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Review

Evidence for a common mucosal immune system in the pig☆

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ABSTRACT

The majority of lymphocytes activated at mucosal sites receive instructions to home back to the local mucosa, but a portion also seed distal mucosa sites. By seeding distal sites with antigen-specific effector or memory lymphocytes, the foundation is laid for the animal's mucosal immune system to respond with a secondary response should to this antigen be encountered at this site in the future. The common mucosal immune system has been studied quite extensively in rodent models but less so in large animal models such as the pig. Reasons for this paucity of reported induction of the common mucosal immune system in this species may be that distal mucosal sites were examined but no induction was observed and therefore it was not reported. However, we suspect that the majority of investigators simply did not sample distal mucosal sites and therefore there is little evidence of immune response induction in the literature. It is our hope that more pig immunologists and infectious disease experts who perform mucosal immunizations or inoculations on pigs will sample distal mucosal sites and report their findings, whether results are positive or negative. In this review, we highlight papers that show that immunization/inoculation using one route triggers mucosal immune system induction locally, systemically, and within at least one distal mucosal site. Only by understanding whether immunizations at one site triggers immunity throughout the common mucosal immune system can we rationally develop vaccines for the pig, and through these works we can gather evidence about the mucosal immune system that may be extrapolated to other livestock species or humans.

1. Introduction

The mucosal-associated immune system (MALT) includes the conjunctiva (conjunctiva-associated lymphoid tissue (CALT)), lacrimal duct-ALT (LDALT), larynx-ALT (LALT), salivary duct-ALT (SDALT), nasal-ALT (NALT), bronchus-ALT (BALT), gut-ALT (GALT) and vaginal (VALT) (Gebert and Pabst, 1999). Although the components of the MALT are anatomically and functionally distinct, they share traits such as organized inductive sites where T cells are presented antigen via antigen-presenting cells (APCs). As with other animals, the majority of porcine pathogens gain entry into the body through mucosal surfaces when ingested or inhaled from the feed, the environment or from fecal contamination. Systemic vaccinations (through intramuscular, intraperitoneal, subcutaneous routes, etc.) generally do not promote mucosal immunity and therefore the animal's immune system can only combat the pathogen after it has gained entry into the body (Mestecky, 1987; Mestecky et al., 1978; Murtaugh, 2014). Thus, because mucosal immunity has the potential to control pathogens at their point of entry, it would be advantageous to develop vaccines that trigger a strong mucosal and systemic immune response rather than simply stimulating the systemic immune system.

For induction of a local mucosal immune response, in the gut for example, antigen is taken up by intestinal DCs which migrate to the mesenteric lymph node (mLN) and lead to antigen-specific T and B lymphocytes activation (Annacker et al., 2005; Fujimoto et al., 2011; Johansson-Lindbom et al., 2005; Schulz et al., 2009). Upon activation, lymphocytes undergo proliferation and differentiation and, in most mammals, these activated clonal lymphocytes exit the mLN via the efferent lymph where they drain into the thoracic cavity and enter into the circulation. The pig has inverted lymph nodes and therefore immigration into the lymph node tissue takes place either by afferent lymph vessels or by high endothelial venules (HEV) and they emigrate directly into the circulation through HEV (Binns and Pabst, 1994; Rothkötter, 2009). Once in

☆ This article belongs to Non-rodent animal models.

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the circulation, the majority of activated lymphocytes home back to site where the antigen was initially encountered (Kiyono and Fukuyama, 2004; Kunisawa et al., 2008; Lefrancois et al., 1999; Svensson et al., 2002).

DCs play a critical role in regulating expression of homing molecules on the surface of activated lymphocytes (Johansson-Lindbom et al., 2003; Stock et al., 2013). In the intestine, migratory CD103 + CD11c + MHCII+ DCs produce retinoic acid (RA) which promotes the expression of α4β7 and CCR9 on activated lymphocytes in mice and humans (Campbell and Butcher, 2002; Johansson-Lindbom et al., 2005, 2003; Mora et al., 2003, 2006; Stagg et al., 2002; Stock et al., 2013; Yokota et al., 2009). The receptor for α4β7, MadCAM, is highly enriched on the endothelium of the vasculature supplying the small intestine such that lymphocytes bearing α4β7 undergo extravasation in these post-capillary venules (Berlin et al., 1993). CCR9+ lymphocytes in turn home to the small intestine epithelial cells that constitutively express the CCR9+ ligand, CCL25 (Kunkel et al., 2000; Lazarus et al., 2003). Homing of IgA producing B cells to diverse mucosal tissues appears to be mediated by CCR10 and the ligand CCL28 (Lazarus et al., 2003). In contrast, skin-derived DCs imprint expression of P- and E-selectin ligands and CCR10 on activated lymphocytes (Campbell and Butcher, 2002; Campbell et al., 2003; Schon et al., 1999). Lung DCs imprint the expression of CCR4 on lymphocytes which promote homing to the lung (Mikhak et al., 2013). Thus, the majority of activated lymphocytes home back to the site of antigen uptake by the DC.

Importantly, a portion of activated lymphocytes seed mucosal tissues outside the local mucosa which is tremendously valuable as distal mucosal sites may also encounter the pathogen (i.e. the source of the antigen) in the future (Brandtzaeg et al., 1999; Campbell et al., 2003; Kunkel and Butcher, 2003). Adoptive transfer experiments in animals has shown that cells obtained from mucosal tissues that have been donated to syngeneic animals preferentially repopulate the recipient's mucosal tissues which is compelling evidence of a common mucosal immune system (Griscelli et al., 1969; Hall et al., 1977; McDermott and Bienenstock, 1979; Weisz-Carrington et al., 1979). This activation by antigen at a mucosal inductive site which leads to effector and/or memory T and B cells in distal mucosal sites is referred to as functional connectiveness and is the basis for the common mucosal immune system (Kiyono and Fukuyama, 2004; Kunisawa et al., 2008; McGhee et al., 1992) (Table 1).

Due to the hostile environment of the gastrointestinal tract and coupled with the propensity of the oral immune system to respond with tolerance to oral antigens, it is a substantial challenge to elicit protective mucosal immune responses in the gut using oral immunizations (Faria et al., 2003; Faria and Weiner, 2005; Strobel and Ferguson, 1985; Strobel and Mowat, 1998). Several physical barriers prevent antigen/pathogen contact with gut-associated lymphoid tissues (GALT) and penetration of the gut wall such as mucous production, peristaltic movement of the gut, secretion of natural antibacterial substances such as lysozyme and host defense peptides which protect the intestinal surface against bacterial penetration, and the extreme pH environment of the stomach and the protease rich environment of the small intestine which compromise the immunogenicity of ingested antigens (Medina and Guzmán, 2000; Pasetti et al., 2011). Also, antibodies or other components in maternal colostrum/milk may interfere with antigen uptake and/or function (Brandtzaeg, 2003; Snoeck et al., 2003). Therefore, if one could design a vaccine to activate the common mucosal immune system, it would be a tremendous advantage to initiate mucosal immunity to oral antigens at respiratory or genital mucosa where the activated lymphocytes would then migrate to the oral mucosa to protect the gastrointestinal tract.

The majority of mucosal vaccines are comprised of replicating, attenuated pathogens which, although effective, have the potential to revert to virulence (www.vetvac.org/index.php). In a disease such as Porcine Respiratory and Reproductive Syndrome Virus (PRRSV), which is economically devastating to a pig barn should an outbreak occur, live-attenuated vaccines are not administered to seronegative herds. Even though it is unlikely that the attenuated virus will revert to virulence, it is considered too great a risk to vaccinate proactively and therefore PRRSV vaccines are administered to pigs in barns that have had an outbreak, and thus these vaccines are not proactively administered (Botner et al., 1997; Hu and Zhang, 2014; Storgaard et al., 1999). One may speculate that the reason why attenuated pathogens are so effective as mucosal vaccines may be that in order to trigger an oral immune response instead of tolerance, the pathogen must traverse the gut wall and/or penetrate the epithelial cells lining the gut wall. However, some researchers have shown that subunit vaccines formulated with adjuvants such as cholera toxin (CT) can trigger mucosal immunity in pigs, and some of these works are described within (Foss and Murtaugh, 1999, 2000; Hyland et al., 2004; Verdonck et al., 2005a,b).

For this review, we present manuscripts with evidence that vaccinations and/or inoculation of pigs at one mucosal site triggers a measurable immune response within the local mucosa, within the blood and within at least one distal mucosal immune site. For example, research wherein pigs have been exposed to replicating but attenuated virus via the intranasal route and which showed virus-specific IgG or IgA antibody production or cell-mediated immune responses within the respiratory mucosa (i.e. local mucosa), oral or vaginal mucosa (distal mucosa) as well as in the blood (systemic immune response) would meet our criteria for reporting. If only a local mucosal immune site and/or serosal response is reported (i.e. intranasal vaccination of piglets reporting antibody production in the bronchoalveolar lavage fluid and/or blood only), the report will not be included in this review. As such, we intend this review to be a thorough examination of the literature that reports evidence for induction of the common mucosal immune response in pigs. We are aware, however, that our approach has several limitations which we will now outline. First, intranasal immunizations may indeed also be peroral if a portion of the vaccine is swallowed. If this is the case, we cannot interpret evidence of antibodies in the gut as truly an induction of a distal mucosal site because the GALT was directly stimulated and therefore should be considered local mucosa. Unfortunately, it is impossible to discern whether sufficient precautions were taken to ensure an exclusively intranasal immunization in these reports and we can only trust what was reported (i.e. that the route was intranasal). But for this reason, we are more confident that the common mucosal immune system was induced if any reports of intranasal immunization also report immunity at a distal site other than the oral mucosal immune system. Second, our approach will be to evaluate protection or immune response through induction of antigen-specific IgG or IgA titers, the presence of antibody-secreting cells (ASCs) and/or induction of antigen-specific cell-mediated immunity (such as induction of IFNγ expression or lymphocyte proliferation), if reported. Unfortunately, the majority of the manuscripts examined here only report antibody production without correlation with the presence of ASCs in that organ. We are thus aware that there is the possibility that IgA and IgG antibodies may be in the blood and transported to the distal mucosal site via receptors such as pIgR or FcRn, respectively (Kaetzel et al., 1991; Raghavan et al., 1993; Stirling et al., 2005). Thirdly, if the antigen is delivered as part of a replicating bacteria, yeast, or virus, the possibility exists that the pathogen/vector can disseminate (unless the pathogen has a strict tissue-tropism) and/or the antigen is presented to distal mucosal sites through the migration of activated DCs. A careful examination of the types of DCs that may take up the antigen

Table 1
Details of the vaccination/immunization regimen showing induction of the common mucosal immune responses from the highlighted literature.

Antigen	Age	Primary	Booster	Challenge	Immune response		Reference
					Local mucosa/systemic	Distal mucosa	
Crude rhoptry proteins derived from *T. gondii* plus Quil-A adjuvant	6–8 week-old	Intranasal	Intranasal vaccine 21 and 42 days later	Oral challenge with 10^3 oocysts (VEG strain) at day 49	-Lymphocytes from PBMCs -↑ proliferation in response to rhoptry proteins, -3/4 pigs had marginal IgG and IgA-rhoptry specific antibody titers in blood and were partially protected from tissue cyst burden.	-Lymphocytes from mLNs showed ↑ proliferation in response to rhoptry proteins	da Cunha et al. (2012)
TGEV	11 days of age	Oral-intranasal		Challenged with TGEV 24 days after primary inoculation	Prior to challenge: -Piglets primed with TGEV were protected, less nasal shedding. -↑ Lymphocyte proliferation in MLN and gut but highest in BLN, -↑ IgA to IgG ASC ratios were detected in the duodenum and jejunum prior to challenge After challenge: -Piglets primed with TGEV ↑ T cells in mLN and gut, lesser extent in BLN and spleen -↑ but transient proliferation of BLN cells -Protected, no virus shedding in nasal secretions, -↑ IgG and IgA ASC in BLN and spleen, -IgA ASC in the gut lamina propria of TGEV-primed pigs correlated with protection against TGEV challenge	-BLN, spleen, mLN and ileum showed a ↑ ratio of IgG to IgA ASC with highest IgG titers in the BLN	Brim et al. (1995); Vancott et al. (1994)
PRCV	11 days of age	Oral-intranasal		Challenged with TGEV 24 days after primary inoculation	Prior to challenge: -No clinical signs of disease, virus shedding in nasal secretions, -↑ Lymphocyte proliferation in mLN and gut but highest in BLN (lower than TGEV primed pigs) -↑ IgG ASC in BLNs, limited number in the ileum. -Very few IgA ASC in duodenum and jejunum with other sites showing negligible IgA ASCs. After challenge: 5/12 pigs developed diarrhea, -↑ IgA ASC in BLN, spleen, duodenum and jejunum -Very few IgA ASC were detected in the ileum and none were detected in the mLN -↑ proliferative responses in BLN after TGEV challenge which correlated with protection against nasal virus shedding	-↑ IgG ASC in the gut lamina propria and mLN but not the BLN (which already had ↑ IgG ASC). -PRCV-specific IgG ASC precursors in BALT of PRCV-primed pigs migrated to the gut in response to TGEV challenge, contributing to partial protection	Brim et al. (1995); Vancott et al. (1994)

A. pleuropneumoniae (APP) (active or inactivated by irradiation)	4-month old	Oral, 10^{11} Active or inactivated APP		Aerosol challenge with 10^8 viable APP at week five		-Non-immunized pigs were severely ill, massive pathology after challenge -Iimmunized with viable or inactivated were clinically normal -↑ neutrophils and lymphocytes into lung after oral immunization -↑ Plasma cells in BAL after oral immunization with either viable or non-viable bacteria -↑ total number of T cells -↑ IgA+ ASCs in BALs after immunization with inactivated bacteria -↑ IgG+ and IgM+ ASCs in pigs orally immunized with viable or nonviable APP	Delventhal et al. (1992); Pabst et al. (1995)
Bovine and simian derived virus-like particles (bsVLP) ± mLT	Gnotobiotic pigs, 3–5 days of age	Intranasal	Intranasal, Two doses bhVLPx2 ± mLT on days 10 and 20	Oral challenge with 10^6 infectious dose $(ID)_{50}$ of virulent Wa HRV on day 21.	Post-challenge: -bsVLP2x+ were not protected against diarrhea -No significantly enhanced virus-neutralizing antibody titers compared to the challenged control pigs.	Prechallenge: -bsVLPx2 + showed ↑ IgM and IgG ASC numbers in the ileum relative to bsVLPx2 Postchallenge: -bsVLPx2+/- showed ↑ antigen-specific IgM, IgA, and IgG ASC in duodenum and ileum	Yuan et al. (2000)
Three doses of bsVLP+ or Three doses of bhVLP2+	Gnotobiotic pigs, 3–5 days of age	Intranasal	Intranasal, Two additional doses of bsVLPx2 + mLT on days 10 and 20	Oral challenge with 10^6 infectious dose $(ID)_{50}$ of virulent Wa HRV on day 21.	Prechallenge: -↑ ASC responses by the bsVLPx3+ regimen in systemic tissues Post-challenge:-bsVLPx3+ or bhVLPx3+ were not protected, -No differences in titers and duration of virus shedding Post-challenge: -↑ IgA and IgG ASC titers in the bhVLPx3+ pigs compared to pre-challenge, except in the bone marrow. -↑ IgA ASC titers in the bhVLPx3+ pigs duodenum and ileum, and -↑ IgG ASC titers in the spleen, peripheral blood, and bone marrow	Prechallenge: -ASC responses induced by the bsVLPx3+ regimen in intestinal tissues was similar to 2 doses -Pigs immunized with bhVLPx3+ ↑ IgG ASC but ↓ IgA ASC in the duodenum and ileum compared to bsVLPx3+ Post-challenge: -↑ IgA and IgG ASC titers in the bsVLPx3+ pigs compared to pre-challenge, except in the duodenum. -↑ IgA ASC titers in the bhVLPx3+ pigs duodenum and ileum, ↑ mLN IgG ASC	Yuan et al. (2000)

Table 1 (*Continued*)

Antigen	Age	Primary	Booster	Challenge	Immune response		Reference
					Local mucosa/systemic	Distal mucosa	
Oral prime:i.n. boost: =AttHRV/bhVLP2+ i.n. prime: oral boost: =bhVLP2+/AttHRV	Gnotobiotic pigs, 3–5 days of age	Orally inoculated with 5×10^7 FFU AttHRV i.n. vaccine with 2 doses of bhVLP plus mLT	i.n. boosts with 2 doses of bhVLP plus mLT Orally inoculated with 5×10^7 FFU AttHRV	At PID 28, challenged orally with $\sim 10^6$ $(ID)_{50}$ of virulent Wa HRV	Oral AttHRV/bhVLP2+: ↓ virus shedding and diarrhea indicating reduced severity of disease i.n. bhVLP2+/AttHRV: -Partial protection against clinical signs of disease -↓ duration of shedding and ↓ peak titers compared to the mLT and mock controls Oral AttHRV/bhVLP2+: -↑ virus-neutralizing titers in the serum relative to other groups	Pre-challenge: -Oral AttHRV/bhVLP2+ regimen ↑ gut IgM, IgA and IgG ASC relative to other groups Post-challenge: -Oral AttHRV/bhVLP2+ very effective in stimulating intestinal IgA ASC responses	Yuan et al. (2001)
Soluble OVA with CpG 2395 plus Polyphosphazene microparticle encapsulating OVA + CpG 2395	Less than 6 h of age	Oral gavage	i.p.-injected with 10 mg OVA plus IFA at 28 days old	N/A	-↑ anti-OVA IgG and IgG1 titers in serum relative to control group, -Trend showing ↑ serum anti-OVA IgM, IgA, IgG, IgG1 and IgG2 over time relative to i.p. control group.	-↑ anti-OVA IgA titers in BALF	Pasternak, et al. (In Press)
CT-B alone, CT-B linked to N-protein from PRRSV or Myc peptide	4-week old	Gavage directly into stomach cavity	Repeat gavage into stomach, 2×10 days apart	NA	-↑ serum IgG and IgA specific for CT-B, -N-protein specific serum IgG in ¾ animals and IgA in ¼ animals.	-CT-fusion constructs induced anti-CT-B IgA at the reproductive tract and in oral saliva, -No antibodies could be detected against N and myc	Hyland et al. (2004)
CT-B subunit + CT adjuvant, Or CT-B subunit + CT adjuvant conjugated to OVA	5–6 weeks old	Gavage directly into stomach	At 7–10-day intervals, repeat 2× more	N/A	-↑ serum anti-CT-B IgA and IgG titers -↑ anti-CT-B IgA titers in jejunum -OVA-CTB plus CT, ↑ serum anti-OVA IgM. -↑ IgA, IgG and IgM ASC in jejunum, -spleen showed ↑ IgM with some IgA and IgG ASC.	-↑ anti-CT-B salivary IgG titers -Modest ↑anti-CT-B salivary IgA titers	Foss and Murtaugh (1999)
A. pleuropneumoniae antigens ApxIA and ApxIIA expressed by replicating *S. cerevisiae*	3-week old	Oral (fed)	Oral, 2×, with 1-week intervals	Intranasal, 1.5×10^9 CFU of *A. pleuropneumoniae* serotype 5 Korean, 1 week after final vaccination	-Lower clinical signs of disease	-↑ IgA anti-ApxIA and ApxIIA in the nasal washes relative to the pig fed vector alone and untreated pigs	Shin et al. (2013)
E. rhusiopathiae expressing the C-terminal portion of the P97 protein of *M. hyponeumoniae*	10 days old, SPF	Oral (fed), 1.0×10^{11} CFU	Fed same dose for a total of 7 days	Intradermal, 5.0×104 CFU of *E. rhusiopathiae* Fujisawa strain, 10 days after the final day of vaccination Then 4 weeks after the final day of vaccination, pigs were challenged intranasally with 1.0×10^8 CCU *M. hyponeumoniae* for 3 consecutive days	After oral challenge with *E. rhusiopathiae*, inoculated group were protected from death and did not show any clinical signs of infection -After intranasal challenge with *M. hyponeumoniae*, inoculated group showed fewer lung lesions.	-↑ P97-specific IgG in BALF, but not IgA	Ogawa et al. (2009)
Live attenuated PRRSV alone with 100 or 1000 μg CpG ODN	20-day old	Intranasal	Intranasal booster 21 days later	NA	-↑ virus–specific IgG2 to IgG1 titers relative to piglets immunized with PRRSV alone -↑ anti-PRRSV-specific IgA in nasal secretions	-↑ anti-PRRSV specific IgA in saliva, and feces	Zhang et al. (2007)

from the local mucosa including their adhesion molecules (i.e. whether they are specific for the nasal-broncho-respiratory tract, digestive tract, etc.) could shed light on antigen migration. Thus, the review will be divided into sections discussing manuscripts which show direct evidence of induction of the common mucosal immune system (i.e. ASC in distal sites and/or induction of cell-mediated immunity) first then followed by manuscripts which only show indirect evidence (i.e. antibody titers). Within these categories, we will discuss non-replicating and replicating vaccines. Finally, please note that we will not include a discussion on the mammary-entero link nor the influence of mammary secretions on gut immune system; for a review on these subjects we suggest the following excellent publications (Bourges et al., 2007, 2008; Levast et al., 2014; Salmon, 2000; Salmon et al., 2009).

2. Direct evidence of induction of the common mucosal immune system

2.1. Non-replicating vaccines

2.1.1. Intranasal administration of rhoptry proteins from Toxoplasma gondii in the presence of Quil-A followed by on oocyte challenge via the oral route (da Cunha et al., 2012)

The parasite *Toxoplasma gondii* most commonly infects by the oral route and can trigger oral toxoplasmosis. da Cunha et al. (2012) immunized pigs intranasally with crude rhoptry proteins of *T. gondii* plus Quil-A (an inexpensive, widely used veterinary adjuvant), Quil-A alone, or saline (da Cunha et al., 2012). The pigs were inoculated intranasally at days 0, 21, and 42 and then orally challenged with 10^3 oocysts (VEG strain) at day 49. Immediately prior to challenge, two pigs immunized with the rhoptry proteins were euthanized and PBMCs and mLNs were harvested. mLNs and PBMCs were stimulated with rhoptry proteins ex vivo and mLN lymphocytes showed higher proliferation (a measure of cell-mediated immunity) relative to PBMCs but because only two pigs were euthanized, statistical analysis could not be performed. The remaining animals were euthanized at day 94 which was several weeks after challenge. From the vaccinated group, three out of the remaining four pigs had marginal IgG and IgA-rhoptry specific antibody titers in blood and they showed a partial protection from tissue cyst burden. They conclude that intranasal immunization with crude rhoptry proteins of *T. gondii* using Quil-A adjuvant in pigs was able to stimulate the common mucosal immune response and partially protect animals from brain cyst formation after oral exposure to *T. gondii* oocytes (da Cunha et al., 2012).

2.2. Replicating vaccines

2.2.1. Oro-nasal administration of attenuated Transmissible Gastroenteritis Coronavirus (TGEV) and porcine respiratory coronavirus followed by an oral TGEV challenge (Brim et al., 1995; Saif, 1996; Vancott et al., 1994)

Transmissible Gastroenteritis Coronavirus (TGEV) is a porcine coronavirus which, although it can infect the respiratory tract, primarily replicates in the intestinal tract causing severe diarrhea, dehydration and death (Brim et al., 1995; Saif et al., 1993). Porcine respiratory coronavirus (PRCV) replicates almost exclusively in the upper and lower respiratory tract of pigs and causes subclinical or mild respiratory infections (Brim et al., 1995; O'Toole et al., 1989; Pensaert et al., 1986; Saif, 1996; Stone et al., 1982). Pigs were inoculated oral-nasally with TGEV or with PRCV at 11 days of age and then piglets were challenged with TGEV by the oral route on day 35 (24 days ppi) (Vancott et al., 1994). Piglets that were oro-nasally exposed to TGEV developed diarrhea and shed virus from rectum with lesser shedding in the nasal passages (Brim et al., 1995; Vancott et al., 1994). These pigs were protected against clinical signs of disease after challenge. In contrast piglets immunized with PRCV showed no clinical signs of disease after primary immunization and they shed virus in nasal secretions only suggesting that PRCV replicated in the respiratory tract and not the gastrointestinal tract (Vancott et al., 1994). PRCV vaccinated animals were only partially protected from TGEV challenge.

The effects of the T cell populations were assessed. Piglets primed with TGEV responded to challenge with a striking increase in the percentages of CD2+, CD4+ and (to a lesser extent CD8+) T cells in mLN and duodenum and ileum (the major replicative sites for TGEV) with a limited increase in the bronchial lymph nodes (BLN) and spleen relative to the number of these cells found in age-matched unexposed pigs (Brim et al., 1995). Proliferative responses in mLN cells from PRCV-inoculated pigs were lower than those in mLN cells from both primary and secondary TGEV-exposed pigs. Despite the fact that the number of CD4+ T cells in the BLNs did not appear to increase in the groups of pigs primed with TGEV and PRCV, the magnitude of TGEV and PRCV-specific proliferation was highest in BLN cells in these groups immediately prior to challenge. In the BLN of PRCV-inoculated pigs, the proliferative responses generally remained high after TGEV challenge which correlated with protection against nasal virus shedding. In contrast, BLN cells from TGEV-reinoculated pigs showed a significant but transient decrease in proliferation that returned to a high level at harvest and these pigs were protected against virus shedding in nasal secretions after challenge. PRCV and TGEV-inoculated pigs as well as TGEV-challenged pigs did not show significant lymphocyte proliferation in response to PRCV or TGEVin any of the lamina propria cells purified from the duodenum, jejunum, and ileum.

When the humoral immune response was measured in pigs oro-nasally primed with TGEV, BLN, spleen, mLN and ileum showed a higher ratio of IgG to IgA ASCs with the highest IgG ASCs observed in the BLN (Vancott et al., 1994). Higher IgA to IgG ASC ratios were detected in the duodenum and jejunum prior to challenge (Vancott et al., 1994). Measurement of ASCs showed that the BLN and spleen had elevated IgA and IgG ASCs after challenge and the presence of IgA ASCs in the gut lamina propria of TGEV-primed pigs at the time of challenge correlated with complete protection against TGEV challenge (Vancott et al., 1994). The immune response generated in piglets primed through the oro-nasal route with PRCV was quite different from the response generated by piglets primed with TGEV. PRCV-exposed pigs had mainly IgG ASCs in BLNs with a limited number in the ileum. The duodenum and jejunum showed a very small number of IgA ASCs whereas other sites had virtually no IgA ASCs. After challenge with TGEV, five of twelve PRCV-primed pigs developed diarrhea and showed an increase in IgA ASCs in BLN, spleen, duodenum and jejunum. Very few IgA ASCs were detected in the ileum and none were detected in the mLN. More importantly, the number of IgG ASCs increased rapidly in the gut lamina propria and mLNs but not the BLN (which already had fairly high IgG ASCs). These data may indicate that PRCV-specific IgG ASC precursors in BALT of PRCV-primed pigs migrated to the gut in response to TGEV challenge, contributing to partial protection. Thus, unlike what has been shown in rabbits and rodents where GALT-derived IgA B cells are the major migratory cells among mucosal lymphoid tissues and which are thought to migrate more readily to BALT than the GALT (McGhee et al., 1992; Sminia et al., 1989), data from Vancott et al. (1994) indicate that virus-specific IgG ASC and memory B cells that rapidly accumulated in the gut lamina propria and mLN of PRCV-primed pigs after TGEV challenge most likely migrated from the BALT (Vancott et al., 1994).

2.2.2. Oral immunization with the Actinobacillus pleuropneumoniae (live or killed) and the impact on lymphocyte subset distribution in the bronchoalveolar space and ASCs in lavage fluid and sera (Delventhal et al., 1992; Pabst et al., 1995)

In these experiments, four month old German Landrace pigs from an SPF breeding farm were used (Delventhal et al., 1992; Pabst et al., 1995). A 'lavage' control group ($n = 5$) were lavaged at week one, five and six (without being immunized) (Delventhal et al., 1992; Pabst et al., 1995). Another 'non-immunization' control group was lavaged then infected by aerosol challenge and lavaged again one week later. The experimental pigs were lavaged followed by orally immunized with 10^{11} active or inactive (irradiated) bacteria (Delventhal et al., 1992; Pabst et al., 1995). All groups (with the exception of the lavage control group) were infected by aerosol challenge with 10^8 viable APP at week five then lavaged one week later. The experimental groups were lavaged at weeks seven and eight as well. Results showed that the nonimmunized pigs were severely ill after infection with APP and showed massive pathology at necropsy within one week after the infection whereas the animals immunized with viable or inactivated appeared clinically normal. The lavage control group showed no significant differences in the numbers of cells in bronchoalveolar (BAL) fluid, the differentials, or lymphocyte subsets. Relative to the animals orally immunized with inactivated bacteria, the pigs immunized with viable bacteria showed a trend toward higher numbers of nucleated cells and an increased proportion of granulocytes, a comparable 40% composition of macrophages, and a constant proportion of about 2–5% lymphoid blasts and plasma cells were found. In the BAL of normal pigs, plasma cells and blasts are rare but the percentage of plasma cells in BAL increased significantly after oral immunization with either viable or non-viable bacteria (Delventhal et al., 1992; Pabst et al., 1995). Immunization with either viable or no-viable bacteria triggered an overall increase in the total number of T cells but the ratio of CD4/CD8 cells remained constant. There was a significant increase in IgA+ ASCs in BALs after immunization with inactivated bacteria but there was a significant increase in IgG+ and IgM+ ASCs in pigs orally immunized with viable or nonviable bacteria. Thus, oral administration of either viable or inactivated *A. pleuropneumoniae* induced an influx of lymphocytes into the bronchoalveolar compartment of pigs, indicating a migration from the gut or lamina propria of the bronchi and trachea into the airways (Delventhal et al., 1992; Pabst et al., 1995).

2.2.3. Gnotobiotic piglets

Gnotobiotic pigs have altered intestinal structure (reduced villous length and increased crypt depth) and proinflammatory cytokine gene expression relative to conventionally reared piglets (Shirkey et al., 2006). These piglets show signs of clinical disease and pathology to Human Rotavirus (HRV) making them a valuable animal model in which to study the mucosal and systemic immune responses to rotavirus (Azevedo et al., 2010; Gonzalez et al., 2004; Iosef et al., 2002; Saif et al., 2003; Ward et al., 1996; Yuan et al., 2005, 1998, 1996).

2.2.3.1. Intranasal administration of rotavirus-like particles with mutant Escherichia coli heat-labile toxin (mLT) adjuvant in gnotobiotic pigs followed by oral challenge (Yuan et al., 2000). Gnotobiotic pigs at 3–5 days of age were administered vaccines via the intranasal route twice with a 10-day interval between immunizations. The vaccine antigens were comprised of Virus-like particles derived from bovine or simian strains (referred to as bsVLP)) with or without 5 μg of mLT (a mucosal adjuvant derived from *E. coli*) (Yuan et al., 2000) (When mLT is included in this vaccine, we will refer to it as bsVLP+. Therefore, pigs immunized twice intranasally with bsVLP without mLT will be referred to as bsVLP but if mLT is included, it will be referred to as bsVLP+. If the dose was repeated twice with mLT, it will be referred to as bsVLPx2+). Control pigs were mock inoculated with saline or mLT alone. At day 21, the IgM, IgA, and IgG ASC numbers in the duodenum and ileum from pigs immunized intranasally with bsVLPx2+ were 2- to 9-fold higher than those pigs immunized without mLT (bsVLPx2). Further, the mean numbers of IgM and IgG ASCs in the ileum of pigs i.n. immunized bsVLPx2+ were significantly higher than those immunized without mLT. These data suggest that intranasal administration of antigens with the mucosal adjuvant promoted a robust mucosal immunity in intestinal sites (Yuan et al., 2000).

On day 21, piglets were challenged orally with 10^6 infectious dose $(ID)_{50}$ of virulent Wa HRV. Compared to animal-specific prechallenge ASC numbers, pigs i.n. immunized bsVLPx2± mLT showed significant induction of antigen-specific ASC responses in the duodenum and ileum indicating induction of a memory response (Yuan et al., 2000). Remarkably, intranasal inoculation of pigs with bsVLPx2+ induced higher numbers of intestinal IgA and IgG ASC and memory B-cell responses than did three oral doses of live attenuated Wa HRV (Yuan et al., 1998, 2000; Yuan and Saif, 2002). Despite these responses, pigs immunized with bsVLP2x+ were not protected against diarrhea nor did they produce significantly enhanced virus-neutralizing antibody titers compared to the challenged control pigs. Clearly, intranasal immunization of gnotobiotic piglets triggered mucosal immunity when mLT adjuvant was included in the vaccine, but not protection.

To determine whether the magnitude of the immune response could be influenced by the number of doses or the subgroup specificity of the VLPs, three to five day old gnotobiotic pigs were vaccinated intranasally with three doses of bsVLP+ (referred to as bsVLP3x+) or three doses of VLPs from bovine strain RF (Labbe et al., 1991) and VLPs from virulent Wa human rotavirus in the presence of mLT (referred to as bhVLPx3+) (Yuan et al., 2000) with the second and third dose being administered at days 10 and 20. The ASC responses induced by the bsVLPx3+ regimen (i.e. with the bovine/simian VLPs) showed similar patterns in intestinal and systemic lymphoid tissues with no significant differences in magnitude compared to pigs i.n. inoculated twice with bsVLP+. Pigs i.n. immunized with bhVLPx3+ (i.e. with the bovine/human VLPs) induced 4- to 7-fold-higher mean numbers of IgG ASC but 6- to 16-fold-lower mean numbers of IgA ASC in the duodenum and ileum compared to bsVLPx3+ pre-challenge suggesting that the strain from which the antigen was derived (human or simian) impacts immunity. After challenge, the mean numbers of IgA and IgG ASC in the pigs i.n. immunized with bsVLPx3+ increased significantly compared to pre-challenge ASC numbers in most tissues, except IgG in the duodenum and IgG and IgA in bone marrow. In contrast, the mean numbers of IgA ASC in the bhVLPx3+ pigs increased significantly in the duodenum and ileum and the IgG ASC increased significantly in the mLN, spleen, peripheral blood, and bone marrow post-challenge suggesting that the source of antigens (human or simian rotavirus) impacts the immune response (Yuan et al., 2000). Again, despite these data showing immunogenicity of the vaccine antigens and induction of memory B cell in the ileum, pigs i.n. immunized with bsVLPx3+ or bhVLPx3+ failed to protect gnotobiotic pigs against rotavirus challenge nor were there significant differences in percentage and mean duration of virus shedding and diarrhea, mean days to onset of shedding, mean peak titer of virus shed, and mean cumulative diarrhea scores. The authors speculate that other studies with different doses, timing of vaccination and/or viral antigens may confer protection (Yuan et al., 2000).

2.2.3.2. Combined oral and intranasal vaccination routes with live attenuated or non-replicating antigens followed by oral challenge with virulent human rotavirus in gnotobiotic pigs (Yuan et al., 2001). Next, this research group explored whether stimulating both the GALT and NALT through a combination of oral and intranasal

vaccination routes would generate superior immunity against rotavirus challenge relative to an either vaccination route alone.

Oral prime:i.n. boost: at three to five days of age, piglets were given sodium bicarbonate to reduce stomach acid and then were orally inoculated with 5×10^7 fluorescent focus-forming units (FFU) of attenuated Wa HRV (AttHRV) followed by i.n. boosting on day 10 and day 20 with bhVLP plus mLT (which will be jointly referred to as AttHRV/bhVLP2+).

i.n. prime: oral boost: at three to five days of age, piglets were i.n. inoculated with bhVLP+ and this priming dose was readministered on ten days later. Twenty-one days post the first inoculation, pigs were given sodium bicarbonate then orally immunized with 5×10^7 FFU AttHRV (which is jointly referred to as bhVLP2+/AttHRV) (Yuan et al., 2001).

Both groups of piglets were orally challenged with virulent Wa HRV seven days after the past inoculation.

Pigs primed nasally then boosted orally (bhVLP2+/AttHRV) showed partial protection against clinical signs of disease, shorter mean duration of virus shedding and lower mean peak virus shedding titers compared to the mLT and mock controls after challenge with virulent Wa HRV. Pigs primed orally then boosted i.n. (bhVLP2+/AttHRV) showed significantly reduced virus shedding (42%) and diarrhea (50%) rates compared to the pigs vaccinated with mLT alone and mock control groups. Of the vaccinated pigs that did shed virus, they had shorter mean duration of shedding and cumulative fecal scores indicating reduced severity of disease.

Results also indicated that the combined vaccine regimens induced virus-specific IgM and IgA ASC responses in all lymphoid tissues, but especially in the intestinal tissues (Yuan et al., 2001). Pigs primed orally then boosted i.n. (bhVLP2+/AttHRV) had significantly higher IgM, IgA and IgG ASC responses in the intestinal lymphoid tissues than the pigs primed nasally then boosted orally (bhVLP2+/AttHRV) (Yuan et al., 2001). In fact, pigs primed orally then boosted i.n. (bhVLP2+/AttHRV) showed the highest mean numbers of intestinal IgA ASCs at challenge among all rotavirus vaccines in gnotobiotic pigs tested by this research group to date, including one to three oral doses of live attenuated Wa HRV and two or three intramuscular doses of inactivated Wa HRV with IFA (Yuan et al., 1996, 2000, 2001). These results suggest that oral priming with live attenuated Wa HRV followed by intranasal boosting with bhVLPs plus mLT was very effective in stimulating intestinal IgA ASC responses (Yuan et al., 2001). Further, pigs primed orally then boosted i.n. showed significantly higher virus-neutralizing geometric mean titers in the serum than all other groups (Yuan et al., 2001). They summarize that priming of the mucosal inductive site at the portal of natural infection with a replicating vaccine, followed by boosting with a non-replicating vaccine at a second mucosal inductive site stimulated the strongest B-cell responses in the intestinal mucosal immune system at challenge and conferred a moderately high protection rate against rotavirus disease (Yuan et al., 2001).

3. Indirect evidence of induction of the common mucosal immune system

3.1. Subunit or killed vaccines

3.1.1. Oral antigen exposure in newborn piglets induces oral immunity in response to systemic vaccination in later life (Pasternak et al. (in press))

For the first 24–36 h after birth, the gut of pigs exists in a semi-permeable state to facilitate uptake of maternal antibodies and other macromolecules and maternal cells (Lecce and Matrone, 1960; Nechvatalova et al., 2011; Nguyen et al., 2007; Rooke and Bland, 2002). Therefore, our laboratory investigated whether a subunit vaccine administered into the stomach cavity within 6 h of birth would allow an antigen to traverse the gut wall and induce oral immunity (and subvert induction of oral tolerance). Without negating stomach acid, piglets were gavaged with a two-part vaccine consisting of soluble OVA (0.5 mg or 0.05 mg) with 50 μg soluble CpG 2395 as well as a polyphosphazene microparticle (MP) encapsulating 0.5 mg OVA + 50 μg CpG 2395 (Pasternak et al. (in press)). Piglets were gavaged at less than 6 h of age with the idea that both the soluble OVA/CpG and the OVA/CpG within the MP would cross the gut wall. Polyphosphazene MPs are water-soluble and will dissolve over time to release the OVA/CpG thus acting like a prime-boost vaccine (Garlapati et al., 2010; Mutwiri et al., 2007, 2005). With the exception of the saline control group, all piglets were i.p.-injected with 10 mg OVA plus Incomplete Freund's Adjuvant (IFA) at 28 days of age and all piglets were euthanized at 49 days of age. The i.p. control group received a saline gavage but was boosted with the i.p. vaccination and therefore act as our primary systemic vaccine control group. Saline control piglets were gavaged and immunized with saline. In serum, we observed significantly higher anti-OVA IgG1 for the group gavaged with 0.05 mg OVA/CpG + MP relative to the saline control group over time and both oral vaccine groups showed higher mean titers over time relative to the i.p. control group for anti-OVA IgM, IgA, IgG, IgG1, and IgG2 although the results were not statistically significant. BALF were collected at time of death and results showed that piglets gavaged with the lowest dose of OVA/CpG + MP showed anti-OVA IgG and IgG1 titers that were statistically higher than the control group, but very low titers of anti-OVA IgA were detected in all groups (Pasternak et al. (in press)). Collectively, these results suggest that oral exposure to OVA triggers low level mucosal immunity at a distal site and they show agreement with similar trials performed in neonatal rat pups and lambs in that a significant local immune response is generated (Buchanan et al., 2013, 2012). Further studies must be undertaken to clarify the precise dose and duration of exposure required for induction of mucosal immunity as pigs grown into adulthood.

3.1.2. Gavage into stomach cavity with CT-B subunit mixed with or conjugated to PRRSV nucleocapsid protein or Myc peptide (Hyland et al., 2004)

Hyland et al., 2004 blocked then negated stomach acid production prior to administering a vaccine directly into the stomach cavity of four week old piglets three times at ten day intervals (Hyland et al., 2004). The oral vaccine was composed of CT-B subunit alone, CT-B co-administered with recombinant PRRSV nucleocapsid (N protein) or myc peptides, or recombinant proteins CT-B genetically fused with N protein or myc protein. CT-B-specific secretory IgA were detected in the intestinal mucosa from all groups but anti-IgA specific for N or myc were only detected in the intestinal mucosa of a minority of animals when fused with CT-B. When the peptides were simply mixed with CT-B, antibodies within the intestinal mucosa were not detected suggesting that linking peptides to CT-B was necessary to generate peptide-specific IgA at local mucosa. Serum antibodies were produced against the CT-B subunit in all pigs as well as weak titers of anti-CT-B antibodies in saliva and vaginal secretions, both of which are distal mucosal sites (i.e. the gavage bypassed the oral cavity and therefore saliva is not considered a local mucosal site). When N protein or myc were fused with CT-B, anti-N and anti-myc IgA antibodies were detected in the serum but not in the saliva or vaginal secretions. IgG anti-Myc was detected in the serum even without conjugation with CT-B, but not at the mucosal sites. Thus oral vaccination with CT-B was able to elicit distal IgA antibody responses in the saliva and reproductive tract, though the magnitude of the response was small relative to local mucosal antibody production and the response did not extend to the unlinked peptides. (Hyland et al., 2004).

3.1.3. Gavage into stomach cavity with CT and CT-B subunit co-administered with Keyhole limpet hemocyanin (KLH) or conjugated to Ovalbumin (Foss and Murtaugh, 1999)

Foss and Murtaugh (1999) blocked and negated stomach acid production prior to administering a vaccine directly into the stomach cavity of five to six week old pigs three times at seven to ten day intervals (Foss and Murtaugh, 1999). Five days after the final vaccine was administered, cells and mucosal secretions were collected. The vaccines consisted of CT (1–100 μg per animal) and recombinant CT-B (1 mg per animal) which did not result in diarrhea or any other clinical signs of toxicity. Oral CT-B alone induced statistically significant induction of anti-CT-B IgG and IgA titers in serum and jejunal mucosa but not in saliva (distal mucosa). When 100 μg CT was added to the vaccine, a robust induction of anti-CT-B IgA and IgG titers were induced in the serum, whereas an examination of the jejunal mucus showed induction of IgA titers and saliva showed induction of IgG titers. The salivary anti-CT-B IgA response also increased modestly. When the jejunal lamina propria was investigated for ASCs, it was found that it contained significantly more IgA-secreting cells than IgG or IgM secreting cells as well as significantly more IgA secreting cells than what was found in the blood and the spleen. The spleen contained more IgM ASCs than lamina propria or peripheral blood mononuclear cells (PBMCs) and less IgA and IgG ASCs than the other two tissues. The large number of IgA secreting cells in lamina propria suggested that anti-CT-B specific antibodies in jejunal mucus were produced in the local tissues.

Additional experiments were conducted to confirm the anti-CT-B IgA measured in intestinal mucus was produced locally. Animals were immunized three times with CT-B plus CT. The lamina propria contained >2000 anti-CT-B specific IgA and >500 anti-CT-B specific IgA ASCs per million total isolated cells. The relative amounts of anti-CT-B IgA and IgG in the adjacent mucus was reflected in the relative number of anti-CT-B IgA and IgG ASCs in the lamina propria, suggesting local production rather than a hematogenous origin. Anti-CT-B IgA and IgG ASCs were also detected in the spleen following oral immunization, but were much fewer in number than in the lamina propria and there was a trend toward increased IgG ASC relative to IgA ASC.

Next, the investigators evaluated whether keyhole limpet hemocyanin (KLH) co-administered with CT-B, or ovalbumin (OVA) conjugated to CT-B by glutaraldehyde in the presence or absence of CT could trigger mucosal and systemic immunity. Oral CT and CT-B resulted in anti-CT-B IgA in the serum but no KLH-specific ASCs were detected in the jejunal lamina propria cells (Foss and Murtaugh, 1999). Animals receiving OVA linked to CT-B had higher levels of IgM-anti-OVA ASCs in jejunum and serum anti-OVA IgM when administered with CT. Unfortunately; they did not report the effect on the distal mucosa (saliva). They conclude that CT is an effective oral immunogen and a safe oral adjuvant in swine at non-toxic doses but it promotes stronger mucosal immunity to linked antigens.

3.2. Replicating vaccines

3.2.1. Feeding (oral exposure) Saccharomyces cerevisia-coding for A. pleuropneumoniae antigens to pigs then challenging intranasally with virulent A. pleuropneumoniae (Shin et al., 2013)

Because the majority of efficacious oral vaccines are comprised of attenuated pathogens, researchers have used yeast such as *Saccharomyces cerevisiae* as live, replicating vaccine vectors to express protein subunits from pathogens of interest (Kim et al., 2010; Shin et al., 2005). Shin and colleagues cloned *A. pleuropneumoniae* antigens ApxIA and ApxIIA into *S. cerevisiae* (Shin et al., 2005). In Shin et al. (2013), three week old piglets were fed 1.5×10^9 Colony forming units (CFU) *S. cerevisiae* expressing ApxIA/ApxIa, *S. cerevisiae* (vector control), or they were left as untreated pigs (controls) (Shin et al., 2013). The oral vaccines were administered three times separated by weekly intervals and blood, nasal washes, and fecal samples were collected weekly. Piglets fed the vector coding for antigens showed significantly higher anti-ApxIA and ApxIIA IgA in the nasal washes relative to the pigs fed vector alone and untreated pigs. One week after the final oral vaccination, sera anti-ApxIIA IgA and IgG were significantly higher in the vaccinated pigs relative to the pigs fed vector control yeast. Representative pigs were challenged intranasally one week after final vaccination with 1.5×10^9 CFU of *A. pleuropneumoniae* (serotype 5 Korean). The vaccinated group showed lower clinical signs of disease compared to the control groups and they showed a reduced number of bacteria that could be reisolated from the lungs, although the reduction was not statistically relevant. The non-treated control and vector control groups showed microscopic lung changes indicative of alveolar wall congestion, inflammatory infiltration and alveoli were filled with edema fluid, and neutrophils indicating acute inflammatory responses. Thus, oral immunization with yeast coding for protein antigens showed a mucosal response in the respiratory tract mucosa which manifested as lowered clinical signs of disease.

3.2.2. Oral vaccination of pigs with a live Erysipelothrix rhusiopathiae vector coding for an Mycoplasma hyponeumoniae antigen then challenged by the intranasal route (Ogawa et al., 2009)

E. rhusiopathiae Koganei (65–0.15 strain) is a bacteria that colonizes the tonsils of pigs and which can be used to protect pigs against erysipelas. Ogawa et al. (2009) created recombinant *E. rhusiopathiae* bacteria expressing the C-terminal portion of the P97 protein of *M. hyponeumoniae* (Ogawa et al., 2009) for use as a vaccine. Eight 10-day old Specific Pathogen Free (SPF) pigs were administered a milk replacer containing live recombinant *E. rhusiopathiae* expressing the *M. hyponeumoniae* protein (1.0×10^{11} CFU/group/day) for seven consecutive days. As controls, two pigs were fed milk replacer containing no bacteria (but unfortunately a vector control group was not included). The pigs were challenged intradermally with 5.0×10^4 CFU of *E. rhusiopathiae* Fujisawa strain 10 days after the final day of vaccination. Results showed that the pigs orally inoculated with the *E. rhusiopathiae* KO-P97/53S strain were protected from death and did not show any clinical signs of infection (such as increased body temperature, skin lesions, reduced activity), whereas the control pigs developed typical signs of erysipelas and died or had to be euthanized within seven days. Five new control pigs (not orally vaccinated) and five pigs randomly chosen from the experimental group were challenged intranasally with a suspension containing 1.0×10^8 color-changing units (CCU) of *M. hyponeumoniae* on three consecutive days four weeks after the final day of vaccination. Four weeks following the challenge exposure, the pigs were euthanized and necropsied. The proportions of their lung surface with pneumonic lesions were determined and the results showed that the median of the proportions of lung surface with pneumonic lesions in the KO-P97/53S group was significantly smaller than those in the control group.

Another vaccination trial was undertaken wherein ten piglets were orally inoculated on three consecutive days with *E. rhusiopathiae* KO-P97/53S strain by feeding pigs with milk replacer containing the bacteria (1.0×10^{12} CFU/group/day). A further nine pigs were orally inoculated with *E. rhusiopathiae* Koganei 65–0.15 (1.0×10^{12} CFU/group/day) as vector controls. Thirteen days after the final vaccination, pigs were each challenged intranasally with a suspension of 2.0×10^8 CCU of *M. hyponeumoniae* on three consecutive days. Twenty-three days after the challenge exposure, the pigs were euthanized and necropsied. The concentration of P97-specific IgG antibodies in BAL fluid obtained at the time of necropsy was significantly higher in the vaccinated group than the control group. These data show agreement with previous studies

showing that vaccinated pigs have a greater concentration of *M. hyponeumoniae*-specific IgG than IgA titers in the BALF (Thacker et al., 2000). Thus, oral vaccination with a replicating pathogen protected pigs from bacteria administered intranasally and promoted induction of antigen-specific humoral immunity in BALF. The authors further showed that gnotobiotic piglets orally inoculated with *E. rhusiopathiae* KO-P97/53S showed evidence of bacteria dissemination to the lung and tracheobronchial lymph nodes after eight days. However, the authors also show that *E. rhusiopathiae* Koganei 65–0.15 strain orally administer to SPF pigs were disseminated to the tonsils (unfortunately they did not specify whether which part of the tonsil they were examining (the pharyngeal, the palantine, etc.)) but not the mesenteric lymph nodes or the lung (the only other organs investigated) after 15 days. These data support the possibility that within these vaccine trials, the bacteria did not disseminate and growth beyond the digestive cavity (Ogawa et al., 2009). Thus antibody titers in BALF are not likely present due to migration the bacteria from the oral cavity to the lungs at the time of infection.

3.2.3. Intranasal administration of attenuated PRRSV with CpG Oligodeoxynucleotide adjuvant (Zhang et al., 2007)

Zhang et al., 2007 immunized pigs intranasally with attenuated PRRSV alone or with CpG oligodeoxynucleotide (ODN) and evaluated the effect on the systemic and mucosal immune response (Zhang et al., 2007). The piglets were administered a primary intranasal vaccine at twenty days of age (reported as day 0), as well as an intranasal booster immunization 21 days later and then serum and secretions were analyzed up for 14 days (up to day 35 post primary immunization (ppi)). On day 35 ppi, piglets immunized with PRRSV plus 100 μg or 1000 μg CpG ODN had serum titers that were approximately 3-fold and 6-fold higher than the total virus-specific IgG in serum from piglets immunized with virus alone or virus plus 100 μg/ml GpC ODN (a control ODN). Further, inclusion of CpG ODN in the vaccine triggered higher overall virus-specific IgG2 to IgG1 titers whereas piglets immunized with PRRSV alone or with GpC ODN (a backbone control ODN) showed higher levels of PRRSV-specific IgG1 than IgG2 on day 35 post-immunization. Thus, the presence of CpG ODN influenced the antigen-specific IgG isotypes of produced. When PRRSV was administered alone or with GpC ODN, there was only weak anti-PRRSV IgA detected in the nasal secretions at day 21 and weak IgA in nasal secretions and oral secretions at day 35. No anti-PRRSV IgA was detected in the secretions from the saline control group for any time point. Saliva, feces and nasal secretions evaluated at day 21 and day 35 days ppi showed significant anti-PRRSV IgA in all three secretions when CpG ODN was included in the vaccine; the highest titers were observed with the highest concentration of CpG ODN. These data suggest that inclusion of the Toll-Like Receptor 9 agonist CpG ODN in an intranasal PRRSV vaccine may promote common mucosal immunity, provided of course that none of the vaccines administered intranasally were not swallowed.

4. Perspectives

The porcine systemic and mucosal immune responses are similar to their counterparts in humans, and immunological tools are available to assess both innate and acquired immunity relevant for vaccine studies. Indeed, a study by Dawson reported that the porcine immune system resembles that of humans for >80% of analyzed parameters, whereas mice were similar in <10% (Dawson, 2011). Pigs are important models for studying respiratory diseases, lung inflammation and cystic fibrosis (Ekser et al., 2012; Elahi et al., 2005; Meurens et al., 2012; Rogers et al., 2008) because the pig and human lung share many physiological features (Swindle et al., 1988) including extensive inter- and intra-lobular connective tissues (Gil et al., 2010). Due to differences in animal size, development and anatomical differences, rodents may not share similar route or dose of infection, disease transmission or periods of latency and therefore they may not be appropriate to predict vaccine efficacy in humans. However, the anatomy and timing of mucosal-associated lymphoid development can be conserved or divergent at distinct mucosal sites across species. For instance, the T-independent response of mouse peritoneal B1 cells has not been confirmed in other species (Bos et al., 1996; Reynolds and Morris, 1983). In rodents there is evidence of gut PP organogenesis development in utero (Adachi et al., 1997) and yet rodents are born with a minimal GALT and they lack circulating B and T cells (Friedberg and Weissman, 1974). In contrast and as detailed in (Levast et al., 2014), piglets are born with a relatively advanced GALT structure complete with Peyer's patches (PP) that undergo further expansion and maturation upon exposure to commensal flora and environmental antigens (Barman et al., 1997; Pabst and Rothkötter, 1999). Thus, conventionally raised piglets may have a gut mucosa that is sufficiently mature to respond to oral vaccines in the period immediately after birth, at least with humoral immunity. Even when comparing the mucosal immune system across large animal species, there are differences in anatomy and function in the mucosal tissues. Ruminants have ileal Peyer's patches that, like the avian bursa, are a primary lymphoid organ necessary for B cell development (Reynolds and Morris, 1983). In contrast, despite sharing similarities such as developing in utero and being comprised of a nearly continuous PP at birth, there is provide compelling evidence that in pigs, IPP are not a primary lymphoid organ but are instead a specialized secondary lymphoid tissue (Butler et al., 2011; Sinkora et al., 2011). In mice and humans, PP are considered important secondary lymphoid organs with B cell lymphogenesis occuring instead in fetal liver and then persisting in the bone marrow (BM) but (Cornes, 1965; Jung et al., 2010). Therefore, it is important not to assume that the common mucosal immune system exists in pigs based on evidence derived from laboratory animals and we instead should look for experimental evidence. Herein we provide some evidence for induction of the common mucosal immune system from local mucosa (i.e. oral or intranasal) to distal mucosa (saliva, genital tract, bronchus and gut, as appropriate) but as of yet we have not found evidence for linking other mucosal sites such as the conjunctiva, etc. Further studies must be undertaken to clarify the impact of mucosal immunization to all other mucosal sites. Once done, we then must define the precise dose and duration of exposure required for induction of mucosal immunity at local as well as distal sites. This knowledge will contribute to our understanding of the underlying mechanisms by which this vaccination strategy promotes immunity and it will have important implications for protecting against infectious diseases in the pigs.

Beyond being an excellent animal model for human immunology, pigs are part of a global food production industry and therefore their health positively impacts Global One Health. For an immunization regimen to be adopted, several factors such as whether the costs and toxicity are low, whether efficacy is high and whether the vaccine is easy to administer must be considered. While controlling costs are always a consideration for livestock operations, it is equally important to consider how effective the vaccine is, especially as pigs are an outbred population. Immunization may not trigger optimal immunity in all of the animals but if enough show a positive response, herd immunity may protect the rest. Further, ease of immunization has a tremendous impact on whether an immunization regimen will be adopted. For instance oral and intranasal immunization are a labor-intensive operation and intravaginal immunization will only protect the females of a litter. Piglets grow very quickly and subsequent booster vaccine will

necessitate that the piglets be snared and then physically or chemically immobilized prior to vaccination, regardless of whether the secondary vaccination takes place via the oral, intranasal or intravaginal routes. Importantly, in the process of breeding, gilts and sows undergo a lordosis response which naturally immobilizes the animals. At this time, oral, intranasal or intravaginal immunization could easily be performed without snaring and without the potential harm to the barn personnel. Again, this will only protect the females of the herd and to a lesser extent protect their offspring through passive immunization in colostrum and milk. All of these considerations must be weighed and found labor and cost-effective before mucosal immunization of pigs will be adopted.

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