Factors affecting induction of peripheral IFN- γ recall response to influenza A virus vaccination
in pigs

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Abstract

While T cell contribution to IAV immunity is appreciated, data comparing methods to evaluate IFN- γ production by IAV-specific T cells elicited following vaccination is limited. To understand the differential immunogenicity between live-attenuated influenza virus (LAIV) and whole-inactivated virus (WIV) vaccines in relation to induction of peripheral T cell responses, ELISpot and ELISA were used to assess IFN- γ production by peripheral lymphocytes following antigen restimulation. Following restimulation, peripheral blood lymphocytes from WIV-vaccinated pigs had a greater quantity of IFN- γ secreting cells (SC) and IFN- γ secreted compared to LAIV vaccinated and non-vaccinated (NV) pigs. Pig age at time of WIV vaccination significantly impacted peripheral IAV-specific IFN- γ recall response, as did the inclusion of adjuvant in the WIV vaccine. Collectively, these data indicate that peripheral IAV-specific IFN- γ recall responses are not predictive of LAIV vaccination status, thus, are unlikely to be a useful surrogate for evaluating LAIV immunogenicity and predicting cross-protection. However, these data suggest that the evaluation of peripheral IFN- γ recall responses may be useful for identifying factors, such as animal age or vaccine formulation, that may impact parenterally-delivered WIV vaccine immunogenicity. Overall, results did not differ based upon the assay used to evaluate IFN- γ recall responses. Therefore, either ELISpot or ELISA could serve as a measure for evaluating IAV-specific IFN- γ cell-mediated immune responses in swine.

Keywords: porcine, immune, influenza, IFN-gamma, cell-mediated immunity, vaccine

Introduction

Influenza A virus (IAV) is a prevalent respiratory disease in swine. Infection is characterized by fever, lethargy, sneezing, coughing, dyspnea, and appetite suppression. In addition to causing primary disease in swine, it predisposes swine to secondary diseases, primarily bacterial infections (Loving et al., 2010; Morens et al., 2008; Rothberg et al., 2008). Currently there are three IAV subtypes that predominantly circulate within the United States swine population, H1N1, H1N2, and H3N2 (Vincent et al., 2008b). A phylogenetic scheme was established to classify the H1 genes of multiple contemporary swine H1 IAV lineages in North America (Vincent et al., 2009). Based upon this classification, there are six H1 clusters [beta (β), gamma (γ), γ -2, delta-1 (δ -1), δ -2 and pandemic (pdm)] as well as H3 IAV circulating in the North American swine population (Anderson et al., 2015; Gauger et al., 2011; Lorusso et al., 2013; Vincent et al., 2009). The large diversity is due to constant evolution of the surface glycoproteins by point mutations (antigenic drift), and genetic reassortments with other circulating viruses (antigenic shift), including introduction of human seasonal IAV that establishes in swine (Nelson et al., 2015a; Nelson et al., 2015c; Vincent et al., 2014). IAV can be zoonotic with noted spillover events to humans and poultry (Andrew et al., 2014; Ma et al., 2008; Nelson et al., 2012; Scholtissek, 1994; Yassine et al., 2007), as well as spillover events from humans to swine (Breum et al., 2013; Nelson et al., 2015b; Nelson et al., 2015c; Rajão et al., 2015; Schaefer et al., 2014; Starick et al., 2012), which contributes significantly to the diversity of circulating strains.

Vaccination can be an effective means to prevent and control disease; however, protection against IAV in swine is complicated by the number of antigenically distinct strains co-

circulating (Sandbulte et al., 2015). The majority of available IAV vaccines currently licensed for use in swine contain whole-inactivated virus (WIV) with two or more H1 and a H3 cluster-type formulated with adjuvant for intramuscular administration (Van Reeth and Ma, 2013). However, WIV vaccines provide limited cross-protection against diverse IAV (Gauger et al., 2014; Gauger et al., 2011; Loving et al., 2013; Sandbulte et al., 2014). Newer vaccine platforms, such as live-attenuated influenza virus (LAIV), can be more effective in providing cross-protection when compared to WIV vaccination (Belshe et al., 2007; Gauger et al., 2014; Hoft et al., 2011). While mucosal antibody likely contributes to the cross-protection associated with LAIV vaccination, cell-mediated immunity (CMI) may play a role in cross-protection as well. CMI as an indicator of LAIV immunogenicity and cross-reactivity has not been extensively explored, particularly in non-rodent species. Interferon-gamma (IFN- γ) is considered a canonical cytokine of the Th1 response and plays a crucial role in the vaccine-induced protection against several infections, including IAV (Bot et al., 1998; Thakur et al., 2012).

Therefore, to assess IAV-specific IFN- γ responses, peripheral blood mononuclear cells (PBMC) were collected from pigs of differing IAV vaccination status to evaluate the induction of peripheral CMI response. Lymphocytes stimulated by antigen presentation in lymph nodes then traffic through the peripheral blood, which makes PBMC a useful specimen to sample and measure CMI responses (Binns et al., 1985). Thus, the objective of this study was to evaluate differential immunogenicity of the WIV and LAIV platforms in swine as it relates to a commonly used peripheral measure of CMI (i.e. production of IFN- γ following antigen restimulation *in vitro*). Two assays frequently used to evaluate IFN- γ production following antigen exposure include enzyme-linked immunosorbent assay (ELISA) and enzyme-linked spot (ELISpot) assay, and both were used in these studies. In addition, we evaluated the impact of age

and WIV vaccine formulation on peripheral IFN- γ recall responses. Overall, our results show that LAIV vaccination induced minimal peripheral IFN- γ responses, and peripheral IFN- γ responses following WIV vaccination were affected by age at time of vaccination and vaccine formulation. These conclusions were made for either IFN- γ assay platform we used for evaluating peripheral recall responses.

Materials and Methods

Experimental animals and vaccine

Three-week-old cross-bred pigs were obtained from a high-health status free of IAV and porcine reproductive and respiratory syndrome virus (PRRSV). Sows in which piglets were derived were free of antibody to *Mycoplasma hyopneumoniae* (ELISA) and porcine circovirus (PCR), which was shared by vendor prior to purchase. Sera from pigs were tested by commercial ELISA kits (MultiS ELISA and Herdcheck PRRS ELISA, IDEXX, Westbrook, ME) to confirm that they were free of IAV nucleoprotein-specific and PRRSV-specific antibodies at the start of the experiment. Upon delivery to the facility, pigs were administered ceftiofur crystalline-free acid (Zoetis Animal Health, New York, NY) and enrofloxacin (Bayer Animal Health, Shawnee Mission, KS) according to manufacturer instructions to reduce potential respiratory bacterial pathogens. Pigs were housed in biosafety level 2 facilities and cared for in compliance with the Institutional Animal Care and Use Committee of the National Animal Disease Center.

For both experiments described, pigs were vaccinated with whole-inactivated virus (WIV), live-attenuated influenza virus (LAIV) or were non-vaccinated (NV). The WIV vaccine contained A/SW/Minnesota/02011/08 (MN08) virus propagated in Madin-Darby Canine Kidney (MDCK) cells, which were maintained in modified Eagle's medium (MEM) supplemented with

antibiotics and 5% fetal bovine serum (FBS) (Atlanta Biologicals; Flowery Branch, GA). At the time of virus propagation, MDCK cells were cultured with Opti-MEM Reduced Serum Medium (Life Technologies; Grand Island, NY) and supplemented with TPCK-trypsin (Sigma, St. Louis, MO), 2mM L-glutamine and 1X antibiotic-antimycotic (Life Technologies; Grand Island, NY) . After approximately 80% cytopathic effect (CPE) was reached, the flask was frozen. Two freeze/thaws were completed prior to centrifugation (30 minutes at 805 x g) and subsequent collection of virus supernatant. Clarified supernatants were then ultracentrifuged (2 h at 140,000 x g through a 25% sucrose cushion) to pellet virus. After centrifugation the virus was resuspended in phosphate-buffered saline (PBS), diluted to 256 hemagglutination units (HAU) and then irradiated using the “sterilize” program twice on a ultraviolet cross-linking chamber (GS Gene Linker; Bio-Rad, Hercules, CA). Vaccine antigen was then frozen at -80° C until day of vaccination. Two blind serial passages on MDCK cells were performed with WIV antigen to confirm inactivation of the virus. Immediately prior to immunization, the WIV was formulated to 128 HAU, mixed with an oil-in water commercial adjuvant used in production animals and prior research by our group (Emulsigen D; MVP Laboratories, Inc., Ralston, NE) at a 1:5 ratio (v/v adjuvant to antigen) for a final 2 mL dose per pig. The LAIV was generated as previously described (Pena et al., 2011). Briefly reverse-genetics was used to rescue a 6+2 reassortant virus with internal genes from A/turkey/Ohio/313053/04 (OH04, H3N2) with mutations introduced in the polymerase genes to render the virus temperature-sensitive (Pena et al., 2011) and surface genes (HA and NA) from MN08.

In experiment 1 (EXP1; Figure 1), a total of 20 pigs were randomly assigned across one of three groups as they arrived off the truck from the farm. A group of pigs were vaccinated intramuscularly with 2 mL of a WIV (MN08) at 4, 7 and 10 weeks of age. A second group of

pigs were vaccinated with 2 mL of LAIV (as described above) at 10^6 50% tissue-culture infectious dose (TCID₅₀) per mL by intranasal inoculation at 4 and 7 weeks of age. A non-vaccinated (NV) group was included for controls.

In experiment 2 (EXP2), (Figures 2, 3, and 4), a total of 48 pigs were randomly assigned across one of five groups as they arrived off the truck from the farm. A group of pigs were vaccinated intramuscularly with 2 mL of WIV (MN08) formulated with adjuvant at 4 weeks of age and subsequently boosted at 7 weeks of age (4/7) or vaccinated at 9 weeks of age and subsequently boosted at 12 weeks of age (9/12) as described (Souza et al., 2016). Pigs in the LAIV group received LAIV as described above at 4 weeks and 7 weeks of age. Pigs in the no adjuvant group were vaccinated intramuscularly with 2 mL of WIV (MN08) formulated without adjuvant. A non-vaccinated (NV) group was included for controls. Figures are presented as dot plots which indicates the number of samples (pigs) used in each recall assay.

Recall Antigens

Live MN08 (δ 1) virus, which was an identical match to the WIV vaccine and had matched surface genes to the LAIV vaccine, was used as homologous recall antigen. Pandemic A/California/04/2009 (CA09, pdm) virus was used as heterologous recall antigen, which is an H1 virus but in a different phylogenetic and antigenic cluster than MN08 (Lorusso et al., 2011). The OH04 (H3N2) virus was also used as heterosubtypic recall virus (different subtype than vaccine antigen), though the internal genes were a complete match to the internal genes of the LAIV and heterologous to the internal genes of the MN08 and CA09 viruses. Viruses were titrated by performing ten-fold serial titrations made in five replicate wells in serum-free media containing TPCK-trypsin (1 μ g/mL) (Sigma, St. Louis, MO). The dilutions were then transferred to confluent MDCK monolayers in a 96-well plate. After the cells were incubated at 37°C in 5%

CO₂ for 48 h, they were fixed and stained for IAV nucleoprotein by immunocytochemistry (Kitikoon et al., 2006). Viral titers were calculated by using the method of Reed and Muench (Reed and Muench, 1938). For use as recall antigen, live viruses were diluted to 2.5×10^6 /mL and 0.1 mL/well was used in assays described below.

Peripheral blood mononuclear cell isolation

To assess peripheral cell-mediated, antigen-specific IFN- γ recall responses, whole blood was collected in sodium citrate CPT tubes (BD Vacutainer, Franklin Lakes, NJ) at 42, 45 and/or 77 days post-vaccination (dpv relative to priming) (Talker et al., 2015) and peripheral blood mononuclear cells (PBMC) were isolated according to the manufacturer's recommendations. PBMC were washed with RPMI-1640 (Life Technologies; Grand Island, NY), passed through a 40 μ m filter and washed once more. PBMC were suspended in complete (c) RPMI (RPMI supplemented with 10% FCS, L-glutamine (Life Technologies; Grand Island, NY), 2-Mercaptoethanol (Sigma; St. Louis, MO), 25 mM HEPES (Sigma; St. Louis, MO), essential amino acids and antibiotics (Sigma; St. Louis, MO). PBMC were enumerated and seeded at 2.5×10^5 cells per well for both the ELISpot and ELISA.

Interferon- γ ELISpot assay

The IFN- γ ELISpot assay was performed according to the manufacturer's recommendations (porcine IFN- γ ELISpot assay; R&D Systems, Minneapolis, MN) as previously described (Braucher et al., 2012). Briefly, 50 μ L of cRPMI was added to each well to pre-wet the ELISpot membrane. Cells were seeded in 0.1 mL and stimulated with 2.5×10^5 TCID₅₀ of live MN08, CA09, or OH04, 0.25 μ g of pokeweed mitogen (Sigma), or MDCK cell BSA-free media in 0.1 mL for a total of 0.25 mL per well. Following an 18 ± 1 h incubation in a 37°C humidified 5% CO₂ incubator, the assay was completed according to manufacturer's

recommendations. Briefly, cells were removed, plates were washed and incubated with secondary antibody, and spots were developed using HRP-substrate provided by the manufacturer. After membranes were dry, plates were scanned and analyzed with S5UV-CTL ImmunoSpot instrumentation and software (Cellular Technology Ltd. Shaker Heights, OH) to count the number of spots per well. The mean spot count of duplicate wells for each treatment for each pig was determined and used to calculate differences between groups.

Interferon- γ ELISA

PBMC were plated in 96-well U-bottom plates (Corning Life Sciences, Tewksbury, MA) and stimulated as described for ELISpot (final volume 0.25 mL). Cells were cultured for 72 ± 2 h, and supernatants were collected (pooled from duplicate treatment wells) and frozen at -80°C . The IFN- γ ELISA assay was performed according to manufacturer's recommendations (Porcine IFN-gamma DuoSet; R&D Systems, Minneapolis MN) and the concentration of IFN- γ for each sample calculated from values obtained using a recombinant IFN- γ protein standard. The optical density at 450 nm and 570 nm was determined using a SpectraMax M5 instrument (Molecular Devices; Sunnyvale, CA) and data acquired with SoftMax Pro 5 software (Molecular Devices; Sunnyvale, CA).

Statistical Analysis

Statistical analysis was completed with GraphPad Prism 6 (GraphPad Prism, GraphPad Software, La Jolla, CA). ELISpot and ELISA data were subjected to a D'Agostino-Pearson omnibus normality test for normal distribution of data. A Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison post-test was performed to evaluate statistical differences and are indicated with connecting bars ($p < 0.05$)

Results

Cellular immune responses following LAIV or WIV vaccination

The number of IFN- γ secreting cells (SC) detected following stimulation with homologous (MN08, δ 1) and heterologous (CA09, pdm) viruses in PBMC from pigs in the LAIV, WIV and NV vaccinated groups were determined by ELISpot and compared to the amount of IFN- γ secreted. In the first study (EXP1) there was a significant increase in the number of peripheral IFN- γ SC following stimulation with homologous virus (MN08, δ 1) or heterologous virus (CA09, pdm) in the WIV group as compared to the LAIV and NV groups at 45 days post-primary vaccination (dpv) (Figure 1A). Similar results were observed when measuring IFN- γ in supernatants following exposure to homologous or heterologous virus (Figure 1B).

After the initial observation comparing IFN- γ recall responses following WIV *versus* LAIV vaccination, a similar study was performed to further validate the initial findings. In the second study (EXP2), at 42 dpv the average number of IFN- γ SC for the WIV group were statistically greater than responses elicited by the LAIV and NV groups (all $p < .04$) (Figure 2A), as observed in EXP1 (Figure 1A; all $p < .04$). The magnitude of IFN- γ SC response to homologous virus was lower in EXP2 (Figure 2A) as compared to EXP1 (Figure 1A). The average number of IFN- γ SC when heterologous virus (CA09, pdm) was used as recall antigen was similar across experiments. In EXP2 (Figure 2B), when heterologous virus was used the amount of IFN- γ secreted was statistically greater than in WIV group over LAIV and NV groups ($p < .02$ for both) (Figure 2B), as observed in EXP1 (Figure 1B; all $p < .03$). Similarly, when heterosubtypic responses were measured in EXP2 the WIV group had statistically greater IFN- γ

secretion than LAIV and NV groups. A statistically significant difference in the amount of IFN- γ secreted in response to both homologous and heterologous virus was detected between WIV and NV groups (Figures 2A and 2B).

There was a decline in average IFN- γ SC recall response for WIV vaccinates over time, as at 42 dpv there were 71 ± 12 IFN- γ SC/ 2.5×10^5 PBMC (Figure 2A) and at 77 dpv there were 28 ± 12 IFN- γ SC/ 2.5×10^5 PBMC (Figure 2C). For LAIV vaccinates, there was not a statistical difference in IFN- γ recall responses over time as at 42 dpv there were 12 ± 6 IFN- γ SC/ 2.5×10^5 PBMC (Figure 2A) and at 77 dpv there were 32 ± 15 IFN- γ SC/ 2.5×10^5 PBMC (Figure 2C). While recall responses following WIV vaccination decreased over time, the recall response following LAIV vaccination remained steady over time, although it was significantly lower compared to responses in WIV vaccinates at 42 dpv. While there was not a statistically significant increase in IFN- γ responses in the LAIV group between 42 to 77 dpv, there was a numerical trend for an increased number of IFN- γ SC over time.

In addition to the ELISpot, an ELISA was used to assess the quantity of IFN- γ secreted by PBMC following stimulation with recall antigen for 72 ± 2 hours. Following stimulation with homologous virus (MN08), there was no statistical difference between WIV and LAIV vaccine groups; however, there was a statistical difference between vaccine groups when either CA09 or OH04 was used as recall antigen (Figure 2B). At 77 dpv there was a statistical difference between the amount of IFN- γ produced by PBMC from WIV vaccinates and the NV group with CA09 or OH04 recall antigens ($p < .05$), while the difference when MN08 was used as recall antigen was statistically insignificant (Figure 2D).

Impact of adjuvant

For evaluation of IFN- γ recall responses following WIV vaccination described in the results above, the WIV was formulated with an adjuvant. Thus, to evaluate the contribution of adjuvant to induction of IAV-specific IFN- γ responses, PBMC from pigs vaccinated at 4 and 7 weeks of age with WIV formulated with adjuvant (WIV) or without adjuvant (No adjuvant) were collected and IFN- γ recall responses were evaluated using ELISpot and ELISA. At 42 dpv an average of 71 ± 12 IFN- γ SC/ 2.5×10^5 PBMC were detected following adjuvanted WIV vaccination, whereas in the group which received non-adjuvanted WIV vaccination an average of 4 ± 3 IFN- γ SC/ 2.5×10^5 PBMC were detected, when homologous virus (MN08) was used as recall antigen (Figure 3A). Similar results were obtained when heterologous virus (CA09) was used as recall antigen, with 76 ± 13 IFN- γ SC/ 2.5×10^5 PBMC detected for adjuvanted WIV group and 4 ± 2 IFN- γ SC/ 2.5×10^5 PBMC for non-adjuvanted WIV group. Moreover, little to no IFN- γ was detected in supernatants of PBMC from pigs given non-adjuvanted WIV, which was similar to the response of non-vaccinated pigs. However, IFN- γ was produced by IAV-stimulated PBMC collected from adjuvanted WIV vaccinates (Figure 3B).

Impact of age

In order to assess the impact of age at the time of vaccination on subsequent IAV-specific IFN- γ recall responses, a study was completed in which pigs were vaccinated at 4 and 7 weeks of age (4/7) or 9 and 12 weeks of age (9/12) with adjuvanted WIV vaccine and peripheral IFN- γ responses were assessed 42 days post-priming. There was a numerical trend for a greater number of IFN- γ SC cells when homologous virus (MN08) was used as recall antigen in pigs vaccinated at 4/7 weeks of age, as compared to pigs vaccinated at 9/12 weeks of age (Figure 4A); however differences were not significantly different. When heterologous virus (CA09) was used as the

recall antigen pigs vaccinated at 4/7 weeks of age had a significant increase in the number of IFN- γ SC (76 ± 13 IFN- γ SC/ 2.5×10^5 PBMC) when compared to pigs vaccinated at 9/12 weeks of age (27 ± 6 IFN- γ SC/ 2.5×10^5 PBMC) ($p=.004$). PBMC from pigs vaccinated at 4/7 weeks of age exhibited a statistically significantly higher number of IFN- γ SC following homologous or heterologous antigen stimulation when compared to NV pigs ($p = .002$ and $.0004$, respectively).

Following stimulation with heterologous (CA09) or heterosubtypic (OH04) recall antigen there was a significant increase in IFN- γ secreted by PBMC from pigs vaccinated at the younger age as compared to the older age (Figure 4B), which was similarly observed in the IFN- γ SC as measured by ELISpots (Figure 4A). Secretion of IFN- γ from cells from 4/7 group stimulated with homologous virus was increased over non-vaccinates, but was numerically increased over cells from pigs in 9/12 group (Figure 4B). Specifically, after stimulation with homologous virus (MN08), PBMC from the 4/7 vaccinates secreted 5240 ± 1246 pg/mL as compared to PBMC from the 9/12 vaccinates which secreted 1928 ± 702 pg/mL IFN- γ . When heterologous virus (CA09) was used as recall antigen, PBMC from the 4/7 vaccinates secreted 6391 ± 1337 pg/mL IFN- γ as compared to PBMC from the 9/12 vaccinates which secreted 857 ± 345 pg/mL IFN- γ . When heterosubtypic OH04 (H3N2) virus was used as recall antigen, PBMC from 4/7 vaccinates secreted 2634 ± 665 pg/mL IFN- γ , as compared to 992 ± 414 pg/mL IFN- γ from 9/12 vaccinates.

Discussion

Influenza A virus (IAV) vaccines are available for use in swine and can be an effective means for prevention and control of IAV. Prior to the licensing of a new replicon particle vaccine (Chen, 2013), only whole-inactivated virus (WIV) vaccines were commercially available to control IAV in swine (Ma and Richt, 2010), and several reviews on different platforms are

available . Due to the large number of antigenic variants circulating in US swine, WIV vaccines currently licensed for use provide limited protection against circulating strains of IAV (Gauger et al., 2011; Kitikoon et al., 2006; Vincent et al., 2010). Live-attenuated influenza virus (LAIV) vaccines have been shown to provide better cross-protection in several experimental studies (Gauger et al., 2014; Hughes et al., 2015; Rajão et al., 2014), although a LAIV vaccine is not yet commercially available for use in swine. While it has been shown that LAIV is protective against heterologous challenge, peripheral antibody responses are limited in predicting cross-protection (Hughes et al., 2015; Loving et al., 2013). Peripheral CMI may be a useful measure to evaluate LAIV vaccine immunogenicity and cross-reactive immunity, and potentially be used as a predictor of cross-protection. Therefore, WIV and LAIV vaccine platforms were used to evaluate the induction of peripheral CMI following immunization, and IFN- γ ELISpot and ELISA were compared to determine if both measured IAV-specific recall responses equally.

This study demonstrated that WIV vaccine formulated with adjuvant induced a stronger peripheral cell-mediated IFN- γ response than LAIV vaccine when evaluated at approximately 6 weeks post-primary vaccination (dpv 42). These results were observed in two independent experiments (Figures 1 and 2), and although there were subtle differences in the WIV vaccination regimen between experiment 1 and 2 (Figures 1 and 2), the trends in responses between the vaccine groups were similar. Previous studies have shown that LAIV vaccination elicits a peripheral T cell response, measured by the number of cells producing IFN- γ after IAV stimulation, with the average IFN- γ spot counts between 60-100 IFN- γ SC (Loving et al., 2012; Rajão et al., 2014; Sandbulte et al., 2014). When compared to other vaccine regimens, such as subunit vaccination (Rajão et al., 2014) or WIV vaccination (Loving et al., 2012), LAIV has been shown to elicit a greater T cell response (reviewed in (Schotsaert et al., 2012). Thus, the

lack of a robust IAV-specific IFN- γ response following LAIV vaccination at approximately 6 weeks post-primary vaccination was somewhat unexpected (Figures 1 and 2). The differences in responses with the LAIV vaccinates in this study *versus* previous studies may be due to study designs and vaccine formulations (Loving et al., 2012; Rajão et al., 2014; Sandbulte et al., 2014). For example, in Loving et. al. 2012 (Loving et al., 2012), LAIV induced a greater peripheral IFN- γ recall response than WIV; however, this was most likely due to the WIV formulation. In the data reported here the WIV vaccines were formulated at 128 HAU, as opposed to 8 HAU in prior work. The change to a higher dose was made because it induced greater antibody responses and reflects a dose used influenza experiments across species. Additionally, the higher antigen load was likely more immunogenic for cell-mediated immune responses, as measured by IFN- γ . In addition, the time between priming and boosting has been different between studies. The changes in vaccine regimen may alter subsequent immune responses, which may contribute to the detected levels of IFN- γ produced by PBMC post-vaccination. Another factor that may have impacted the response in the peripheral blood for LAIV in this study was the number of cells used in the ELISpot (Loving et al., 2012; Rajão et al., 2014; Sandbulte et al., 2014). In this study 2.5×10^5 PBMC/well were used as compared to other studies, which used 5×10^5 PBMC/well. Therefore, when interpreting data, vaccine formulation and assay cell number should be taken into consideration to truly assess the factors that may contribute to the outcome of a study.

At 77 days post primary vaccination the IFN- γ recall responses began to decline in WIV vaccinates suggesting that peripheral CMI was decreasing over time (Figure 2A and 2C). This decline in IAV-specific IFN- γ response measured in the periphery following WIV vaccination may be important in terms of duration of protection. The number of IAV-specific IFN- γ SC in LAIV vaccinates did not decline during this time period (Figure 2A *versus* 2C). It has been

shown that LAIV can induce a protective local immune response, and a memory response capable of conferring homologous and heterologous cross-protection upon viral challenge, which in terms of effective vaccination to influenza is important (Hughes et al., 2015; Loving et al., 2012; Powell et al., 2007). The decreased response in the WIV vaccinate group may be due to a waning effector T cell population and/or a lack of an established peripheral memory IAV-specific T cell population. While the weak IAV-specific IFN- γ response in the LAIV vaccinate group may also be due to a lack of an established peripheral IAV-specific T cell population, it is more likely the result of sampling of a peripheral site *versus* a local, pulmonary site. Previous publications have shown that LAIV vaccines can induce a protective cellular immune response (Belshe et al., 2007; Hoft et al., 2011; Pena et al., 2011; Strutt et al., 2013). However, our results indicate that if this is the case, there are not a large number of vaccine-induced, IAV-specific IFN- γ producing T cells in circulation. LAIV vaccines are delivered by the intranasal route, thus, IAV-specific T cells likely reside at the respiratory mucosa or draining lymph nodes as opposed to the periphery (Vincent et al., 2007). It has been shown that long-lived cytotoxic T lymphocyte memory can establish in mucosal tissues after mucosal immunization as opposed to systemic immunization (Gallichan and Rosenthal, 1996). Recent work has shown that multifunctional T cells are elicited into the periphery following intratracheal influenza challenge (Talker et al., 2015), however, there is likely to be differences in peripheral T cell responses following infection *versus* LAIV vaccination. One purpose for research reported here was to explore the possibility of peripheral IFN- γ recall responses serving as a surrogate of immunogenicity and cross-protection following LAIV vaccination, not necessarily characterizing the full cell-mediated immune response following LAIV vaccination. It will be informative in future work to examine sites of immune induction, such as the draining lymph node and lungs, to delineate the

mechanisms of protection following LAIV vaccination. Regardless of the critical role of IFN- γ in Th1 mediated immunity, measurement of this cytokine alone is not sufficient as an immune correlate of protection for IAV (Bot et al., 1998; Thakur et al., 2012). Mucosal IAV-specific antibody induced following LAIV vaccination is associated with heterologous protection (Hughes et al., 2015), and pulmonary T cells likely contribute to protection as well.

In addition to evaluating differences in IFN- γ recall responses between WIV and LAIV vaccinates, the impact of age at WIV vaccination on peripheral IFN- γ recall responses was also assessed (Figure 4). The age at which WIV vaccine was administered had a significant impact on subsequent peripheral IAV-specific IFN- γ recall response, regardless of the assay used to make this determination (Figure 4). Adjuvanted WIV vaccine was more immunogenic when administered to pigs at 4 and 7 weeks of age when compared to pigs vaccinated at 9 and 12 weeks of age as the number of IFN- γ SC and the amount of IFN- γ produced in recall assay was significantly increased in the 4/7 group compared to the NV group, while the 9/12 group was not statistically increased over NV group (Figure 4). In addition to the peripheral IFN- γ response reported here, serum hemagglutination-inhibition (HI) titers to vaccine antigen were greater in pigs vaccinated at 4 and 7 weeks-of-age *versus* 9 and 12 weeks-of-age (Souza et al., 2016). Collating these results suggests that overall, the adaptive immune response to adjuvanted WIV vaccination was greater when administered at a younger age, as opposed to any skewing towards a particular type of response (Th1 *versus* Th2). It is unclear to us as to why the younger pigs had an increased response when compared to pigs vaccinated at a greater age. Prior work has shown that CD4/CD8 double-positive T cells from pigs vaccinated at 6 and 9 weeks of age produce IFN- γ after *in vitro* exposure to virus (Platt et al., 2011), though we did not phenotype the IFN- γ producers in this study. Extrathymic T cells in pigs that express both CD4 and CD8 α (double-

positive) have been characterized as memory cells that are MHC-II restricted, but maintain functions similar to cytotoxic T lymphocytes (Zuckermann, 1999). Analysis of the cell type producing IFN- γ may provide insight into any skewing of immune response associated with vaccination at different ages, perhaps there's a difference in induction of CTL's versus double-positive memory T cells that could provide mechanistic insight. Pigs would have likely been protected against homologous challenge given the antibody titers; however, vaccine associated enhanced respiratory disease (VAERD) occurred following challenge with the heterologous virus (Souza et al., 2016).

The induction of a detectable peripheral CMI response required addition of adjuvant to the WIV antigen, as IFN- γ recall responses were not detected when pigs were given WIV without adjuvant (Figure 3). Formulation of inactivated vaccines with adjuvants can have benefits, such as enhancing antibody responses in situations where the WIV alone is poorly immunogenic or in cases when mass quantities of vaccine antigen is needed for a naïve population (Brown, 2010; Reed et al., 2013). Adjuvants can activate the innate immune system, which has been shown to enhance both antibody and cell-mediated adaptive immune responses (Moser and Leo, 2010; Pasquale et al., 2015). Data indicates that vaccines containing adjuvant can elicit stronger, more persistent and broader immune response against H3N2 strains than non-adjuvanted vaccines (Baz et al., 2012). Administration of WIV with adjuvant was necessary to induce a peripheral IFN- γ response at 4/7 weeks of age as no response was observed in pigs given no adjuvant. VAERD has been associated with adjuvanted vaccine/mismatch and subsequent IAV heterologous challenge (Gauger et al., 2011; Vincent et al., 2008a). Thus, although WIV vaccines have been reported to provide protection against homologous challenge, VAERD can potentiate as a problem, causing severe lung lesions, in comparison to alternative

vaccine platforms, such as LAIV (Souza et al., 2016). Thus, when choosing vaccine platform to use this should be taken into consideration.

In regards to different assay platforms evaluated in the current study, the conclusions drawn were the same regardless of assay platform used (ELISpot *versus* ELISA). There was only a single instance when the conclusion based on statistical differences in the peripheral IFN- γ recall response between WIV and LAIV vaccinates was different when comparing the two platforms. Specifically, in EXP2 there was no statistical difference between the amounts of IFN- γ secreted by PBMC from WIV vaccinates when compared to LAIV vaccinates following restimulation with homologous MN08 virus (Figure 2B). However, there was a difference in the number of IFN- γ SC for the same comparison (Figure 2A). It is worth noting that the ELISpot does not quantify the amount of secreted IFN- γ , only the number of antigen-specific cells. While LAIV vaccinates may have fewer IFN- γ SC in the periphery, each cell may be producing more IFN- γ which could explain the difference sometimes observed between assays. However, in general, there was not a major difference in the conclusions drawn when comparing the two recall assays, which has been observed for IFN- γ recall responses in mice (Favre et al., 1997). The use of ELISA eliminates the need for advanced imaging systems and spot counting software, as ELISAs rely primarily on spectrophotometers commonly found in biological research laboratories. While either assay may be appropriate when testing IFN- γ based cell-mediated immunity for swine, investigators may need to make comparisons for their particular disease/vaccine.

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Figure Legends

Figure 1 Peripheral IAV-specific IFN- γ recall response following WIV and LAIV vaccination.

Groups of pigs were vaccinated intramuscularly with WIV (MN08, δ 1) or intranasally with LAIV as described in the materials and methods for Exp1. Peripheral blood mononuclear cells (PBMC) were collected at 45 days post-initial vaccination (dpv) for evaluation of IFN- γ recall responses. (A) Number of IFN- γ SC in PBMC collected at 45 dpv following stimulation with homologous virus, MN08 (δ 1), or heterologous virus, CA09 (pdm) was determined by ELISpot. (B) Quantity of secreted IFN- γ protein produced by PBMC collected at 42 dpv following in vitro stimulation with homologous virus, MN08 (δ 1), or heterologous virus, CA09 (pdm) was determined by ELISA. Each individual point represents the response of a single pig in the indicated group with mean $\pm$ SEM indicated. A Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison post-test was performed to evaluate statistical differences and are indicated with connecting bars ($p < 0.05$).

Figure 2 Peripheral IAV-specific IFN- γ recall response following WIV and LAIV vaccination.

Groups of pigs were vaccinated intramuscularly with WIV (MN08, δ 1) or intranasally with LAIV as described in the materials and methods for Exp2. Peripheral blood mononuclear cells (PBMC) were collected at 42 (A and B) and 77 (C and D) days post initial vaccination (dpv) for evaluation of IFN- γ recall responses (A, C). Number of peripheral IFN- γ SC following stimulation with homologous virus, MN08 (δ 1), or heterologous virus, CA09 (pdm) was determined by ELISpot. (B, D). Quantity of secreted IFN- γ protein produced by PBMC following stimulation homologous virus, MN08 (δ 1), heterologous, CA09 (pdm), or

heterosubtypic virus, OH04 (H3N2) was determined by ELISA. Each individual point represents the response of a single pig in the indicated group with mean $\pm$ SEM indicated. A Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison post-test was performed to evaluate statistical differences and are indicated with connecting bars ($p < 0.05$)

Figure 3 WIV formulated with adjuvant was required to elicit a peripheral IAV-specific IFN- γ recall response in pigs. Groups of pigs were vaccinated intramuscularly with WIV formulated with adjuvant (WIV) or without adjuvant (no adjuvant) as described in the materials and methods. PBMC were collected at 42 dpv for evaluation of IFN- γ recall responses. (A) Number of IFN- γ SC in PBMC collected at 42 dpv following stimulation with homologous virus, MN08 (δ 1), or heterologous virus, CA09 (pdm) virus was determined by ELISpot. (B) Quantity of secreted IFN- γ protein produced by PBMC collected at 42 dpv following stimulation with homologous virus, MN08 (δ 1), heterologous virus, CA09 (pdm), or heterosubtypic virus, OH04 (H3N2) determined by ELISA. Each individual point represents the response of a single pig in the indicated group with mean $\pm$ SEM indicated. A Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison post-test was performed to evaluate statistical differences and are indicated with connecting bars ($p < 0.05$)

Figure 4 Age at time of WIV vaccination significantly impacted peripheral IAV-specific IFN- γ recall responses. Groups of pigs were vaccinated intramuscularly with adjuvanted WIV (MN08, δ 1) at 4 and 7 weeks of age (4/7) or 9 and 12 weeks of age (9/12) as described in the materials and methods. Peripheral blood mononuclear cells (PBMC) were collected at 42 days post initial vaccination (dpv) for evaluation of IFN- γ recall responses (A) Number of IFN- γ SC in PBMC

following stimulation with homologous virus, MN08 ($\delta 1$), or heterologous virus, CA09 (pdm) virus was determined by ELISpot. (B) Quantity of secreted IFN- γ protein produced by PBMC following stimulation with homologous virus, MN08 ($\delta 1$), heterologous virus, CA09 (pdm), or heterosubtypic virus, OH04 (H3N2) determined by ELISA. Each individual point represents the response of a single pig in the indicated group with mean $\pm$ SEM indicated. A Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison post-test was performed to evaluate statistical differences and are indicated with connecting bars ($p < 0.05$)

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