responses were readily detected from the TBLN cells of single-dose LAIV-vaccinated pigs. Data captured from the TBLN, which drain the site of infection and intranasal LAIV vaccination, are a valuable complement to data from peripheral blood cells. Both vaccines, particularly LAIV, elicited effector or memory cells that were available to respond to infection. Taken together, these data indicate that cellular immunity induced by the LAIV vaccine was not impeded by MDA or by the antigenic divergence between H3N2 strains. Cellular immunity may have been the primary defense against heterologous infection in the MDA(+) LAIV group, which was deficient in pre-existing mucosal antibodies and serum HI antibodies.

Our data highlight the concept that LAIV vaccines confer broad protection whether or not they induce detectable amounts of systemic functional antibodies. In our model, LAIV-induced immunity supplied multiple cross-reactive factors (mucosal antibodies and T cells before infection and anamnestic systemic antibody response after infection). When one factor was minimized (i.e. MDA interference with mucosal antibody induction), other mediators provided cross-protection. On the other hand, our WIV primed an immune response that had adverse effects to the host respiratory tract when pigs were infected with a mismatched virus. These components complicate the development of feasible antemortem correlates of protection from either vaccine platform, and additional work is required to define assays that can be applied in diagnostic settings. However, the results are further evidence of the need for critical review of WIV vaccination practices in sows and further development of LAIV as an influenza vaccine platform for the swine industry.

Materials and methods

Viruses and vaccine preparation

Antigen for the WIV vaccine was wild type A/swine/Texas/4199-2/1998 (H3N2) (TX98), grown in Madin-Darby canine kidney (MDCK) cells or 9-day-old fertilized hen eggs. The WIV vaccine for sows contained MDCK-grown virus, while the WIV vaccine for piglets contained egg-grown virus. Clarified virus supernatants from infected culture were ultracentrifuged to pellet virus (2 h at 140,000g through a 25% sucrose cushion). Virus was resuspended in PBS and UV-irradiated using the “sterilize” setting in a UV cross-linking chamber (GS Gene Linker; Bio-Rad, Hercules, CA). Inactivation of the virus was confirmed by two serial passages on MDCK cells. A commercial adjuvant (Emulsigen D; MVP Laboratories, Inc., Ralston, NE) was added at a 1:4 volume ratio, bringing the final mixture to a hemagglutination (HA) titer of 128. The LAIV was generated via reverse genetics as previously described (Solorzano et al., 2005). The attenuated vaccine virus contained an NS1 gene with a 3' premature termination, producing a protein of 126 amino acids with a carboxy-terminal truncation (NS1Δ126 TX98). The remaining seven gene segments were from wild-type TX98. The challenge virus, A/sw/Colorado/23619/99 (H3N2) (CO99), was cultivated in MDCK cells. The TX98 and CO99 viruses were shown previously to have limited serologic cross-reactivity (Richt et al., 2003) and therefore considered heterologous.

Experimental design

Sows at a high-health herd free of IAV and porcine reproductive and respiratory syndrome virus (PRRSV) were vaccinated intramuscularly (neck) with the TX98 WIV in 3 doses, once before breeding and twice during mid-late gestation. 2 weeks before farrowing, vaccinated and non-vaccinated sows from the same source were delivered to Veterinary Resources Inc. (VRI, Ames, IA), which provided animal care and housing through the remainder of the study. 1 week-old piglets were bled to evaluate transfer of existing mucosal antibodies and serum HI antibodies.

Experimental treatment groups.

<table>
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<tr>
<th>Treatment group</th>
<th>MDA status</th>
<th>Piglet vaccine</th>
<th>Challenge virus</th>
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<td>None</td>
<td>Sham</td>
<td>4</td>
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<td>CO99</td>
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<td>LAIV, 2 doses</td>
<td>CO99</td>
<td>8</td>
</tr>
<tr>
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<td>CO99</td>
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<td>NC</td>
<td>4</td>
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<td>NC</td>
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<tr>
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<td>+</td>
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<td>NC</td>
<td>8</td>
</tr>
<tr>
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<td>−</td>
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<td>NC</td>
<td>8</td>
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<tr>
<td>MDA(+) LAIVx1*</td>
<td>+</td>
<td>LAIV, 1 dose</td>
<td>NC</td>
<td>8</td>
</tr>
</tbody>
</table>

a MDA-positive pigs were from sows that had received 3 doses of TX98 WIV vaccine. MDA-negative pigs were from non-vaccinated sows of the same source farm.
b Vaccines are derived from A/Texas/4199-2/1998 (H3N2).
c A/sw/Colorado/23619/99 (H3N2).
d NC, not challenged. Subgroups marked with asterisks were euthanized at 0 dpi for pre-challenge specimens.
rooms. Booster doses were administered 14 days later to the WIV groups and designated LAV groups. Extra groups of pigs in some treatments were moved to separate housing at the USDA-ARS National Animal Disease Center (Ames, IA) for collection of pre-challenge specimens (Table 1). At 50–55 days of age [0 days post-infection (dpi)], pigs were each inoculated intratracheally (2 ml) and intranasally (1 ml) with virus suspension containing 10^3.7 TCID_50/ml. Non-challenged control groups were housed in separate isolation rooms. Pig studies at VRI and NADC were conducted in compliance with their respective Institutional Animal Care and Use Committees.

**Clinical observation, sampling, and pathology**

CO99 virus infected pigs were observed daily for clinical signs. Three criteria (labored breathing, lethargy, and coughing) were scored for severity on a scale of 1–4 and reported as a composite clinical score. Rectal temperatures were recorded daily. Nasal swabs were taken at 0, 1, 3, and 5 days postinfection (dpi), placed in 2 ml minimal essential medium (MEM), and stored at −80 °C. Animals were euthanized humanely at 5 dpi with a lethal dose of pentobarbital. At necropsy, each lung was lavaged with 50 ml of MEM to obtain bronchoalveolar lavage fluid (BAL). BAL samples were confirmed negative for nucleic acid of porcine circovirus 2, PRRSV, and Mycoplasma hyopneumoniae, by quantitative PCR. Nasal swabs were processed and analyzed by virus isolation as described previously (Vincent et al., 2012). TCID_50 virus titers in BAL samples and each virus isolation-positive nasal swab sample were determined as described previously (Vincent et al., 2012). Macrophagic and microscopic lung lesions, and microscopic lesions of trachea were analyzed and scored as described previously (Gauger et al., 2012). IAV was detected in lung tissues by a previously described immunohistochemical method (Braucher et al., 2012).

**Serologic and mucosal antibody assays**

Serum samples were collected at pre-weaning (~12 days post-vaccination [dpv]), first vaccination (0 dpv), second vaccination (14 dpv), 27 dpv, 34 dpv, and 40 dpv (necropsy). For use in the hemagglutination inhibition (HI) antibody assay, sera were treated with receptor-destroying enzyme (Sigma-Aldrich, St. Louis, MO) to remove nonspecific inhibitors and with turkey erythrocytes to remove non-specific agglutinins and heat inactivated at 56 °C. HI assays were performed with egg-grown TX98 and CO99 viral antigens and turkey erythrocytes, using standard techniques (Palmer et al., 1975). Enzyme-linked immunosorbent assays (ELISA) to detect total IgG and IgA antibodies against whole virus preparations of TX98 and CO99 present in serum and BAL were performed as described previously (Gauger et al., 2011), with the following modifications. Serum samples were diluted to 1:2000 for the IgG ELISA. BAL samples were diluted to 1:4 for IgG and IgA ELISAs. Antibody levels were reported as the mean OD for duplicate wells.

**Multi-parameter flow cytometry analysis of IAV-specific T cells**

Peripheral blood mononuclear cells (PBMC) were stimulated by ex vivo culture with virus and tested for response markers by multi-parameter flow cytometry, using a previously described method (Platt et al., 2011). Briefly, PBMC of each pig were incubated for 4 days in the presence of TX98, CO99, or supernatant of mock-infected MDCK cells. Antibodies to the CD4, CD8 and γδ TCR markers were used with fluorochrome-conjugated secondary antibodies to label T cell subsets. The activation marker CD25 and intracellular cytokines interferon-γ (IFN-γ) and interleukin-10 (IL-10) were labeled as measures of antigen-specific responses. The net increase in the percentage of a subset to express each response marker was calculated by subtracting the percentage of mock-stimulated cells from that of virus-stimulated cells. If the net result was negative, it was adjusted to 0.

**ELISPOT analysis of IAV-specific T cells**

Lymphocytes were dissociated from fresh tracheobronchial lymph nodes (TBLN) by mechanical homogenization in 15 ml plastic tissue grinder tubes (Kendall Healthcare Products). Following homogenization, samples were run through a 40 μm filter screen to eliminate debris. The cell suspension was overlayed onto Histopaque 1077 gradient (Sigma-Aldrich) and buffy coats isolated. Cells were washed twice, enumerated, and resuspended for seeding into ELISpot plates. The ELISpot assay for IFN-γ secreting cells (IFN-γ SCs) was performed as previously described (Braucher et al., 2012) and according to the manufacturer’s recommendation (Porcine IFN-gamma ELISpot Kit, R&D Systems, Minneapolis, MN). Briefly, antibody-coated membrane plates were seeded with 5 × 10^5 cells per well and stimulated with 0.1 ml of 5 × 10^6 TCID_50/ml live TX98, CO99 virus, or uninfected MDCK culture supernatant in triplicate. Following an 18–20 h incubation period, plates were developed according to manufacturer’s recommendations. Dry plates were scanned and analyzed with SSUV CTL-ImmuSpot instrumentation and software (Cellular Technology Ltd., Shaker Heights, OH). Individual pig responses were calculated as the average number of spots per triplicate well within a stimulation treatment.

**Statistical analysis**

Macroscopic and microscopic pneumonia scores, log10-transformed BAL and nasal swab virus titers, mean ODs for ELISAs, and T-cell results (flow cytometry and ELISpot) were analyzed using analysis of variance (ANOVA) and the Tukey test for pairwise comparisons between groups, with P values < 0.05 considered significant (Prism software; GraphPad, La Jolla, CA).

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**Appendix A. Supplementary material**

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.06.027.
References


