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Immune responses in the uterine mucosa: clues for vaccine development in pigs

Pooja Choudhary[1†], Donaldson Magloire[1,2†], Glenn Hamonic[1] and Heather L Wilson[1,2*]

[1]Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan, Saskatoon, SK, Canada, [2]Department of Veterinary Microbiology Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada

The immune system in the upper reproductive tract (URT) protects against sexually transmitted pathogens, while at the same time providing immune tolerance responses against allogenic sperm and the developing fetus. The uterine environment is also responsive to hormonal variations during the estrus cycle, although the most likely timing of exposure to pathogens is during estrus and breeding when the cervix is semi-permissive. The goal for intrauterine immunization would be to induce local or systemic immunity and/or to promote colostral/lactogenic immunity that will passively protect suckling offspring. The developing fetus is not the vaccine target. This minireview article focuses on the immune response induced in the pig uterus (uterine body and uterine horns) with some comparative references to other livestock species, mice, and humans.

1 Introduction

Unlike many other large livestock animals, pigs are not immobilized in headgates during handling; therefore, delivery of vaccines can be difficult and a potential safety hazard, especially in terms of the risk of needle-stick injuries (1). Before immunization can take place, the animals need to be snared or moved into crates, both of which require more than one person. Like other agriculture sectors, the pig industry is experiencing a labor shortage, and novel ideas to eradicate inefficiencies within the barns may help bridge these gaps. To that end, vaccines that can be administered without needles and at a time when animals are immobilized (without the need for multiple workers) would be well-received by the swine industry. Sows and gilts are bred during estrus when the cervix is semi-permissive and, upon being exposed to the boar or boar pheromones, they become temporarily rigid in preparation for mounting, referred to as lordosis (2). Because they are temporarily immobilized, the sows and gilts are safe to immunize and other barn personnel are not required to snare the animals, meaning that two tasks can be accomplished at once

(breeding and immunization) with reduced laborers. Further, because breeding takes place more than two times per year, the timing of intrauterine immunization is optimal for induction of immunity (3–5). Reports on the innate and adaptive immune response in the uterus support the hypothesis that the uterus may be amenable to an immune induction site, although care should be taken to avoid induction of non-tolerizing response to sperm and seminal fluid constituents.

2 Innate immune responses in the uterus and uterine horns

It has lately been appreciated that the uterus and uterine horns have their own microbiome (6). The gut microbiome in the sow can be modulated in response to antimicrobials, reproductive stages, feed and supplements, pathogen exposure as well as vaccines (reviewed in (7)). The impact of these factors on the sow uterine microbiome needs to be explored, as does the potential impact that an intrauterine vaccine may have on the microbiome of the uterus and other sites.

The uterine microenvironment is under immune surveillance and is reactive to foreign antigens (8). Sperm deposited in the uterus triggers a natural immune response, which serves both to clear the uterus of excess sperm (9, 10) as well to accommodate the embryo for implantation (11). Studies in livestock bred by AI showed that spermatozoa, seminal plasma, and extender trigger rapid and transient neutrophil infiltration into the lumen and an inflammatory response, complete with cytokine and chemokine induction (12–15). Studies in pigs show that semen extender and seminal plasma alone induced interleukin (IL)-10, transforming growth factor (TGF)-β, IL-8, and tumor necrosis factor (TNF)-α gene expression but that when combined with spermatozoa, the expression of these genes is reduced (16). Sperm has been shown to promote immune tolerance via signaling through Toll-like receptor (TLR) 4 (17, 18). Furthermore, seminal fluid antigens activate regulatory T cells in the uterus draining lymph node, which dampens the immune response in the uterus to tolerate the antigens present in the embryo (19).. Others show that semen extender triggers induction of granulocyte-macrophage colony-stimulating factor (GM-CSF) and a corresponding increase in major histocompatibility complex (MHC) class II-positive cells in the uterine lamina propria and directly basolateral to the epithelial layer (20). The extent to which a vaccine coupled with artificial insemination will influence the sperm and seminal fluid's role in evoking or dampening an immune response has not been fully elucidated.

Recruitment of antigen-presenting cells (APC) to the lumen or the uterine tissue may be key to an effective intrauterine (i.u.) vaccine that is delivered in combination with a semen extender. Boar semen combined with three adjuvants (poly I:C, host defense peptide, and polyphosphazene (Triple Adjuvant; TriAdj)) administered to the uterus was shown to trigger changes in localized gene expression and cellular recruitment *in vivo* and greatly increased the number of neutrophils in the uterine lumen

(12, 21). These data suggest that sperm, semen, semen extender, and adjuvants may augment local immune response and, therefore, may influence the immune response to intrauterine vaccines. Our results in pigs have shown that vaccines coupled with sperm at the time of breeding does not affect reproduction, but the effect of multiple breedings/vaccinations should investigate whether anti-sperm antibodies do increase (22). Caution should be used to formulate the vaccine so that it does not trigger the induction of anti-sperm antibodies, which may cause infertility (23).

3 The uterus as an immune induction site

Intrauterine immunization is a novel approach and currently, published data have been limited to rodents and pigs. In mice, prior i.u. exposure to live *Chlamydia trachomatis* (Ct) generated protective immunity against subsequent challenge, suggesting that the uterus can act as an immune induction site (24, 25). Further, immunization with ultraviolet light (UV)-inactivated Ct (UV-Ct) complexed with charge-switching synthetic adjuvant biodegradable nanoparticles (cSAPs) elicited long-lived protection in conventional and humanized mice (24). Mice immunized with UV-Ct alone generated regulatory T cells and an accumulation of tolerogenic CD11b⁻CD103⁺ dendritic cells (DCs) that exacerbated subsequent Ct infection, whereas mice immunized with UV-Ct-cSAP exhibited elevated immunogenic uterine CD11b⁺CD103⁻ DCs that led to effector T cells seeding the uterine mucosa with resident memory T (T$_{RM}$) cells (24). These data suggest that the inclusion of mucosal adjuvants may be critically required for protective i.u. immunization.

The hormonal state may also impact the immune response as although DCs in the decidua of pregnant mice retain responsiveness to pro-inflammatory stimuli and migration capacity towards CCL21, these cells are prevented from trafficking to the draining lymph node (LN), possibly to promote T cell tolerance to fetal antigens (26). These data suggest that DCs may have region-specific or timing/hormone-specific responses. Understanding the mechanism of action of adjuvants and the cells they target should continue to be a research focus for i.u. vaccines.

Recent studies in pigs show that the uterus can act as a site of booster immunization and/or as an immune induction site. In a previous study, sows that had been immunized repeatedly with the Porcine ParvoShield L5E Swine Vaccine® (Elanco Animal Health) by the intramuscular (i.m.) route at each parity were bred with semen alone or semen plus BEI-inactivated porcine parvovirus (1 × 10⁷ TCID₅₀ PPV (NADL-7) formulated with a combination adjuvant (TriAdj; host defense peptide, poly I:C, and polyphosphazene) (21). This vaccine was not spermicidal. Positive control sows received i.m. ParvoShield® vaccine as they entered farrowing crates. Serum antibody titres against viral protein 2 (VP2, one of the capsid proteins of PPV) were comparable between the positive control sows and sows immunized by the uterine route, suggesting that the uterus could act as a site of booster immunization to an inactivated virus (21). When sows were bred

with the immunogenic recombinant spike protein from a porcine epidemic diarrheal virus (rPEDVS) plus TriAdj adjuvants, the anti-PEDVS serum and uterine antibodies were low in the i.u.-vaccinated gilts (22). These data suggest that a single primary immunization delivered into the uterus may not be sufficient to evoke a systemic or mucosal humoral immune response (21). Importantly, there was no difference in the viable fetus/corpus luteum ratio after 30 days between i.u.-vaccinated and control sows, suggesting that the i.u. vaccines did not impact fetal development.

To assess whether multiple i.u. vaccines could trigger a robust immune response, gilts were bred with heat-inactivated extended semen containing rPEDVS formulated with TriAdj. The gilts returned to estrus after 21 days and they were rebred with the same inactivated semen and vaccine, suggesting that i.u. immunization did not impact hormonal cycling. When they returned to estrus again, they were bred with live semen plus the vaccine (22). Control gilts were administered semen alone at second estrus following common industrial breeding practices. Litter weights and the number of live to non-viable piglets were comparable, indicating that the three-times-administered i.u.-vaccine did not appear to impact fertility. The i.u.-vaccinated gilts showed significant PEDVS-specific serum, colostral, and uterine antibody titers, and low-level colostral PEDVS-neutralizing antibodies. Serum from piglets born from i.u immunized gilts showed increased antibody titers compared to control piglets (22), which showed that i.u vaccines can induce higher maternal antibodies. Piglets born to i.u.-vaccinated gilts received partial passive protection from PEDV infection 3 days after birth but eventually succumbed to the disease (22). Collectively, these data indicate that the porcine uterus could act as an immune induction site, but that more than one dose is needed, at least when TriAdj is used as the vaccine adjuvant (22). In a follow-up trial, the rPEDVS vaccine was formulated with polymeric poly-(lactide-co-glycolide) (PGLA)-nanoparticle (NP) including a muramyl dipeptide analog and a monophosphoryl lipid A (MPLA) analog as adjuvants (NP-PEDVS) (27). The gilts responded with significant induction of serum anti-PEDVS-IgG following the single dose after 30 days, suggesting that an NP vaccine may be suitable for primary i.u. vaccination. Collectively, these experiments indicate that the pig uterus can act as an immune inductive site when the vaccine is administered at breeding, but that the use of robust adjuvants (that are formulated to not be spermicidal) may be critical to vaccine efficacy. In addition to rodents and pigs, it would be interesting to see the results on other animals that breed via AI such as cattle and horses.

Intradermal (i.d.) vaccination is also an alternative to i.m. immunization which results have shown can induce a comparable or better immune response (28). In some instances, i.d. immunization requires needles administered using the Mantoux technique, which requires 5–15° injection angle into the skin going approx. 1 mm deep (29–31). This technique requires persons be trained and it can be difficult to use on a non-anesthetized animal (31, 32). Administration of i.d. vaccines using bifurcated needles or multipuncture devices can be complicated by uneven antigen delivery (33). In contrast, i.u. immunization takes advantage of breeding practices and does not require specialized skills from the administrator.

4 Mechanism of action

4.1 Uterine epithelial cell pattern recognition receptors

As in other mammals, the porcine uterus/uterine horn is lined with a single layer of simple columnar cells with tight junctions between the cells to control passage, and the tight junctions are regulated by the hormonal state, cytokines, growth factors, TLR agonists, and pathogens (34–36). The underlying endometrial layer has a superficial functional layer (*stratum functionale*) and a deeper basal layer (*stratum basale*) with glandular epithelial cells forming tubular glands that spiral into the tissue (37, 38). The endometrium undergoes changes in the branching of the glands and growth, including changes in endometrial thickness and epithelial cell height in response to the estrus cycle (37, 39, 40).

The epithelial and endometrial layer of the uterus expresses several pattern recognition receptors that may play a role in the uterine immune response. TLRs are membrane-spanning receptors on the uterine epithelial cell surface that identify the pathogen-associated molecular patterns (PAMPs) present in bacterial, fungal, and viral pathogens and initiate innate immune responses (41, 42). Analysis of mRNA expression levels performed on isolated and cultured pig primary uterine epithelial cells showed that TLRs 1-7 and 9, NOD1, NOD2, NLRP3, NLRP6, NLRX1, RIG1, MDA5, and LGP2 are expressed (43). Polarized primary uterine epithelial cells (UECs) stimulated with TLR3, TLR4, and TLR9 ligands showed induced secretion of IL-6, IL-13, and IL-10, respectively, indicating that these receptors were functional (43). Polarized uterine epithelial cells stimulated with a TLR3 agonist showed increased expression of interferon (IFN)-β, TNF-α, IL-8, CCL2, CCL3, CCL4, and CCL-20 (21). Further, laser-captured uterine epithelial cells obtained one day after being bred with semen plus an adjuvant cocktail containing a TLR3 agonist showed significantly increased CCL2, suggesting that pig uterine epithelial cells are responsive to immune stimuli (21). Immunohistofluorescence and immunofluorescence performed on pig uterine tissue and in polarized pig primary UECs indicated that TLR3 and TLR9 localizes to the apical cell surface, whereas TLR4 localizes to the intracellular space (43). Surface localization of TLR3 in pig uterine epithelial cells shows agreement with uterine epithelial cells in humans (44, 45) and rabbits (46); continued research in this area shows that the 'canonical' localization patterns of TLRs may not be conserved across the cell and/or tissue types and may also vary in response to stimulation, age, disease, or cellular environment (reviewed in (43)).

4.2 Immune cells in the uterine lumen and endothelium

Cells in the uterine lumen and endometrium are sensitive to changes in the hormonal environment. We limited the scope of this mini-review to the estrus cycle when the cervix may be permissive to vaccines. Immune cells in the endometrium are primarily

lymphocytes, with some macrophages and APCs and large numbers of neutrophils; these levels tend to be at their highest during estrus (38, 40). Macrophages, DCs, lymphocytes, and granulocytes migrate from the blood to subepithelial tissue where they may persist (20, 47). Neutrophils are found close to the basal lamina of the surface epithelium and the subepithelial capillaries at pre-estrus and estrus (48, 49). They migrate into the uterine lumen after breeding where they eliminate a large number of spermatozoa and microbes present in boar semen (47, 50) and they usually die after 24 hours (20).

Uterine APCs, such as macrophages and DCs, are present throughout the endometrium during estrus; however, at other stages of the estrus cycle, they are found deeper in the lamina propria and rarely reside directly below the surface epithelium (40, 51). In mice, macrophages can be identified by the expression of the cell surface F4/80 and can be positive or negative for the surface marker CD11c (52, 53).. Macrophages are the most abundant professional APC in the human uterus (54). DCS can be characterized into 3 major subsets: plasmacytoid DC (pDC), conventional DC1 (cDC1), and conventional DC2 (cDC2) populations based on cell-surface markers and transcription factors such as interferon regulatory factors 8 and 4 (IRF8 and IRF4) (55–57). Once DCs capture antigens, they become mature and migrate to lymphoid structures to present antigens to T cells (58). DCs in the uterus are characterized as having high levels of major histocompatibility complex class II. In mice, cDC1 cells in nonlymphoid tissue, such as the uterine tissue, can be either $CD103^+CD11b^-$ cDC1 and $CD11b^+$ cDC2 (59). $CD103^+$ cDC1 has two principal functions, i.e., priming $CD8^+$ cells by cross presentation and induction of tolerance (60). pDC1 cells mainly produce high type I IFN in response to viral infection (61) whereas $CD11b^+$ cDC2 drives the $CD4^+$ T helper 2 (TH2) and 17 (TH17) response (62). In pigs, subsets of DCs are identified and characterized as cDC1: $CD135^+CD14^-CD172a^{low}CADM1^+wCD11R1^+$ cells; cDC2: $CD135^+CD14^-CD172a^+CADM1^+CD115^+wCD11R1^+CD1^+$ cells; and pDCs: $CD4^+CD135^+CD172a^+CD123^+CD303^+$ (63).

In pigs, plasma cells are dispersed throughout the endometrium with a predominance of IgG-secreting plasma cells (38, 38). The most prevalent cell type at all stages of the estrus cycle is the $CD2^+$ cell (48) with $CD8^+$ cells being present more frequently than $CD4^+$ T cells in the surface epithelium compared to the $CD4^+$, and more $CD4^+$ cells than $CD8^+$ cells in the glandular connective tissue (51). In the connective tissue of the subepithelial layer, there is no significant effect of the estrus cycle stage on the numbers of $CD2^+$, $CD4^+$, and $CD8^+$ cells (49).

4.3 Antigen presentation

For an immune response to occur, APCs need to internalize the antigen, process it, and present it to T cells on MHCI or MHCII proteins (64, 65). The quality and direction of the adaptive response depends on how the APCs react to the adjuvant, leading to the secretion of select cytokines (66, 67).. In the human uterus, antigen presentation on MHC class II can be performed by professional APCs as well as uterine epithelial cells (68, 69). In contrast, in pigs,

SLA-DRA gene expression was not detected in any uterine epithelial cells, indicating that pigs do not express the porcine equivalent of MHC class II (21). These data suggest that there are species-specific differences between the roles of epithelial cells in immune activation in the uterus and that pig uterine epithelial cells do not act as APCs.

The upper reproductive tract does not contain the mucosal-associated lymphoid tissue (MALT) thought to be critically required for the induction of an immune response (70). There is limited evidence that the pig's upper reproductive tract has lymphoid aggregates that may be acting as a limited MALT. One study showed that in 3 out of 6 sows studied, aggregations of lymphocytes were noted in the subepithelial connective tissue of the cervix (https://stud.epsilon.slu.se/12300/1/edstrom_k_171031.pdf). Another study referred to an unpublished observation that reported 50% of ancestral multiparous sows had a few small lymphocyte aggregations in the endometrium at weaning (71). Other studies show that lymphoid aggregates are present throughout the uterus, usually within the surface and in the glandular epithelium, and vary in size throughout the estrous cycle stages (72, 73). In the glandular epithelium, $CD4^+$ cells are absent, $CD8^+$ cells typically increase during estrus whereas $CD2^+$ cells are at most during estrus and early diestrus. On the surface epithelium, more $CD4^+$ are found over $CD8^+$ cells during estrous, whilst $CD2^+$ cells are in high numbers both during estrus and early diestrus (73).

The human uterus has lymphoid aggregates in the endometrial tissue at the basal and functional area of the uterus near the uterine epithelial glands (74). These aggregates are comprised of $CD19^+$ B cells surrounded by numerous (and primarily $CD8^+$) T cells and an outer layer of monocytes or macrophages (75, 76). The size of the lymphoid aggregates varies with the estrus cycle. It appears to be the largest during the secretory and proliferative phases; this is consistent with an increase in the immune cell trafficking into the endometrium, which may contribute to the larger lymphoid aggregate in the secretory phase (75, 77).

In the subepithelial stromal layer of the endometrium of the bovine uterus, isolated lymphoid nodules and aggregates predominantly comprised of clusters of B and T-lymphocytes have been reported (78, 79) (80). B lymphocytes were also observed as a small aggregate deep in the stroma or adjacent to blood vessels in the myometrial layer of the uterus, possibly recruited to the mucosal surfaces in response to the chemokines secreted during infection (81–83). These studies indicate there may be species-specific differences in how vaccines in the uterine lumen mediate the immune response, and/or it is possible that uterine vaccination would require transport of the vaccine across the epithelial barrier.

4.4 Transport of molecules across the epithelial barrier

Tight junctions between UECs limit the transport of molecules across the epithelial barrier. The predominant method for transporting macromolecules across an epithelial cell wall would be through pinocytosis, which involves transporting

macromolecules into or across the cell using vesicles. Pinocytosis is a form of endocytosis that is not receptor-mediated and is therefore non-specific. Pinocytosis involves the internalization of the plasma membrane to form a vesicle that contains extracellular fluid, and any molecules present in that fluid. Studies in other epithelial cell barriers such as in alveoli and the intestine have shown that pinocytosis occurs in a non-specific fashion and transports macromolecules across the epithelial cells at a rate proportional to their size (84) and that negatively charged nanoparticles are more efficiently transported (85). Although there is limited data on the mechanisms of pinocytosis by UECs and how the size or charge of particles impacts their transport, there is evidence of molecules being transported in a luminal to basolateral direction, which could be used by intrauterine vaccines (86).

Another mechanism of transport across the uterine epithelial cell barrier may include receptor-mediated transport using antibody transporters. For example, despite its name, neonatal Fc receptor (FcRN) is expressed by both porcine and human UECs into adulthood (87, 88). IgG can be bi-directionally transported between neutral environments through FcRN-mediated transport (89, 90). The transfer of IgG by FcRN in the human female reproductive tract has been confirmed (87). It is possible that by binding to the vaccine antigen, FcRN-IgG transportation could deliver the antigen and possibly the associated vaccine components across the epithelial wall. Further, polymeric immunoglobulin receptor (pIgR)-mediated transport for the secretion of sIgA has been well described and is known to be carried out by uterine epithelial cells (88), although IgA is not the predominant immunoglobulin secreted into the uterine lumen. Thus, antibodies bound by these receptors could transport coupled antigens across the epithelial barrier. Future studies should investigate where the vaccine components localize and are taken up by innate immune cells when they are formulated as soluble or particulate vaccines.

A summary of how intrauterine immunization can impact the pig industry and factors that may influence immune activation are presented in Table 1.

5 Discussion

The uterus is known to exhibit inflammatory responses and there is evidence that it can act as an immune induction site. Studies are needed to determine whether intrauterine vaccines trigger immune cell recruitment into the lumen, which is critical for induction of immunity, or whether the antigen traverses the uterine wall to trigger immunity. How the antigen traverses the uterine wall (i.e., via paracellular transport or transcytosis, uptake by dendritic cells extending dendrites into the lumen, etc.) and whether the antigen is presented to draining LNs or lymphoid aggregates should also be investigated. Once it is clear how the uterus acts as an immune induction site, vaccines can be formulated to exploit this mechanism of action. Coupling breeding with vaccination should reduce the number of personnel required for handling and would not require any special training, making it a potentially important new route of immunization for the pig industry. The effect of multiple rounds of i.u. immunization on

TABLE 1 Impact of intrauterine immunization.

Impact on industry		Factors that may influence immune activation
Positive attributes	Potential challenges	
•Needle-free •Safe to immunize during lordosis response •Coupling breeding with immunization reduces labor requirements	•Assess long-term impact on fertility •Must ensure sperm are not targeted and no negative effect on microbiome	•Uterine epithelial cell pattern recognition receptors •Hormonal changes in transport receptors, cell numbers, and cell localization •Site of immune activation is not yet clear. •Uterus has limited lymphoid aggregates instead of MALT •UECs do not present antigens to T cells

sperm tolerance and the uterine microbiome must be investigated further, with each new vaccine formulation.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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