EVALUATING THE INNATE IMMUNE RESPONSE OF THE PORCINE UTERUS TO VACCINE ADJUVANTS FOR THE DEVELOPMENT OF AN INTRAUTERINE VACCINE

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By

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ABSTRACT

Mucosal vaccination is a widely sought after method of vaccination for both its ease of delivery and because it can stimulate a strong local mucosal immune response. As the vast majority of pathogens enter the body through mucosal surfaces, a strong mucosal immune response has the potential to eliminate pathogens before them crossing the epithelial barrier, and can, in the case of the uterus, protect the developing fetuses from pathogens. For a mucosal vaccine composed of inactivated antigens to be effective, it must be formulated with potent adjuvants that will initiate a strong immune response in the uterus. This thesis aimed to evaluate the capacity of uterine epithelial cells to respond to vaccine adjuvants and to subsequently initiate and modulate the local uterine immune response of an intrauterine vaccine in sows. Because uterine epithelial cells (UECs) are the first point of contact for an intrauterine vaccine, we established an *in vitro* model using primary uterine epithelial cells to enable us to evaluate what receptors are present on these cells that could be targeted by the adjuvants in an intrauterine vaccine. Previous research showed that a triple adjuvant combination including polyI:C, a host defense peptide (HDP) and polyphosphazene (PCEP) administered as part of a mucosal vaccine induced strong humoral immunity. We tested these adjuvants as well as muramyl dipeptide and LPS alone and in combination with each other in the *in vitro* system. *In vitro* stimulations resulted with polyI:C alone and in combination with other potential adjuvants induced significant pro-inflammatory cytokines and chemokines, which was not further improved by the inclusion of HDP and PCEP. To determine if the observed response *in vitro* resulted in similar responses *in vivo,* the triple adjuvant combination was administered *in vivo* into the uterus of sows in combination with a standard semen dose. Twenty four hours post-breeding, epithelial cells had significantly increased CCL2 expression in response to the adjuvants combined with semen compared to the response

generated to semen alone. Additionally animals bred with adjuvants included in the semen dose had a significant decrease in the proportion of γδ T cells and monocytes in the blood, however, there was no significant increase of these cells in the lumen indicating the γδ T cells were not being recruited into the uterine lumen. Following evaluation of infiltrated monocytes and macrophages in the tissue, no significant difference was observed in these cells levels compared to animals bred with semen alone. Lastly, returning to the *in vitro* UEC model, I took steps to quantify whether secretions from stimulated uterine epithelial cells impacted monocyte to DC differentiation or moDC activation. Secretions from triple adjuvant-stimulated uterine epithelial cells were not able to induce recruitment of monocytes nor did the secretions impact on the moDCs capacity to endocytose antigen, mature in response to stimulation or induce proliferation in T cells. The established primary UEC culture system established and characterized was observed to closely resemble the behaviour of UECs *in vivo* for studying the responses of adjuvants and could be further utilized for continuing the develop UECs role in the uterine immune response to stimulants and pathogens. Although uterine epithelial cells respond to vaccine adjuvants, the magnitude of this response is unable to significantly impact the immune response to vaccine adjuvants delivered by the intrauterine route in a semen dose.

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TABLE OF CONTENTS

PERMISSION TO USE i
ABSTRACT ii
ACKNOWLEDGEMENTS iv
TABLE OF CONTENTS v
LIST OF TABLES vii
LIST OF FIGURES viii
LIST OF ABBREVIATIONS x
1 INTRODUCTION AND LITERATURE REVIEW 1
1.1 The porcine reproductive tract 1
1.1.1 Gross and microscopic anatomy 1
1.1.2 Estrus cycle 5
1.1.3 Breeding in the swine industry 8
1.1.4 Early pregnancy and placentation 10
1.1.5 Transport of molecules across the epithelial barrier 12
1.2 Immunology 15
1.2.1 Cells of the immune system 15
1.2.2 Tissues of the immune system 25
1.2.3 Innate immunity 27
1.2.4 Epithelial cells in innate immunity 29
1.2.5 Toll-like receptors 31
1.2.6 NOD-Like receptors 33
1.2.7 RIG-I-Like receptors 34
1.2.8 C-type lectin receptors 35
1.2.9 Cytokines 36
1.2.10 Chemokines 37
1.2.11 Antigen uptake and presentation 39
1.2.12 Hormonal control of the immune response 41
1.3 Vaccines 42
1.3.1 Mucosal vaccines 43
1.3.2 Vaccine adjuvants 44
1.3.3 Delivering intrauterine vaccines in sows 52

2 HYPOTHESIS AND RESEARCH OBJECTIVES ... 54
3 CHARACTERIZATION OF PORCINE UTERINE EPITHELIAL CELLS AND ESTABLISHMENT OF A POLARIZED CULTURE SYSTEM. ... 55
3.1 Introduction ... 57
3.2 Materials and Methods ... 59
3.3 Results ... 68
3.4 Discussion ... 80
3.5 Supplemental Figures ... 86
3.6 Transition statement ... 87
4 THE INNATE IMMUNE RESPONSE IN POLARIZED UTERINE EPITHELIAL CELLS TO VACCINE ADJUVANTS ALONE AND IN COMBINATION. ... 88
4.1 Introduction ... 90
4.2 Materials and Methods ... 92
4.3 Results ... 101
4.4 Discussion ... 111
4.5 Supplemental Figures ... 118
4.6 Transition statement ... 121
5 MONOCYTE RECRUITMENT AND DIFFERENTIATION IN RESPONSE TO UTERINE EPITHELIAL CELL SECRETIONS *IN VITRO*. ... 122
5.1 Introduction ... 124
5.2 Materials and Methods ... 125
5.3 Results ... 132
5.4 Discussion ... 142
6 GENERAL DISCUSSION AND FUTURE DIRECTIONS ... 147
REFERENCES ... 154
APPENDIX ... 172
A.1 Additional publications during my studies ... 172
A.2 Oral presentations given during my studies ... 182
A.3 Scholarships and Fellowships received during my studies ... 182

LIST OF TABLES

Table	Page
Table 3-1. Primer names, sequences, and annealing temperature for characterization of uterine epithelial cells.	64
Table 3-2. Antibody targets and concentrations used for IHF and immunofluorescence assays.	66
Table 3-3. Antibodies and recombinant standards used by Bioplex ELISA.	68
Table 3-4. Mean fold changes and standard deviations of fold changes relative to LN expression presented in Figure 3-2.	73
Table 4-1. Primer names, sequences, annealing temperature, and target sequence used in all qPCR experiments.	96
Table 4-2. Antibodies used in FCM analysis, final concentrations, and suppliers.	99
Table 5-1. Antibodies, final concentrations, and suppliers of all flow cytometry antibodies used.	130

LIST OF FIGURES

Figure 1-1. Diagram of the porcine reproductive tract.	1
Figure 1-2. Schematic of the porcine estrous cycle displaying the relative hormonal levels through each stage of the cycle.	7
Figure 1-3. Haematopoietic cell pathways in generating the cells of the immune system.	16
Figure 1-4. Innate immune roles that have been described for epithelial cells.	31
Figure 1-5. Schematic demonstrating the mode of action of adjuvants in generating an immune response.	46
Figure 3-1. Representative IHF of UT and IF of UECs for tight junction proteins.	70
Figure 3-2. Relative expression of several PRRs by LC-UE, UT, and TES compared to LN mRNA expression.	72
Figure 3-3. Relative expression of several PRRs by primary UECs compared to PBMCs	75
Figure 3-4. Representative IHF of UT for select TLRs.	77
Figure 3-5. Representative IF of primary UECs on transwell membranes for select TLRs.	78
Figure 3-6. Cytokine secretion of primary UECs in response to stimulation with polyI:C, LPS, CpG, and MDP.	80
Supplemental Figure 3-1. H&E staining of UT pre (A) and post (B) enzymatic digestion with pancreatin and dispase at 4°C overnight.	86
Supplemental Figure 3-2. Frozen UT slices were cut to a thickness of 14µm and stained with cresyl violet.	86
Figure 4-1. Schematic timeline of hormonal synchronization method for fixed time artificial insemination of sows.	94
Figure 4-2. Gene expression heat map of polarized uterine epithelial cells (UECs) stimulated with multiple adjuvant components alone and in combination.	102
Figure 4-3. Changes in the primary uterine epithelial cell (UEC) transepithelial electrical resistance TEER stimulated with multiple adjuvant components.	103

Figure 4-4. Gene expression of uterine tissue (UTE) and laser captured uterine epithelia (LC-UE) of sows 24 hours following breeding.	105
Figure 4-5. Uterine flush cell counts and immunotyping of luminal cell populations of sows 24 hours after breeding.	107
Figure 4-6. Blood immunotyping of T cells, B cells, and monocytes of sows before and 24 hours after breeding.	108
Figure 4-7. Representative immunohistofluorescence of CD163 positive cells (A) in uterine tissue 24 hours after breeding.	110
Supplemental Figure 4-1. Gating strategy used for T cell, B cell, and monocyte immunotyping stains from the blood.	117
Supplemental Figure 4-2. Gating strategy used for myeloid cell immunotyping stain used for luminal cell populations.	118
Supplemental Figure 4-3. Haemotoxylin and Eosin stained tissue following breeding with semen alone or semen containing a triple adjuvant combination.	119
Figure 5-1. Flowchart for moDC differentiation and subsequent experiments and analysis.	128
Figure 5-2. Recruitment of monocytes across a membrane in a micro chemotaxis chamber in response to supernatants from uterine epithelial cells.	132
Figure 5-3. Expression levels of CD1, CD16, MHCII, and CD80/86 of monocyte-derived dendritic cells after 6 days and 8 days.	134
Figure 5-4. Effect of maturation on the expression levels of CD1, CD16, MHCII, and CD80/86 by monocyte-derived dendritic cells (moDCs).	136
Figure 5-5. Endocytosis of FITC dextran by monocyte-derived dendritic cells (moDCs) when differentiated in differing conditions.	138
Figure 5-6. The proliferation of T cells in response to co-culture with Pam3CSK4 stimulated monocyte-derived dendritic cells (moDCs).	140

LIST OF ABBREVIATIONS

AI	Artificial insemination
APC	Antigen-presenting cell
BALT	Bronchus-associated lymphoid tissue
BCR	B cell receptor
BIR	Baculovirus inhibitor of apoptosis protein repeat
BSA	Bovine serum albumin
CARD	Caspase recruitment domain
CCAC	Canadian Council on Animal Care
CD	Cluster of differentiation
cDC	Conventional dendritic cells
CLP	Common lymphoid progenitor
CLR	C-Type lectin receptors
CMP	Common myeloid progenitor
CpG	CpG oligodeoxynucleotide
CT	Cycle threshold
CTL	Cytotoxic T lymphocytes
DAMP	Damage-associated molecular patterns
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic cell
E2	Estradiol
eCG	Equine chorionic gonadotropin
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FCM	Flow cytometry
FcRN	Neonatal Fc receptor
FITC	Fluorescein isothiocyanate
FSH	Follicle stimulating hormone
GALT	Gut-associated lymphoid tissue
GMP	Granulocyte-macrophage progenitor

H&E	Hematoxylin and eosin
HDP	Host defence peptide
HP	Haptoglobin
HSC	Haematopoietic stem cell
IFN	Interferon
Ig	Immunoglobulin
IHF	Immunohistofluorescence
IL	Interleukin
IM	Intramuscular
IN	Intranasal
IPS-1	IFN-β promoter stimulator 1
ISCOM	Immune stimulating complexes
LCM	Laser-capture microdissection
LC-UE	Laser-captured uterine epithelial cells
LGP2	Laboratory of genetics and physiology 2
LH	Luteinizing hormone
LN	Lymph node
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MALT	Mucosa-associated lymphoid tissues
MDA5	Melanoma differentiation-associated protein 5
MDP	Muramyl dipeptide
MEP	Megakaryocyte-erythrocyte progenitor
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
moDC	Monocyte-derived dendritic cell
MPL	Monophosphoryl Lipid A
MPP	Multipotent progenitor
MyD88	Myeloid differentiation primary response 88
NAIP	NLR family inhibitory protein

NALT	Nasopharynx-associated lymphoid tissue
NK	Natural killer cells
NLR	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
OCT	Optimal cutting temperature compound
ODN	Oligodeoxynucleotide
P4	Progesterone
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCAI	Post-cervical artificial insemination
pDC	Plasmacytoid dendritic cells
PET	Polyethylene terephthalate
Pig-MAP	Major acute phase protein
pIgR	Polymeric immunoglobulin receptor
PRRs	Pattern recognition receptors
PYD	Pyrin domain
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RIG	Retinoic acid-inducible gene
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptors
RT	Room temperature
SO	Semen only
STA	Semen plus TriAdj
TBS	Tris-buffered saline
TBST	Tris-buffered saline tween
TCR	T cell receptor
TEER	Transepithelial electrical resistance
TES	Porcine testicular tissue

Tfh	Follicular T cell
Th	Helper T cells
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain containing adapter protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM	TRIF related adaptor molecule
Treg	Regulatory T cell
TriAdj	Triple adjuvant combination
TRIF	TIR-domain-containing adapter-inducing IFN-β
UEC	Uterine epithelial cell
UEC CA	Uterine epithelial cell unstimulated supernatant
UEC Tri	Uterine epithelial cell triple adjuvant combination stimulated supernatant
UT	Uterine tissue
ZO-1	Zona occludens-1

1 INTRODUCTION AND LITERATURE REVIEW

1.1 The porcine reproductive tract

1.1.1 Gross and microscopic anatomy

The porcine reproductive tract can be divided into two distinct regions, the lower and upper reproductive tract, each composed of multiple tubular organs and structures (shown in Figure 1-1). The lower reproductive tract is composed of the internal organs (the vagina and the cervix) and the external sex organ (the vulva). The upper reproductive tract is composed of the uterine horns, the oviducts, and the ovaries. My description of the reproductive tract will begin with the vagina and progress into the body discussing the gross anatomy, as well as the microscopic anatomy of these tissues and the localization of relevant immune cells within these tissues.

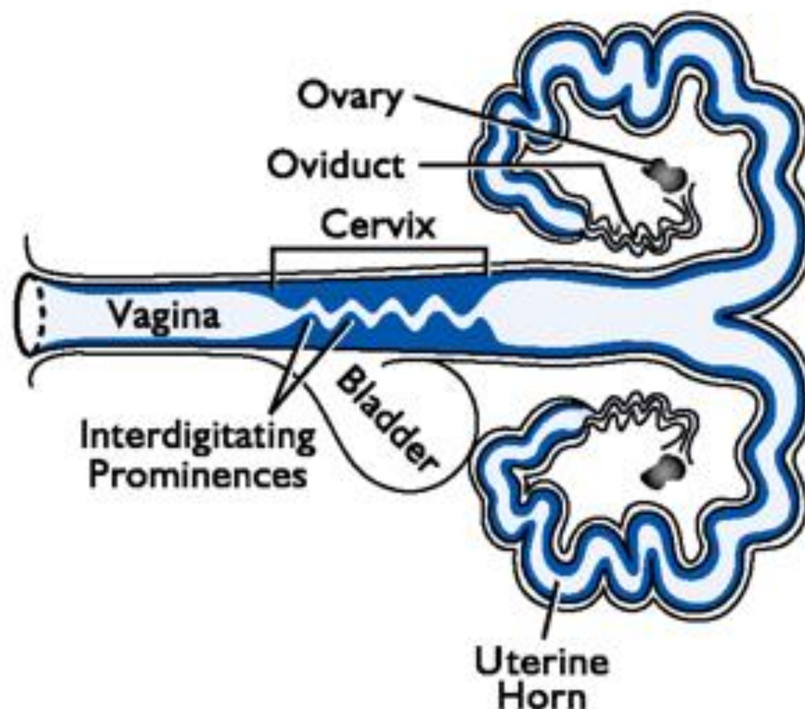

Figure 1-1. Diagram of the porcine reproductive tract taken from (Senger, 2005).

The vagina of the pig is approximately 20 cm long, it connects the external sex organs to the cervix, and it has a poorly defined muscular layer (Tarocco and Kirkwood, 2002). The porcine vagina houses the urethral opening within the vaginal cavity resulting in a single urogenital opening, unlike what is observed in human females who have two distinct canals. The outer layer of cells in the vagina is stratified squamous epithelia that form the epithelial barrier of the vagina which, at approximately 116 µm thick, forms a layer as thick as porcine skin (Squier et al., 2008). Underneath the epithelia is the *lamina propria* which is comprised of loose connective tissue with a muscular layer below, composed of both an inner circular and outer longitudinal muscle fibers (Gruber et al., 2011). As the gilt/sow ages, both the musculature and folds or evaginations of the epithelial layer become more pronounced (Bal and Getty, 1972). Lastly, the final layers of tissue of the vagina surrounding the muscular layers are the *tunica adventitia* which is a connective tissue at the caudal portion of the vagina, and *tunica serosa* at the cranial portion of the vagina (Lorenzen et al., 2015). Notable cell populations within the vagina include the large distribution of IgA-producing plasma cells distributed adjacent to the epithelial layer evenly throughout the vagina. Less numerous IgG and IgM-producing plasma cells are located deeper into the *lamina propria*, often in the proximity of the vasculature (Hussein et al., 1983). Although they have not been well described in pigs, Langerhans cells (a subset of dendritic cells) reside within the vaginal epithelial layer and finally, within the *lamina propria* reside lymphocytes, dendritic cells (DCs) and macrophages (Wira et al., 2005a).

Continuing deeper into the reproductive tract of gilts and sows, the following organ is the cervix, which (unlike some other species such as humans and cows) lacks a fornix, and has a continuous transition from the vagina to the cervix. The cervix of pigs is up to 25 cm long, and it

is a muscular tissue which forms interdigitating prominences in the cervical cavity (Edstrom, 2009). Interestingly, the epithelium throughout the cervix is predominantly stratified squamous epithelium through its length, however towards the endocervix; the epithelium begins to change to a columnar epithelium or pseudostratified columnar epithelium (Hussein et al., 1983). Similar to the what is observed in the vagina, below the epithelial layer is a *lamina propria* surrounded by an inner circular and an outer longitudinal muscular layer encased by a serosa (Edstrom, 2009). A notable semi-structure within the porcine cervix is the presence of lymphoid aggregates. No organized lymphoid tissues are present in the reproductive tract. However, clustering of B cells surrounded by primarily $CD8^+$ T cells is distributed below the epithelium of the cervix and uterus (Edstrom, 2009; Lorenzen et al., 2015). Additionally, both IgA and IgG plasma cells are located throughout the cervix, primarily basolateral to the epithelial layer, with some plasma cells located within the intraepithelial spaces (Hussein et al., 1983). Throughout the cervix, there are large numbers of lymphocytes and macrophages, with minimal neutrophils (Edstrom, 2009).

The porcine uterus is a true bicornuate uterus with a short uterine bifurcation immediately following the cervix, which splits the uterus into two highly developed uterine horns (60 cm long in gilts and up to 100 cm in non-pregnant sows) that are suspended from the abdomen by the broad ligament (Edstrom, 2009). The outer layer of the porcine uterus is composed of a columnar epithelium across the entirety of the uterus; however, unlike the vagina and cervix, there are also glandular epithelial cells which form tubular glands that spiral into the tissue (Hussein et al., 1983). A highly vascularized layer of connective tissue can be found below the epithelial layer, which together makes up the endometrium. In response to the estrus cycle, the endometrium changes the branching of the glands and growth including changes in endometrial thickness and epithelial cell height (Edstrom, 2009; Kaeoket et al., 2002b; Lorenzen et al., 2015). The *lamina propria* is

surrounded by an inner circular and outer longitudinal muscular layer surrounded by serosa (Senger, 2005). The lymphoid aggregate semi-structures are present throughout the endometrium and are typically located directly basolateral to the glandular epithelium (Lorenzen et al., 2015). Immune cells in the endometrium are primarily lymphocytes, and although numbers will vary throughout the estrus cycle, typically $CD8^+$ T cells are present in high numbers below the surface epithelium, with more $CD4^+$ T cells deeper within the connective tissue (Kaeoket et al., 2002a). Plasma cells are dispersed throughout the endometrium with a predominance for IgG-secreting plasma cells (Hussein et al., 1983). Lastly, the distribution of uterine antigen presenting cells (APCs) such as macrophages and DCs are present throughout the endometrium during estrus, however at other stages of the estrous cycle, they are found deeper in the *lamina propria* and rarely reside directly below the surface epithelium (Kaeoket et al., 2002a; Kaeoket et al., 2002b).

The distal sections of the porcine female reproductive tract are the oviducts (which can be separated into three distinct segments) and the ovaries. The three segments of the oviduct beginning at the uterus are the isthmus, followed by the thicker ampulla, and terminating with the funnel-shaped infundibulum which surrounds the ovary (Edstrom, 2009). The epithelial layer is made up of simple columnar epithelium, with intermittent ciliated columnar epithelium throughout the oviduct which is present on “fern-like” folds that extend into the lumen of the oviduct (Hussein et al., 1983; Senger, 2005). The presence of more mucosal folds in the ampulla and a thicker muscular wall in the isthmus differentiate the isthmus and ampulla (Senger, 2005). Below the epithelial layer is the *lamina propria* of connective tissue which is much thicker in the ampulla compared to the isthmus, which has a thin *lamina propria* and substantially thicker musculature (Özen et al., 2012). Similar to all other regions of the female reproductive tract, the *lamina propria* is surrounded by both an inner circular and outer longitudinal muscle layer and lastly surrounded

by a serosa (Senger, 2005). As is observed in the uterus, lymphocytes are the most numerous immune cell in all segments of the oviduct, and they are located directly below the epithelium and dispersed throughout the *lamina propria* (Jiwakanon et al., 2005). Additionally, although sub-epithelial lymphocyte numbers remain consistent between sections of the oviduct, the lymphocytes in the *lamina propria* are less numerous in the isthmus compared to the ampulla and infundibulum (Jiwakanon et al., 2005). Both IgG and IgA plasma cells are dispersed through the *lamina propria* and are most numerous in the infundibulum, with equal proportions of IgA and IgG-secreting plasma cells (Hussein et al., 1983; Jiwakanon et al., 2005). Lastly, macrophages are found primarily in proximity to the epithelial layer and are evenly distributed between all sections of the oviduct (Jiwakanon et al., 2005).

1.1.2 Estrus cycle

Gilts reach puberty between 5-7 months of age, and they are frequently bred on their second or third detectable estrus following puberty. The estrus cycle in pigs is on average 21 days (summarised in Figure 1-2) but can range from 18-24 days, split between the follicular phase (further split into proestrus and estrus) and the luteal phase (further split into metestrus and diestrus) (Soede et al., 2011). Like humans, pigs are spontaneous ovulators, have a continuous cycle, and in the case of pigs will enter into estrus 4-6 days after weaning (Lorenzen et al., 2015). Each stage of the estrus cycle is controlled by several reproductive hormones, of which I will focus on progesterone (P4), estradiol (E2), follicle stimulating hormone (FSH) and luteinizing hormone (LH).

The follicular phase of the estrus cycle (5-7 days) is the phase in which pre-ovulatory follicles are present, and estradiol levels are high. This phase can be subdivided into proestrus (~3 days) and estrus (~2 days) with ovulation occurring during estrus. At the onset of proestrus, antral follicles (4-6 mm diameter) will begin to develop in the ovary in response to an increased frequency and a lower amplitude pattern of release of FSH and LH from the pituitary gland (Michiko et al., 2010). At this stage, luteolysis has occurred, and therefore, P4 levels are decreasing, which allows for the increasing FSH and LH levels. Increased FSH and LH levels and the subsequent growth in follicle size allows the follicles to begin secreting increasing levels of E2 (Soede et al., 2011). It has been shown that the FSH is primarily responsible for the development of antral follicles (3-6 mm) and that LH is the primary hormone for developing the pre-ovulatory follicles (7-9 mm) (Cooper et al., 1990). As E2 levels increase, the gilt/sow moves into estrus, where peak E2 levels occur, and the animal is sexually receptive. As E2 reaches peak level, approximately 48 hours before ovulation, the threshold of the positive feedback loop on gonadotropin production is attained resulting in a large surge of secreted LH that will allow the follicles to develop into pre-ovulatory follicles (Michiko et al., 2010; Senger, 2005). Increased E2 levels result in several behavioural and physiological responses in the gilt/sow to show her sexually receptive behaviour, including lordosis (mating posture; stands rigid with an arched back and cocked ears), in addition to external genitalia swelling and increased mucus production (Soede et al., 2011). Lastly, ovulation occurs (approximately 40 hours after LH surge), which will result in luteinization of ovulated follicles, continuing the drop in E2 production and the beginning of the luteal phase of the estrus cycle.

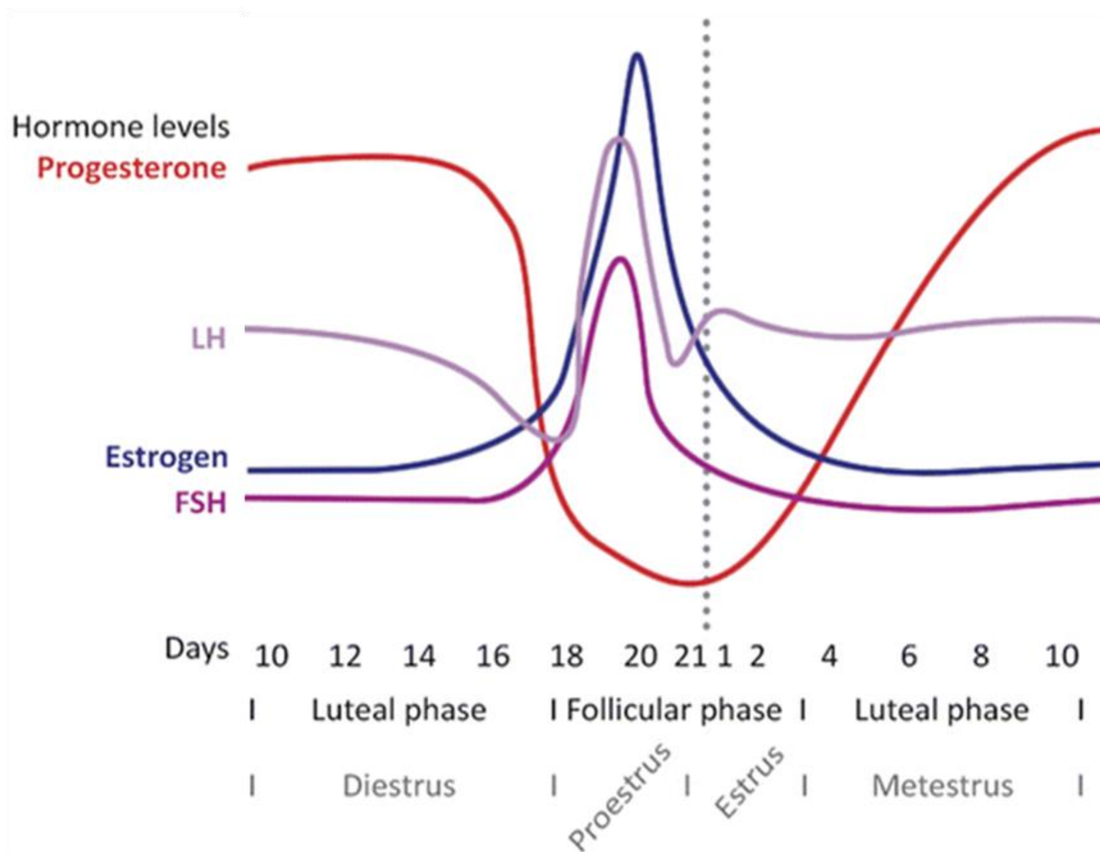

Figure 1-2. Schematic of the porcine estrous cycle displaying the relative hormonal levels through each stage of the cycle, modified from (Lorenzen et al., 2015).

The luteal phase of the cycle (16 days) is marked by high levels of P4, and the formation and maintenance of the *corpus luteum*. This phase can be separated into metestrus (3 days) and diestrus (13 days) (Edstrom, 2009). During metestrus, the ovulated follicles (now *corpus hemorrhagicum*) will develop into *corpus luteum* and begin producing large quantities of P4, which will ultimately peak in serum levels during diestrus (Senger, 2005). Additionally, there is a minor increase in FSH following ovulation as the pre-ovulatory follicles suppression of FSH is lifted, resulting in the initiation of follicular development and development of a new pool of small and medium follicles (Soede et al., 2011). During diestrus, pigs reach their peak level of P4 8-9 days post-ovulation. At 15 days post-ovulation, luteolysis occurs, resulting in a steep decline in

serum P4 levels, provided pregnancy did not occur at the previous ovulation (Michiko et al., 2010). Interestingly, differences in the serum FSH levels of gilts during the luteal phase directly relate to ovulation rates, with animals that have higher FSH levels during the luteal phase having higher ovulation rates (Naber et al., 2003). Additionally, even during detectable FSH surges during the luteal phase, follicular development remains limited to producing follicles of 3-4 mm (Hazeleger et al., 2005). As the P4 levels decrease following luteolysis at the termination of diestrus, gonadotropin levels increase initiating follicular development to progress beyond 4 mm and begin the proestrus phase of the following cycle.

1.1.3 Breeding in the swine industry

In an effort to reduce costs, breeding in the swine industry is under continuous pressure to reduce the number of boars, all while increasing the number of live piglets born. Because of this drive for increased profitability, most commercial swine barns have adopted artificial insemination (AI) as the primary method of breeding (Knox, 2016). AI reduces the number of sperm cells used per breeding, removes the need to maintain breeding boars in barns and maintains the latest genetic lines by using semen sourced from the genetics companies or boar studs. Unlike with bovine AI, frozen boar sperm results in reduced fertility, thus requiring the use of fresh semen for AI in pigs but with the addition of semen extenders, chilled AI doses remain effective for 5-7 days (Gerrits et al., 2005). Currently, barns that utilize AI only require a boar for estrus detection and to initiate the lordosis response for breeding. However, use of a boar can be eliminated through the use of an androstenone spray without any detrimental impact on fertility (Miller et al., 2003). An additional benefit of AI is the screening of semen doses for diseases before use and the inclusion of antibiotics such as gentamicin in the semen dose resulting in a healthier sow population.

Breeding sows by classical AI is performed during estrus, generally when the gilt/sow is in lordosis. A foam-tipped catheter that is inserted so that the foam tip is lodged in the cervix and the semen dose is deposited into the catheter over a 3-5 minute period with pressure being applied to the back of the animal. The catheter is often left in place for a few minutes to limit the amount of extended semen that flows back (McNamara et al., 2013). AI doses used in classical AI contain between 2.5-3 billion sperm in 75-100 ml volume, which results in each boar producing 8-20 AI doses per ejaculate (Knox, 2016). Breeding is carried out relative to estrus detection, with most barns doing estrus detections once per day and performing the first insemination at first detection or the following estrus detection and a second breeding 24 hours after the initial insemination provided the animal is still in estrus (McNamara et al., 2013). Currently, classical AI is the predominant method of breeding at commercial swine barns of all sizes.

Post-cervical AI (PCAI) involves the delivery of the semen dose directly into the uterus through the use of a modified catheter that includes a flexible inner catheter that can be threaded through the pig's cervix. Advantages of using PCAI include reducing the sperm numbers per semen dose to 1.5-2 billion sperm in a reduced volume, resulting in 13-30 semen doses per boar ejaculate (Hernández-Caravaca et al., 2012). Additionally, PCAI is regularly performed in the absence of a boar and without the gilt/sow being immobilized by the lordosis response (Knox, 2016). PCAI does have its challenges and limitations, including difficulty in passing the inner catheter through the cervix of gilts and decreased fertility observed on some farms due to internal injury resulting from inadequate training (Roca et al., 2006). Currently, PCAI represents a small proportion of the breeding method in the United States and Canada; a study from 2007 found that only 6% of barns were using this method of insemination in the United States (McNamara et al., 2013).

Further modifications can be made to AI by synchronizing estrus and ovulation through the hormonal treatment of gilts and sows. The most basic synchronization protocol in pigs uses an oral altrenogest (Regumate) treatment of the animals with one dose being delivered every 24 hours for a minimum of 14 days (Davis et al., 1985). Treatment with altrenogest, which is an oral progestin, will suppress the FSH and LH secretions necessary to progress from diestrus into proestrus and estrus. Therefore, once the daily treatment is halted, animals will enter estrus within 5-7 days as FSH and LH secretions will increase as altrenogest levels drop initiating follicular growth. Synchronization protocols can also be enhanced for increased accuracy in predicting ovulation to the point that only a single insemination is required; this is referred to as fixed-time AI. These protocols utilize oral progestin treatments, however, following the withdrawal of the oral progestin, gilts/sows are given equine chorionic gonadotropin (eCG) to stimulate follicular development, followed by administration of porcine LH 80 hours after eCG. This injection of LH mimics the LH surge that initiates ovulation (Degenstein et al., 2008). Fixed-time AI synchronization results in ovulation occurring between 35.6-42.5 h (sows) or 35.6-52.1 h (gilts) after LH treatment compared to 36.1-83.8 h (gilts) in a control group in gilts and sows and has been successfully used for fixed-time AI in sows (Cassar et al., 2005; Degenstein et al., 2008). Use of fixed-time AI protocols with either classical AI or PCAI allow for further reductions in sperm needed per breeding by only requiring a single insemination. However, these methods have not yet been embraced by the industry.

1.1.4 Early pregnancy and placentation

The first stages of pregnancy and placentation of embryos involve several mechanisms to ensure the pregnancy is established by ensuring the *corpus luteum* does not undergo luteolysis,

and that implantation can occur. Swine have the least invasive placenta of placental mammals, as the conceptus develops in an epitheliochorial placenta. Gestation in pigs lasts for 114 days, and a commercial barn sow will give birth to over 23 piglets per year on average. The number of piglets born per sow per years is under constant pressure to increase every year and has had a two piglet per year increase from 2005-2010 (Knauer and Hostetler, 2013).

Following insemination, the spermatozoa will fertilize the egg in the ampulla, and the resulting conceptus will proceed through the isthmus into the uterus by the eight-cell stage on day two after fertilization (Senger, 2005). Over the following three days, the conceptus will progress from the eight-cell stage to the morula, to the blastocyst and lastly hatch from the zona pellucida by day 6 of gestation. After hatching, the pig conceptus begins rapid elongation going from a spherical shape, to a tubular shape and as it continues to grow a filamentous shape that is between 80 – 100 cm long by day 16 (Bazer and Johnson, 2014). During this stage the free-floating conceptus will begin to secrete E2 causing the prostaglandin F2alpha, produced by uterine epithelial cells and the cause of luteolysis, to be directed towards the uterine lumen preventing it from reaching the *corpus luteum* and initiating luteolysis (Bazer, 2013). The secretion of E2 by the conceptus is the maternal recognition signal; to successfully prevent luteolysis and allow the pregnancy to be maintained, there must be a minimum of 2 embryos per uterine horn. Shortly after maternal recognition of pregnancy, the embryos will attach by day 14-18 of gestation (Senger, 2005).

As pigs have an epitheliochorial placenta, the uterine epithelia remain intact throughout gestation, therefore implantation in pigs involves attachment of the placenta to the uterine epithelium. The process of attachment begins around day 12-14 of gestation and embryos will be fully attached to the uterine wall by day 18, this is the result of decreased secretion of anti-adhesive

compounds by the uterine epithelium (primarily mucins) (Fuller et al., 2009). As the diffuse placenta forms, the chorionic villi spread across the entire surface of the porcine placenta. These villi will form interdigitating microvilli into the endometrium without penetrating the luminal epithelium. The chorionic villi will not form at the openings of uterine glands and at these locations dome-like structures will form in the placenta called areolae, where large amounts of nutrients can be absorbed (Kridli et al., 2016). Because the uterine epithelia remain intact throughout gestation, there is no contact between the maternal blood supply and six layers of cells/tissue separate the maternal blood supply and fetal blood supply, unlike in human placentas where the placenta is in direct contact with the maternal blood (Bertasoli et al., 2015). Consequently, fetal piglets receive no transfer of immunoglobulins (Ig), and all maternal transfer of antibodies must be to be carried out after birth through colostrum and milk.

1.1.5 Transport of molecules across the epithelial barrier

For molecules or pathogens to enter the body proper from the uterus and for molecules to enter the uterine lumen, they are required to cross the epithelial barrier through transport or by accessing damaged tissue. As uterine epithelial cells (UECs) establish tight junctions between adjacent cells, molecules are unable to diffuse across these junctions and therefore require specific mechanisms to pass through the epithelial cells. Although the transport of molecules has not been well characterized in porcine UECs, there are several defined mechanisms in which molecules can transfer across an epithelial barrier which an intrauterine vaccine could utilize.

The predominant method for transporting macromolecules across an epithelial cell wall is 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. In alveolar epithelial cells, studies have been carried out regarding epithelial pinocytosis in an apical to basolateral direction and found strong evidence that pinocytosis occurs in a non-specific fashion and transports macromolecules of varying size across the epithelial cells at a rate proportional to their size (Bertasoli et al., 2015). Additionally, transport of both positively and negatively charged nanoparticles have been characterized using intestinal epithelial cell lines *in vitro,* and it was determined that negatively charged particles were more efficiently transported by pinocytosis regardless of size (100 nm vs. 50 nm). Both the charge and size of molecules impact their transport across epithelial cells (Bannunah et al., 2014). Although there is limited data on the mechanisms of pinocytosis by UECs and how size or charge of particles impact their transport, there is evidence of molecules being transported in a luminal to basolateral direction (Leroy et al., 1976).

One mechanism of receptor-mediated transport across UECs that could be utilized by a vaccine would be through the utilization of antibody transporters; however, this mechanism would require the gilt or sow to secrete IgG specific for the antigen into the uterine lumen. IgG is transported by the neonatal Fc receptor (FcRN) which historically was believed to only transport IgG from an acidic to a non-acidic environment allowing for transport of maternal IgG from milk or colostrum in a neonates gut (Rodewald and Kraehenbuhl, 1984). However, several studies have determined that FcRN mediated transport can bi-directionally transport IgG between neutral environments (Dickinson et al., 1999). Additionally, FcRN has been found expressed by both porcine and human UECs, and the transfer of IgG by FcRN in the human female reproductive tract has been confirmed (Li et al., 2011). By binding to the vaccine antigen, the FcRN-IgG transport

could deliver the antigen and possibly the associated vaccine components across the epithelial wall. This method of transport may be very effective for booster responses after a primary immunization triggers antigen-specific IgG production. Polymeric immunoglobulin receptor (pIgR) mediated transport for the secretion of sIgA has been well described and is known to be carried out by rat uterine epithelial cells (Richardson et al., 1995), although IgA is not the predominant immunoglobulin secreted into the uterine lumen.

1.2 Immunology

The immune system encompasses all mechanisms present within an organism that protects against pathogens. This system can be separated into two branches of immunity, the innate immune response (a non-specific first line of defence of the immune system), and the adaptive immune system (a targeted and antigen-specific response that includes a memory of previously encountered pathogens). Both arms of the immune system are crucial for the elimination of pathogens and for initiating a strong immune response to a vaccine. To generate a strong immune response, the innate immune response first detects the pathogen (or vaccine) and recruits the necessary innate immune cells, such as neutrophils, NK cells, and APCs, to that location. APCs will pick up the vaccine antigen and migrate towards a secondary lymphoid organ where they can present the antigen to the cells of the adaptive immune response where effector and memory antigen-specific lymphocytes are activated. As the focus of this thesis is on the innate immune response towards vaccines, the review will focus on the innate immune system, introducing components of the adaptive immune system where appropriate.

1.2.1 Cells of the immune system

All of the main cells of the immune system a derived from a common progenitor cell, the haematopoietic stem cell (HSC), which resides at low levels in the bone marrow and is self-renewable (cell differentiation pathway summarized in Figure 1-3) (Orkin and Zon, 2008). This HSC differentiates into either a common myeloid progenitor (source of all myeloid cells and erythrocytes) or a common lymphoid progenitor (source of all B and T cells). These progenitors can be differentiated by expression of the interleukin (IL)-7 receptor by the common lymphoid progenitor (Akashi et al., 2000). As these stem cells have the potential to differentiate into any

immune cell, these cells require environmental signals to determine which differentiation pathway to undergo, and this is dictated by the HSCs environment or niche (Orkin and Zon, 2008). The cells surrounding HSCs that make up their niches inside bone marrow have complicated interactions with HSCs which are still under scientific scrutiny. Niche cells include osteoblastic cells, mesenchymal stromal cells, endothelial cells, and several other cells, which all contribute to the generation of immune cells (Morrison and Scadden, 2014). However, scientists have determined that osteoblasts support differentiation towards B lymphocytes and that macrophages support differentiation towards erythrocytes (Chow et al., 2013; Zhu et al., 2007).

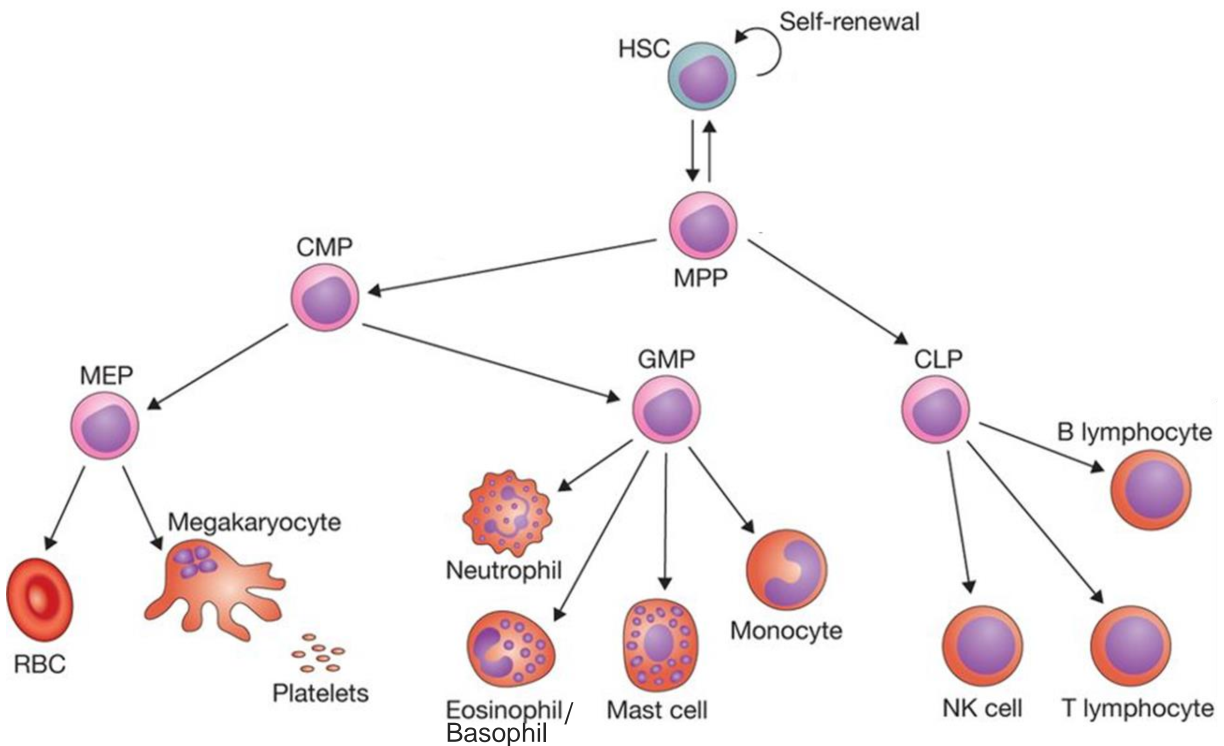

Figure 1-3. Haematopoietic cell pathways in generating the cells of the immune system, modified from (Wahlster and Daley, 2016). Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitors; HSC, haematopoietic stem cell; MEP, megakaryocyte-erythrocyte progenitor; MPP, multipotent progenitor; NK, natural killer; RBC, red blood cell.

The granulocytes include basophils, eosinophils, and neutrophils, are all derived from the myeloid lineage with a common granulocyte-macrophage progenitor. These cells can all be easily distinguished from other cells by their multi-lobed nuclei and the high numbers of cytoplasmic granules present (Kindt et al., 2007). Basophils are the least numerous granulocyte in the blood of pigs, and their cytoplasmic granules are primarily composed of heparin and histamine (Karalyan et al., 2012). Basophils play an important role in inflammation, through recruiting Th2-type T cells and eosinophils and inflammatory macrophages through antagonism of the prostaglandin receptor (Schwartz et al., 2016). Eosinophils, which are recruited to tissue by basophils, are more numerous then basophils and their granules are filled with histamines, peroxidase, ribonuclease and deoxyribonucleases among other proteins (Mair et al., 2014). Although eosinophils can be phagocytes, they are more typically associated with the immune response to multicellular parasites mediated through IgE binding to the parasites (Butterworth, 1985). Eosinophils can also stimulate the release of histamine from both basophils and mast cells as a result of the eosinophil peroxidase (Flier et al., 1991). In addition to basophils and eosinophils pro-inflammatory or anti-parasitic activity, these granulocytes have been identified to be essential contributors to allergic inflammation as a result of their IgE receptor-mediated activation (Stone et al., 2010).

Neutrophils are the most numerous myeloid cell present in the blood, and they are the main cell produced in the bone marrow. They circulate in the blood for only for a few hours before entering the tissue where they are short-lived and survive for only 2-3 days (Borregaard, 2010). Similar to the other granulocytes, neutrophils are filled with numerous granules that can be further differentiated into the primary granules that contain peroxidase and secondary or tertiary granules that are peroxidase negative (Borregaard and Cowland, 1997). Neutrophils play an important role as the first cellular line of defence within the innate immune system, and they are recruited by IL-

8 (also referred to as CXCL-8). Once recruited to the site of IL-8 secretion, they phagocytose bacteria and fungi (Borregaard, 2010). Once neutrophils have phagocytosed microbes, they release their granular contents, which can include several proteases, myeloperoxidase, and the NADPH oxidase into the phagosome and efficiently kill the microbe (Segal, 2004). Neutrophils also kill extracellular microbes through the production of neutrophil extracellular traps, composed of granule proteins and decondensed DNA that bind, trap and kill microorganisms (Brinkmann et al., 2004). Activated neutrophils can secrete several chemokines for recruitment of other immune cells, pro-inflammatory cytokines, such as IL-6 and IFNγ, and in some circumstances, they have been observed to secrete anti-inflammatory cytokines (Mantovani et al., 2011). It is noteworthy to point out that, following insemination in gilts and sows, there is a large influx of neutrophils that will eliminate any microbial contaminants in semen, in addition to phagocytosing spermatozoa present within the uterine lumen (Katila, 2012).

Monocytes are mononuclear myeloid cells that represent 5-10% of the immune cells present in pig blood and as APCs are one of the cell types that link the innate and adaptive immune systems (Karalyan et al., 2012). Independent of their role as APCs and immune modulators, circulating monocytes recruited to tissue will undergo differentiation into pro-inflammatory macrophages and, in select circumstances, into DCs (Geissmann et al., 2010). Monocytes are considered phagocytic cells; however, unlike neutrophils, monocytes can present microbial antigens to T cells through the expression of major histocompatibility complex (MHC) class II on their surface (Randolph et al., 2008). In both humans and mice, monocytes have been characterized as classical monocytes ($CD14^{+}CD16^{-}$) and non-classical monocytes ($CD14^{+}CD16^{+}$) which differ in function, as the classical monocytes are considered to be inflammatory monocytes and non-classical monocytes are considered the patrolling subset (Boyette et al., 2017). In swine, there has

been no identification of CD16$^-$ monocytes (Piriou-Guzylack and Salmon, 2008), indicating the classical vs. non-classical classification of monocyte roles is not appropriate in swine immunology. However, two populations of swine monocytes have been identified and can be differentiated by the presence or absence in the expression of the scavenger molecule CD163, and they are present in equal abundance in blood, unlike the subtypes in humans and mice (Fairbairn et al., 2013). The characterization of CD163$^+$ and CD163$^-$ monocytes is ongoing, however, CD163$^+$ monocytes have been shown to induce antigen-specific T cell proliferation more efficiently, which may be the result of the increased SLA DR (an MHC class II protein in swine) and costimulatory molecule expression by CD163$^+$ cells (Chamorro et al., 2005). Monocytes also express a wide range of both pro-inflammatory and anti-inflammatory cytokines in addition to chemokines in response to different stimuli, allowing for monocytes to modulate the response to stimulation or infection (Vignola et al., 1998).

Macrophages are phagocytic cells that are important for both antigen presentation, inflammation, and clearance of dead cells. Macrophages are generated from monocytes upon entry to tissue, or granulocyte-macrophage progenitors directly, and can account for up to 10% of the cells in tissue (Mair et al., 2014). Macrophages are primed in tissue, and they are dependent on the cytokines present in tissue to direct their differentiation to either an M1 phenotype (classical activation, interferon (IFN)-γ induced) or an M2 phenotype (IL-4 and IL-13 induced) (Gordon and Martinez, 2010). Both M1 and M2 macrophages can be activated by pathogen-associated molecular patterns (PAMPs) binding to the macrophages pattern recognition receptors (PRRs). The function in the immune response of the two subtypes of macrophage differs significantly as M1 macrophages are inflammatory and produce nitric oxide and pro-inflammatory cytokines, as opposed to M2 macrophages that produce anti-inflammatory and Th2-type cytokines (Mills,

2012). M2 macrophages can also be further classified as M2a, M2b or M2c macrophages and have different functions in the immune response including complement-induced inflammation, Th2-type T cell activation, or tissue remodelling respectively (Martinez and Gordon, 2014). Interestingly, when endocytic and phagocytic capabilities of M1 and M2 macrophage were compared, M2 macrophages were more efficient at endocytosis of dextran, and M1 macrophages were more efficient at phagocytosis of *E. coli* (Tarique et al., 2015).

DCs are the professional APCs of the immune system and are derived from granulocyte-macrophage progenitors, and they can be derived from monocytes under specific conditions (Orkin and Zon, 2008). There are several types of DCs, however, in general, they can be categorized as a conventional DCs (cDC) (which are considered the professional APCs and they have high levels of MHC class II expression) or they can be categorized as plasmacytoid DCs (pDC) (which are considered professional IFN-α producers and have low levels of MHC class II expression)(Summerfield and McCullough, 2009). Members of the cDC family include blood cDCs, skin DCs, mucosal tissue DCs, and the *in vitro* generated bone marrow-derived DCs or monocyte-derived DCs (moDCs). In the body, DCs are present within tissues at potential entry sites of pathogens (mucosal surfaces and within the skin), and both cDCs and pDCs together represent less than 1% of immune cells in the blood. Because of the low levels of circulating DCs, significant quantities of blood are required for their isolation and subsequent characterization (Summerfield et al., 2003). Following stimulation with PAMPs, DCs become activated and initially induce increased endocytosis quickly followed by maturation (West et al., 2004) PAMP-induced pDCs express significant amounts of IFN-α and tumour necrosis factor (TNF)-α (Guzylack-Piriou et al., 2004). Because of the inherent difficulties in isolating tissue DCs and the low levels of DCs in blood, *in vitro* characterizations of DCs are typically carried out with moDCs

that in pigs closely resemble blood cDC. However, moDCs express higher levels of CD1, CD14, a minor increase in CD80/86, and they exhibit greater endocytic activity (Facci et al., 2010). A significant obstacle in DC studies in pigs is the lack of a distinct DC marker, as several of the DC markers are shared by macrophages and B cells, resulting in difficulties differentiating a purified population (Mair et al., 2014).

Cells derived from the common lymphoid progenitor (CLP) are natural killer (NK) cells, B cells, and all T cells, with B and T cells being the cells of the adaptive immune response. Although NK cells are lymphocytes from the same progenitor as the cells of the adaptive immune response, they are considered members of the innate immune response because they lyse virus-infected cells and several cancerous cells without the need for antigen presentation and priming (Vivier et al., 2008). NK cells have inhibitory receptors that bind to MHC class I and when NK cells encounter cells with downregulated MHC class I (as is often the case in virus-infected or abnormal cells), they will initiate their cytolytic activity through the release of lysosomes containing granzymes and perforin to lyse the infected or abnormal cells (Topham and Hewitt, 2009). Also, NKs express several PRRs, and upon stimulation with PAMPs from viruses, bacteria, or parasites; they induce several pro-inflammatory cytokines activating other immune cells (Lodoen and Lanier, 2006). Additionally, porcine NK function can be modulated by disease states, as infection with several viral pathogens including African Swine Fever virus and porcine respiratory and reproductive syndrome virus can decrease NK lytic activity (Cao et al., 2013; Norley and Wardley, 1983). NK cell numbers are high in pig blood (15% of lymphocytes) and high in the spleen (12% of splenocytes), and similar to other species, they are widely spread through several tissues, including the uterus (Denyer et al., 2006; King et al., 2002).

B lymphocytes are so named because they were identified to mature in the bursa of chickens (Cooper et al., 1965) and not because they mature in the bone marrow of several mammalian species, including pigs. B cells are the immunoglobulin (Ig) producing cells of the adaptive immune response (Sinkora and Sinkorova, 2014). B cells can be identified by the B cell receptor (BCR), which is an Ig bound to the outer membrane of the cell. The Ig which comprised the BCR on each B cell differs based on the epitope or antigen each B cell specifically targets. During maturation, B cells in the bone marrow acquire their antigen specificity by modifications in the variable region of Ig and any Igs that bind "self" epitopes are eliminated (LeBien and Tedder, 2008). These antigen-specific B cells move from the bone marrow and circulate within the blood and lymphoid organs such as the lymph nodes, spleen, or mucosal-associated lymphoid tissue until the BCR binds its antigen and can initiate B cell activation (LeBien and Tedder, 2008). To complete B cell activation, the B cell presents the antigen in MHC class II to its corresponding Th2-type T cell (which will be expanded on below) which, in turn, secrete cytokines that activate the B cell and initiate its proliferation and differentiation into plasma cells (Parker, 1993). Activated B cells form germinal centers in the B cell zones of lymphoid tissues to proliferate, undergo plasma cell differentiation, undergo somatic hypermutation, and to become memory B cells (LeBien and Tedder, 2008). Somatic hypermutation is the process that enables B cells to acquire a greater affinity for their specific antigen and to undergo class switching between the different Ig subtypes. Plasma cells are the primary Ig producing cells derived from activated B cells and will migrate from the germinal center throughout the body into tissue secreting large amounts of Ig. In some studies, they have been found to survive and secrete antibody for over one year (Slifka et al., 1998).

T cells are the other branch of lymphocytes that make up the adaptive immune response, and they are so named because they mature within the thymus (Adkins et al., 1987). T cells can be further categorized into helper T cells (Th; $CD4^+$), cytotoxic T cells (CTLs; $CD8^+$), and double positive T cells (memory T cells in pigs, $CD4^+CD8^+$) that all express the αβ chain of the T cell receptor (TCR) or γδ T cells that express the γδ chains of the TCR (Gerner et al., 2015). All T cells express the TCR (either the αβ chains or the γδ chains), which similar to the specificity of the BCR, is variable between all T cells. During maturation in the thymus, all "self" binding TCRs are eliminated before exiting from the thymus (Sinkora and Butler, 2016).

The TCR of Th cells identifies their specific antigen presented on MHC class II of APCs. Following activation, CD4+ T cells differentiate into a specific Th or T cell subpopulation based on the local cytokine environment (Th1, Th2, Th17, Treg or Tfh). A naïve Th cell will commit to the Th1 phenotype in response to IL-12 and IFN-γ stimulation during activation, which in turn results in Th1-type T cells that secrete high levels of IFN-γ and TNF-α to activate the inflammatory M1 macrophages (Murphy and Reiner, 2002). For the generation of Th2-type T cells, the naïve Th cell will be activated in an IL-4 environment which will initiate the Th-2 type phenotype, with the Th cells secreting IL-4, IL-5 and IL-13 to induce B cell proliferation and differentiation (Murphy and Reiner, 2002). Similar to Th2-type T cells, follicular T cells (Tfh) also play a role in supporting B cell support and differentiation within the germinal center (Webb and Linterman, 2017), and research on these cells is an active area of research with little data being available on these cells in pigs. The differentiation of naïve Th cells into Tfh-type T cells is poorly characterized, however the differentiation occurs upon interaction with an antigen presented in MHC class II on an APC, and results in increased CTLA-4 co-stimulatory receptor expression on the surface of the Tfh-type T cell, which results in limited clonal expansion of the population (Webb and Linterman, 2017).

Naïve Th cells can differentiate towards a Th17-type T cell through activation in the presence of IL-12 and TGF-β; these Th17-type T cells secrete high levels of IL-17A and TNF-α (Gerner et al., 2015). Th17-type Th cell's role in immunity is still being determined, but they have been implicated in autoimmunity and a neutrophil dominated inflammatory response (Miossec et al., 2009). Lastly, Naïve Th cells can differentiate into regulatory T cells (Treg), which as opposed to the other Th cells will supress the responses of other immune cells, and are generated from naïve Th cell upon stimulation from IL-2 and TGF-β (Käser et al., 2012). Tregs can be further differentiated by the expression of Foxp3 and CD25 (high or low expression), and interestingly unlike human and mice Tregs which all express IL-10, only the CD25+ low Tregs secrete IL-10 (Käser et al., 2012). Following activation, T cells will also undergo clonal expansion resulting in high levels of antigen-specific T cells to eliminate pathogens.

The αβ TCR of CTLs have a similar function to the TCR of Th cells, however, the TCR binding to MHC class I-bound antigen drives immunity against intracellular pathogens viruses and cancers (Kindt et al., 2007). CTLs are activated through binding the antigen in the MHC class I groove with their TCR, and they undergo clonal expansion and disperse through the body in response to secretion of type I IFNs and IL-12 (Pennock et al., 2013). As every nucleated cell in the body expresses MHC class I, infected cells will display the antigen of intracellular pathogens in this complex, which can be recognized by the activated cognate CTLs. CTL-mediated killing of target cells occurs through several mechanisms, but most commonly once the TCR interacts with its cognate receptor it will initiate the release of granzymes (of which there are several types) that upon uptake will initiate death cascades in the target cell that can result in either apoptosis or necrosis (Barry and Bleackley, 2002).

γδ T cells are a unique cell type that are considered cells of both the innate and adaptive immune system as they respond through both the γδ TCR in addition to other non-antigen specific receptors (Bonneville et al., 2010). Interestingly, circulating γδ T cells are found at high levels in blood in pigs (30%), unlike in humans and rodents (<5% in blood), and this high level of circulating cells may be associated with the number of variable δ genes (36 in pigs vs. 10 in humans) (Sinkora and Butler, 2016; Takamatsu et al., 2006). The precise role of γδ T cells is complicated and they have been implicated in induction of cytotoxicity, production of Th1 and Th2 cytokines, non-specific responses to pathogens through PRRs, and one small subset was found to present antigen via MHC class II (Takamatsu et al., 2006; Takamatsu et al., 2002). There are likely several subsets of porcine γδ T cells that have not yet been identified, and more research is required to differentiate these subsets and their specific roles.

1.2.2 Tissues of the immune system

The immune system is not only composed of specific immune cells, but primary and secondary lymphoid organs also play a role in immunity. The primary lymphoid organs are the bone marrow and the thymus. They are the major source of haematopoiesis in the body, they are the site for B and T cell maturation, and they are critical for the development and maintenance of the immune system. The secondary lymphoid organs are the spleen, lymph nodes, and mucosa-associated lymphoid tissues (MALT) and they are the sites where the adaptive immune response will generate a response to a particular antigen (Kipps, 2010). The secondary lymphoid organs are also the main site of B cell class switching and where the generation of memory cells occurs. The spleen is a major site of immunity for blood-borne pathogens as it continuously filters the blood of the body. The spleen has three primary zones based on its cellular content; the white pulp, the

marginal zone, and the red pulp (Mebius and Kraal, 2005). The white pulp regions are where the lymphocytes are located and are subdivided into T cell zones, which are near the vasculature and B cell zones or germinal centers (Kipps, 2010). As matured APCs move through the vasculature and reach the white pulp of the spleen, they come into contact with their cognate T cells present in the T cell zone (primarily $CD4^+$ Th cells) initiating the development of the adaptive immune response (Mebius and Kraal, 2005). Upon activation, T cells move towards the edge of the B cell zone allowing the cells to come in contact with the cognate B cell and initiate the activation, differentiation and clonal expansion of B cells within the germinal center (Mebius and Kraal, 2005). The red pulp zone contains few lymphocytes, and the main immune cells present are DCs and macrophages (Kipps, 2010). The function of the red pulp zone is primarily for the filtering of blood and recycling of RBCs by macrophages. However, it also contains large numbers of plasma cells originating from the white pulp, that migrate to the red pulp to secrete Ig into the blood (Mebius and Kraal, 2005). And lastly, the marginal zone separates the white and red pulp zones, and it contains large numbers of memory B and T cells (Kipps, 2010).

Lymph nodes are small organs spread throughout the tissues of the body where antigens sourced from tissue are presented on APCs to naïve T cells, and B cells become activated (Kipps, 2010). Lymph nodes are comprised of several lobules, comprising of T and B cell zones, similar to the white pulp of the spleen (Willard-Mack, 2006). Similar to what happens in the spleen, APCs enter the lymph nodes, and they home to the T cell regions to present their antigen to their cognate T cell. Once activated, the Th2-type T cells move to the periphery of a B cell zone to activate the cognate B cell (Willard-Mack, 2006). The other Th T cells and CTLs exit the lymph node as effector cells or remain in the lymph node where they continue to proliferate and mature.

The smallest of the secondary lymphoid organs are the MALT, which is located at mucosal surfaces and have been studied the most in the gastrointestinal tract (gut-associated lymphoid tissue; GALT) and the respiratory tract (nasopharynx-associated lymphoid tissue; NALT and bronchus-associated lymphoid tissue; BALT) (Cesta, 2006). There is no known MALT in the uterus on any mammal, and therefore antigen-specific immune responses are generated within the draining lymph node of the uterus. Although the basic structure of the MALT can differ based on the mucosal surface, generally the MALT is a small follicle with distinct T cell zone and B cell zone, and it is predominantly involved in the generation of a Th2-type T cell response and IgA class switching of B cells (Cesta, 2006). In addition to the secondary lymphoid organs, there is a network of capillaries and vessels connecting the secondary lymphoid organs that collects interstitial fluid from the tissue where it drains into the lymph nodes and continues transporting through several lymph nodes until eventually draining into the circulatory system (Swartz, 2001). These networks are used by leukocytes to travel from the tissue through several lymph nodes to initiate the development of antigen-specific immunity and can eventually re-enter the bloodstream.

1.2.3 Innate immunity

The innate immune system is the first line of defence against pathogens, and innate immune cells and their anti-microbial molecules act in an antigen non-specific capacity. A major component of the innate immune response includes PRRs, which PAMPs present on bacteria, fungi, and viruses. PRRs are categorized based on their protein structure into Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and C-type lectin receptors (CLRs) (Kumar et al., 2011). Although these receptors were originally identified as expressed by macrophages,

DCs and other cells of the immune system, they are also expressed on epithelial cells and fibroblasts (Mair et al., 2014; Yao et al., 2015). Several soluble compounds that are also considered part of the innate immune system include the components of the complement system, acute phase proteins, and host defence peptides (HDPs, or innate defence regulators) (Mair et al., 2014).

The complement system is composed of over 30 serum proteins that can be either soluble or membrane-bound that initiate a signal cascade upon activation that ultimately leads to a wide range of responses including chemotaxis of other immune cells, cell lysis or B and T cell regulation (Carroll, 2004). There are 3 primary pathways in which the complement signal occurs; the classical pathway, which binds of IgG or IgM bound to the surface of a pathogen, the alternative pathway, which is initiated through carbohydrates or lipids found on pathogens surface, and the lectin pathway, which is initiated through mannose-binding lectins binding the surface of pathogens (Sarma and Ward, 2011). The most common result of all three cascade pathways is the formation of a membrane attack complex (MAC) on the surface of the pathogen, which forces a pore into the cell membrane resulting in cell lysis. Additionally, complement can recruit inflammatory immune cells through the interaction of complement proteins binding to chemotaxis receptors expressed on inflammatory cells (Carroll, 2004).

Acute phase proteins are present in the blood, and they increase significantly in response to inflammation. Major acute phase proteins in pigs include pig-major acute phase protein (pig-MAP), haptoglobin (HP), and several other minor acute phase proteins (Mair et al., 2014). Liver hepatocytes will secrete acute phase proteins in response to pro-inflammatory cytokines (Kindt et al., 2007). The specific roles of each acute phase protein can differ between species studied, however, they can act in both anti-inflammatory capacities or inflammatory capacities through

activation of complement (Gruys et al., 2005). These proteins can be used as a diagnostic tool for determining inflammation or stressors of animals (Piñeiro et al., 2007).

HDPs are secreted by epithelial cells and phagocytes at sites of infection or mucosal surfaces, and they act as antimicrobial agents directly or through induction of immune cell recruitment (Mair et al., 2014). HDPs can be classified as defensins or cathelicidins, and all cathelicidins are produced as inactive precursors (Nijnik and Hancock, 2009). A large number of cathelicidins have been identified in pigs, and they are expressed primarily by neutrophils or other immune cells (Nijnik and Hancock, 2009). Defensins can be separated into three subtypes of α, β, and θ defensins of which only β-defensins have been identified in pigs, and they are expressed by epithelial cells, exclusively. HDP mediated killing of microbes occurs by the lysis of microbial membranes and has been proposed to occur by the defensin forming pores within microbial membranes leading to lysis, or alternatively by coating the membrane surface in HDPs destabilizing the membrane resulting in a detergent-like activity on the membrane (Kumar et al., 2018). Although the original activity of HDPs was considered limited to microbial killing, several studies have indicated they have the potential to modulate or influence the responses of other immune cells (Dybvig et al., 2011; Nijnik and Hancock, 2009).

1.2.4 Epithelial cells in innate immunity

Although epithelial cells are not derived from the HSCs and are not considered immune cells, they play several important roles in the immune system and in protecting the body cavity from infection (summarised in Figure 1-4). Epithelial cells are the outermost layer of cells of all mucosal surfaces and the skin. They form tight junctions between adjacent cells that form a barrier

preventing the entry of pathogens into the body (Nusrat et al., 2000). More directly, epithelial cells impact the immune system through the expression of several PRRs, and they induce cytokine and chemokine secretion in response to PAMP stimulation, resulting in the initiation of immune responses of pathogens at the epithelial barriers (Wira et al., 2005b). Additionally, as discrete mucosal surfaces have different levels of contact with microbes (commensal or pathogens), they have adapted to respond or not to microbes respond as appropriate. For example, mucosal epithelial cells at mucosal surfaces with high commensal bacterial load tend to be less sensitive to the induction of an inflammatory response to bacteria (McClure and Massari, 2014). This regulation is partially accomplished based on select localization of PRR, such as expressing PRRs on the basolateral surface to promote a response to pathogens once they have crossed the epithelial barrier (Yu and Gao, 2015). Epithelial cells have been shown to suppress DC maturation selectively and therefore; they may play a role in the modulation of the response of immune cells (Ochiel et al., 2010). In addition to cytokine and chemokine secretion in response to PRR signalling, porcine mucosal surface epithelial cells secrete β-defensins to eliminate microbes directly (Nijnik and Hancock, 2009). In humans and mice, it has been determined that select epithelial cells can express MHC class II and to present antigen directly to T cells (Mulder et al., 2011; Wallace et al., 2001; Wira et al., 2005c). However, thus far, no study in pigs has detected MHC class II expression by epithelial cells (Mair et al., 2014) thereby highlighting species-specific differences between the role of epithelial cells in immune activation.

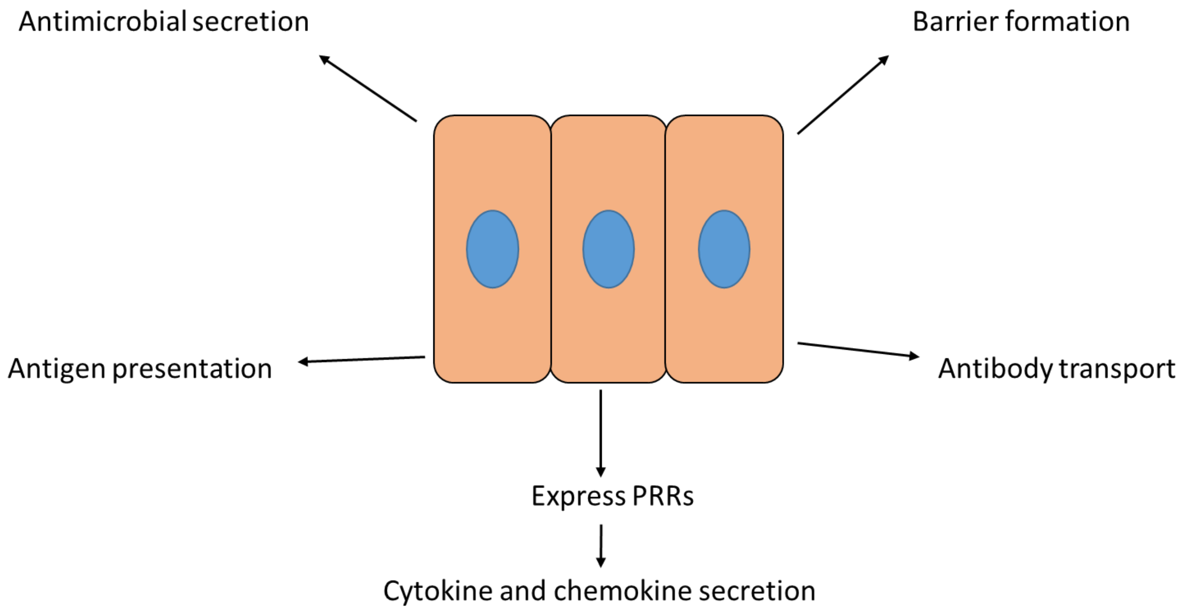

Figure 1-4. Innate immune roles that have been described for epithelial cells.

1.2.5 Toll-like receptors

TLRs are the most widely studied family of PRRs to date with ten members identified in most mammalian species, including pigs, and an additional two receptors identified in mice and rats (Kawai and Akira, 2007). TLRs are transmembrane proteins with a domain composed of leucine-rich repeats that are the location of PAMP binding and can bind bacterial, viral, and fungal PAMPs (Kumar et al., 2011). Originally, TLRs were categorized as extracellular or endosomal in localization with TLR 1,2,4,5 and 6 localized to the outer membrane and TLR3, 7, 8 and 9 localized to the endosome (Akira et al., 2006). However, the subcellular localization appears not to be as fixed as previously believed with several cells, particularly epithelial cells, expressing non-canonical TLR localization (Hamonic et al., 2018b). A more effective categorization of TLRs is based on their ligands as TLRs bind nucleotides, lipids, or protein. TLRs that bind nucleotides

include TLR3 that binds dsRNA, TLR7 and TLR8 that binds ssRNA and TLR9 that binds unmethylated CpG DNA motifs (Kawai and Akira, 2007). The lipid-binding TLRs include TLR2, which forms dimers with TLR1 and TLR6 to bind peptidoglycan, lipopeptides, and fungal zymosan, and TLR4 that binds LPS (Kawai and Akira, 2007). TLR5 binds the protein flagellin. The ligand for TLR10 is unknown, however, evidence indicates that it acts as a negative regulator of the signalling pathway of the other TLRs (Jiang et al., 2016). Following binding of each ligand, TIR domain-containing adapters are recruited to each TLR to initiate the signalling cascades that culminate in cytokine and chemokine expression. The specific TIR domain-containing adapter recruited to the activated TLR differs, however it will be one or a combination of myeloid differentiation primary response 88 (MyD88), TIR-domain-containing adapter-inducing IFN-β (TRIF), TIR domain-containing adapter protein (TIRAP) or TRIF related adaptor molecule (TRAM) (Kawai and Akira, 2007). All TLRs recruit MyD88 except TLR3, which undergoes a MyD88-independent pathway by signalling through TRIF (Kawai and Akira, 2007). Generally, if the signal is initiated by MyD88, the resulting response will be the induction of pro-inflammatory cytokines TNFα, IL-1β, and IL-6, and signalling initiated through TRIF results in the induction of type I IFNs, IFN-α and IFN-β (Akira et al., 2006). Induction of these pathways induces chemokines to recruit APCs, neutrophils, NKs, and T cells, with the response varying between cell types. For example, pDCs induce much higher levels of IFNs through a modified MyD88-signalling cascade compared to cDCs(Kawasaki and Kawai, 2014). TLR induction in response to ligands may be controlled both by the downstream signalling and the subcellular localization of the receptors. For example, basolateral or intracellular localization of TLR4 on epithelial cells may reduce TLR activation in response to commensal flora but promote TLR4 activation to commensals that have been taken up by epithelial cells or that have traversed the epithelial cell membrane.

1.2.6 NOD-Like receptors

NLRs are the largest family of PRRs containing over 20 cytoplasmic receptors, which also bind their ligands through a leucine-rich repeat. All NLRs contain a NOD region and also contain a variable N terminal region, which further dictates the sub-family of NLR (Mair et al., 2014). NLRs can be subdivided into one of 3 main subfamilies; the NLRC sub-family, which all contain a caspase recruitment domain (CARD); the NLRP sub-family, which contains a pyrin domain (PYD); and the NLRB sub-family, which contains a baculovirus inhibitor of apoptosis protein repeat (BIR) (Mair et al., 2014). The NLRC sub-family contains NOD1 and NOD2, which both bind peptidoglycans and respond to stimulation by induction of proinflammatory cytokines, chemokines for neutrophil and APC recruitment, and IFNs (Kumar et al., 2011). NLRX1 binds to viral RNA and typically results in increased TNF-α secretion (Tattoli et al., 2008); however, it has been identified as a suppressor of IFNs generated in response to viral infection (Allen et al., 2011). The NLRP sub-family is most commonly associated with the formation of the inflammasome, although one member of the NLRC sub-family (NLRC4 which is absent in pigs) is also associated with the inflammasome (Sakuma et al., 2017). The NLRP sub-family can be stimulated by several PAMPs and damage associated molecular patterns (DAMPs). The NLRP sub-family contains NLRP1-14, however, the majority of research has focused on the NLRP1 and NLRP3 inflammasome formation and subsequent activation of caspase 1 (Lupfer and Kanneganti, 2013). The active inflammasome cleaves inactive pro-IL-1β leading to release of the active form of the protein, IL-1β (Monie, 2013). Interestingly, NLRP6 and NLRP12 appear to suppress the pro-inflammatory response generated towards TLR signalling, again demonstrating an anti-inflammatory role in members of the NLRs (Anand et al., 2012; Lupfer and Kanneganti, 2013).

Lastly, the NLRB sub-family, which has only one identified member in humans (NLR family inhibitory protein (NAIP), similar to mouse NAIP1) has been implicated in the response to bacterial type III secretion system and upon activation induce inflammasome formation and IL-1β secretion (Yang et al., 2013). A recent study suggests that pigs lack a functional NAIP gene (Sakuma et al., 2017).

1.2.7 RIG-I-Like receptors

RLRs are also cytosolic receptors comprised of 3 members, retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) which recognize viral dsRNA through an RNA helicase domain (Kumar et al., 2011). Both RIG-I and MDA5 initiate secretion of pro-inflammatory cytokines and type I IFNs through a CARD domain. LGP2 lacks a CARD domain and is believed to modulate the response of RIG-I and MDA5 (Szabo and Rajnavolgyi, 2013). Both RIG-I and MDA5 rely on the central adapter protein IFN-β promoter stimulator 1 (IPS-1) to signal induction of both IFNs and pro-inflammatory cytokines (Eisenächer and Krug, 2012). It is important to note that although both RIG-I and MDA5 bind dsRNA, they interact with different dsRNAs (MDA5 interact with longer dsRNA and RIG-I with shorter dsRNA), as they have been implicated as inducers of IFN in response to different viruses, and in rare occasions the same virus (west nile virus) (Eisenächer and Krug, 2012). LGP2 modulation of RIG-I and MDA5 responses is still under investigation as several studies have produced conflicting reports, showing both positive and negative regulation of the RLR response to viral infection (Rothenfusser et al., 2005; Satoh et al., 2010).

1.2.8 C-type lectin receptors

CLRs are a large family of PRRs that include both soluble cytosolic receptors as well as transmembrane receptors, with most studies being directed towards the membrane-bound receptors as PRRs (Geijtenbeek and Gringhuis, 2009). In general, CLRs bind carbohydrates or have a carbohydrate recognition domain that is dependent on Ca^{2+} for carbohydrate recognition and binding (Zelensky and Gready, 2005). Although CLRs can recognize ligands from the bacteria, virus, and parasites, they primarily recognize ligands from fungi and can be categorized by their ligand affinity, i.e. mannose-binding receptors (group I CLR), or the asialoglycoprotein receptor family (group II CLR) (Geijtenbeek and Gringhuis, 2009). Porcine CLRs have been poorly studied, and research has been primarily focused on their identification with little information concerning their signal cascades uncovered. CD205 is a type I CLR that has been identified in pigs which is typically expressed by DCs, and although no specific ligand has been identified, it is known to play an important role in antigen capture or endocytosis (Flores-Mendoza et al., 2012). Pig type II CLRs include Dectin-1 (or CLEC7A), DC-SIGN and Langerin among a few others (Mair et al., 2014). Dectin-1 is an important receptor for recognition and binding of β-glucans which are present on fungi, plants, and bacteria. Upon stimulation with a wide variety of β-glucans, Dectin-1 positive cells respond with increased cytokine expression of TNF-α and IL-1β; however, the signal cascade has not been identified (Sonck et al., 2010). Langerin expression by Langerhans cells has been confirmed in porcine skin (Nfon et al., 2008). Although functional studies have not been carried out in swine, langerin has been associated with amplified endocytosis and antigen presentation (Geijtenbeek and Gringhuis, 2009; Nfon et al., 2008). DC-SIGN which is a CLR expressed on APCs has been characterized in humans and has been implicated in the induction of IL-10 and Th differentiation towards Th1-type and Th17-type T cells (Geijtenbeek

and Gringhuis, 2009; Huang et al., 2009). More research on CLRs is required to better identify their ligands and their function on APC uptake and presentation of antigens in addition to the secretion of cytokines and chemokines.

1.2.9 Cytokines

Cytokines are a diverse group of small proteins that act as a means of immunological communication. Binding to their specific receptors on cells, cytokines can mediate cell proliferation, inflammation, tissue repair differentiation and maturation of immune cells among other roles in both the innate and adaptive immune systems (Murtaugh, 1994). Cytokines are diverse and they can be separated into several non-mutually exclusive functional families including; pro-inflammatory cytokines, anti-inflammatory cytokines, IFNs, and colony-stimulating factors (Dinarello, 2007). The pro-inflammatory cytokines generally induce the function of local enzymes responsible for NO synthesis, increased expression of endothelial adhesion molecules to assist leukocytes entering the tissue from the blood, or induced synthesis of platelet-activating factor, which must be tightly regulated to prevent tissue damage (Dinarello, 2000). Pro-inflammatory cytokines include TNF-α, IL-6 and the IL-1 family (including Il-1β), and they can trigger increased expression of other pro-inflammatory cytokines such as IFN-γ pretreatment of murine macrophages induces TNF-α (Sol et al., 2008). Anti-inflammatory cytokines tend to suppress or regulate pro-inflammatory cytokine function, however, they often possess other functional roles, such as IL-10 and IL-13 which also promote B cell activation (Dinarello, 2000). IFNs are antiviral cytokines that can be differentiated into sub-families based on their receptors and functional activity; type I IFN including IFNα, IFNβ, IFNκ, IFNω, and IFNε, Type II IFN including IFNγ, or type III IFNs including IFNλ (Capobianchi et al., 2015). The

primary function of type I IFNs is in preventing viral replication and spread which is accomplished through the reduced proliferation of cells, induction of antimicrobial states in infected cells, increased antigen presentation and activation of both B and T cells (Ivashkiv and Donlin, 2014). IFNγ induces and activates M1 macrophages, induces expression of pro-inflammatory cytokines and is very effective in suppressing the proliferation of virus-infected cells, inducing upregulation of MHC class I and II expression in immune cells (Capobianchi et al., 2015), and in some instances, inducing MHC class II in epithelial cells (Mulder et al., 2011). Lastly, the type III IFN, IFNλ has a similar function as type I IFNs that is limited to primarily epithelial cells that express its unique receptor (Donnelly and Kotenko, 2010). Finally, Colony-stimulating factors, which are so named because they differentiate bone marrow cells into myeloid cells, can be categorized as granulocyte/macrophage-, macrophage-, and granulocyte-colony stimulating factor (GM-, M-, G-CSF, respectively) (Hamilton, 2008). These cytokines play important roles as haematopoietic growth factors, however, there is evidence that they may also possess pro-inflammatory activity (Hamilton, 2008). It is difficult to analyze the responses of cytokines in isolation as their functional responses may differ based on other cytokines present in the environment, as is seen with IL-12 and IL-18 which together induce IFNγ and a Th1-type T cell response, however, IL-18 alone will induce a Th2-type T cell response (Nakanishi et al., 2001).

1.2.10 Chemokines

Chemokines are a specific family of cytokines that are associated with cellular migration, and are subdivided based on the spacing of their N terminal cysteines (Laing and Secombes, 2004). The four sub-families are based on the spacing of the first two cysteine residues are termed CXC, CC, C, and CX_3C. Additionally, the names of all chemokines indicate the sub-family (ex. CXCL8,

CCL2, CL1 or CX_3CL1). The chemokine receptors also indicate which sub-family binds the receptor through its naming, such as CXCR1 which binds certain CXC chemokines or CCR2, which binds certain CC chemokines (Rossi and Zlotnik, 2000). It should be noted that because most chemokines were discovered and described prior to the sub-family based naming system, they are commonly still referred to as their previous names with a notable example being CXCL8, which was originally named IL-8 (Laing and Secombes, 2004). Chemokines are widely expressed by several cells in the body, not limited to the immune system, but most chemokine receptors are expressed on immune cells or endothelial cells (Murphy, 2019). The CXC family of chemokines contains 16 members in humans (not all have been described in pig), with the most notable member being CXCL8 which is a potent recruiter of neutrophils and it promotes angiogenesis by enhancing endothelial cell proliferation and survival (Li et al., 2003). Interestingly, CXCL8 is lacking in both mice and rat genomes (Rossi and Zlotnik, 2000). The CC family of chemokines is the most numerous family, with 28 members identified in humans, and they are primarily responsible for the recruitment of mononuclear cells, including both myeloid and lymphocyte cells (Laing and Secombes, 2004). Notable CC chemokines include CCL2, a potent monocyte recruitment chemokine that interacts with CCR2 on monocytes with a similar potency as CXCL8 recruits neutrophils. CCL2 has also been implicated in the recruitment of memory T cells and induction of histamine release from basophils (Rollins, 1997). Other monocyte chemoattractants include CCL3, CCL4, CCL7, CCL8, CCL12 and CCL13 which bind to various CC receptors, however, none of these chemokines recruit monocytes as effectively as CCL2 when evaluated in humans (Uguccioni et al., 1995). CCL20 recruits immature DCs through CCR6 binding to the site of inflammation, although it may also play a role in the recruitment of memory T cells and B cells (Schutyser et al., 2003). Additionally, CCL27 and CCL28 bind to CCR10 and recruit mucosal B and T cells, as well

as IgA-secreting plasma cells (Mohan et al., 2017). Interestingly despite having a similar structure, CCL28 but not CCL27 has antimicrobicidal activity against both Gram-negative and Gram-positive bacteria (Hieshima et al., 2003). Lastly, C and CX_3C chemokines such as CL1, CL2 and CX_3CL1 have been identified in humans but not yet identified in pigs recruit lymphocytes (C) or lymphocytes and monocytes (CX_3C)(Laing and Secombes, 2004).

1.2.11 Antigen uptake and presentation

Antigen uptake and presentation is a crucial link between the innate and adaptive immune systems. APCs process and present antigen that prime T cells leading to the specificity and subsequent memory associated with the adaptive immune system. I will focus on DCs although the subsequent descriptions apply to all APCs. Antigen uptake into the DCs can occur through a variety of methods, including receptor-mediated endocytosis, pinocytosis, or phagocytosis (Guermonprez et al., 2002). Receptor-mediated endocytosis requires antigen to bind to a receptor on the outer surface of the DC which initiates the formation of an endocytic vesicle at the site of the receptor thereby internalizing the antigen. The common receptors that initiate endocytosis in DCs are Fc receptors that bind the conserved region of Ig, scavenger receptors that bind lipoproteins, and CLRs that bind carbohydrates (Guermonprez et al., 2002). DCs constitutively undergo high levels of pinocytosis, resulting in DCs constantly sampling the fluid that surrounds them for potential antigen into pinocytic vesicles (Sallusto et al., 1995). During phagocytosis, antigens bind to a receptor (often the same receptors as receptor-mediated endocytosis) which triggers rearrangement of the cytoskeleton to engulf the targeted antigen, cell or pathogen, forming a phagocytic vesicle (Guermonprez et al., 2002). Proteolytic enzymes enter these vesicles and cleave proteins into small 13-18 amino acid fragments. These small antigenic peptides are then

trafficked to vesicles containing MHC class II proteins where the antigen is loaded onto the MHC class II complex groove before moving to the surface of the cell (Lanzavecchia, 1996). Simultaneously, the DC will initiate maturation through the activation of several receptors including TLRs, cytokine receptors or Fc receptors, which results in decreased endocytic activity, increased MHC class II expression and increased co-stimulatory molecule expression (Guermonprez et al., 2002). Further, during maturation, DCs will switch from expressing CCR6 to CCR7, resulting in the chemotaxis of DCs towards secondary lymphoid organs where the DCs can present the antigen to T cells (Dieu et al., 1998). Once the mature DC has reached the regions of T cells in the lymph node, spleen, or lymphoid tissues, the cognate T cell will bind the MHC class II antigen complex with its specific αβ TCR, followed by CD4 anchoring to the MHC class II molecule (Guermonprez et al., 2002). The binding of the TCR and MHC class II is insufficient to activate the T cell, and further interaction of co-stimulatory molecules is required. The costimulatory signal is typically an interaction of CD80 or CD86 on the DC interacting with CTLA4 on the T cell (Lim et al., 2012). Although CD80 or CD86 are often used as the measurement of maturation in a DC, there is an ever-increasing list of possible co-stimulatory molecules on the DC and receptors on the T cell which can lead to differential T cell activation (Chen and Flies, 2013). Additionally based on the cytokines being produced by the DC in response to potential PRRs or immunostimulatory molecule, the DC can secrete the necessary cytokines for development of a specific Th-type immune response, (such as secretion of IL-4 or IL-13 to generate a Th2-type T cell response) (Burakova et al., 2018). As the uterus has no MALT, trafficking of the mature DCs and presentation of antigen to T cells occurs in the draining lymph node present in the broad ligament (Woodrow et al., 2012).

1.2.12 Hormonal control of the immune response

Because the sexually mature uterus is under hormonal control, it is important to note how the hormonal environment impacts the immune system at different stages of the estrus cycle. The estrus cycle in pigs has been shown to alter the immune cell populations present within the endometrium. Lymphocytes including plasma cells and T cells were shown to be at their highest level within the endometrium during estrus and metestrus, and they decreased to half their peak level during the other stages of the cycle (Hussein et al., 1983; Kaeoket et al., 2002b). Macrophages are present at their highest levels in the endometrium during proestrus and estrus, and their levels dropped back to basal levels within 70 hr following ovulation (Kaeoket et al., 2002b). It is not unexpected that the endometrium will have its highest levels of all immune cells around the period of estrus, as at this stage of the cycle when breeding occurs, the gilt/sow would be primed to eliminate a large number of microbes present in boar semen (Gączarzewicz et al., 2016).

E2 or P4 stimulation of pig UECs allow them to respond to infection or stimulation. For example, studies in pigs show that pig UECs were more susceptible to infection by *Chlamydia suis* infection during an E2 dominant phase of the estrus cycle. (Guseva et al., 2003). Additionally, in humans, UECs had higher bactericidal activity through increased defensin expression and decreased expression of pro-inflammatory cytokines in an E2 dominant environment (Fahey et al., 2008). It is becoming clear that the complicated interactions of the immune system, and other cells that may impact the immune system, are sensitive to changes in the hormonal environment. However, more studies are required in the pig to gain a better understanding of the impact the hormonal environment has on the immune response.

1.3 Vaccines

Vaccination is one of the greatest advancements in protecting humans and animals from infectious disease and is considered one of the most cost-effective measures for preventing disease in a population (Rémy et al., 2015). Vaccination involves exposing an individual or animal to a pathogen in a weakened, killed, or partially inactivated state. This exposure enables the immune system to generate a strong antigen-specific adaptive immune response, subsequently protecting the individual from infection when exposed to the live pathogen. Vaccination first began in the 18th century with Edward Jenner, who developed a vaccine for protection against smallpox. In the intervening 300 years, vaccination techniques have advanced dramatically to provide safe and effective means to protect against a wide array of pathogens (Plotkin, 2014). In general, vaccines are composed of 3 types of components; 1. antigen(s) which may be whole, live pathogen (typically attenuated), killed whole pathogen, or sub-units (proteins/toxins) derived from the pathogen; 2. adjuvants that are molecules that modulate or enhance the immune response to the antigen; and 3. stabilizers and preservatives, which aid in long term storage and allow for multi-dose vaccine vials. Live attenuated vaccines are the most effective at inducing a response in the immune system, however, they also carry the greatest safety concern with the possibility of incomplete attenuation leading to disease or the possibility of reversion back to wild type (Bastola et al., 2017). Alternatively subunit vaccines are considered safer as there is no whole pathogen delivered (and therefore no danger of reversion to virulence), however, they are typically less immunogenic and require adjuvants to increase their immunogenicity (Bastola et al., 2017). Vaccines can also differ based on their route of delivery either being injected (generally intramuscular (IM)) as a parenteral vaccine or delivered to a mucosal surface as a mucosal vaccine (Srivastava et al., 2015). Although there are multiple types of vaccines each with their advantages

and disadvantages, the goal of all vaccines is to generate high enough population-level immunity that results in decreasing the circulating pathogen levels that protect those most at risk through herd immunity (Fine, 1993).

1.3.1 Mucosal vaccines

As the majority of pathogens enter the body through mucosal surfaces, generating a strong mucosal immune response can result in the elimination of pathogens before crossing the epithelial barrier. The goal of mucosal vaccines is the efficient induction of mucosal immune responses to block colonization and invasion, as opposed to parenteral vaccines that are less pre-disposed to generating a strong mucosal response (Neutra and Kozlowski, 2006). There are exceptions to this with select parenteral vaccines that can generate strong mucosal immune responses (Clements and Freytag, 2016; Pasternak et al., 2017). When subcutaneous and intranasal (IN) vaccination was compared directly in a vaccine against respiratory syncytial virus, mice that had been vaccinated by IN route alone had significantly lower viral RNA copies in the lung following challenge compared to both subcutaneous vaccination and combinations of IN and subcutaneous routes (Mapletoft et al., 2010). In addition to inducing a strong mucosal immune response, mucosal vaccines eliminate needle-stick injuries, are generally delivered more easily (less invasive) and potentially have fewer side effects (Srivastava et al., 2015). There are still significant challenges associated with mucosal vaccines including overcoming the tolerance response generated to antigens and sites with commensal flora, unknown dosages of vaccine crossing the epithelial barrier, and dilution of vaccines in mucosal fluid requiring large doses (Neutra and Kozlowski, 2006). Mucosal vaccines can be delivered to any mucosal surface, however, the ease of delivery can make select mucosal surfaces difficult to vaccinate and because of this mucosal vaccines are

limited to the IN, ocular, or oral route (Gerdts et al., 2006). An important strategy in selecting a mucosal vaccines route of delivery is to consider what mucosal surfaces the pathogen will infect, as mucosal surface where a response is generated will generate the strongest immune response. Although there is evidence of a common mucosal immune system in the pig (Wilson and Obradovic, 2015), current evidence suggests that select compartments are shared with others, such as genitourinary mucosal immunity being compartmentalized with the respiratory tract, but not always the gastrointestinal tract (Russell and Mestecky, 2000). With all these considerations, mucosal vaccines would result in several benefits above parenteral vaccines, however, they require careful selection of adjuvants and delivery methods to develop safe, effective economically viable vaccines.

1.3.2 Vaccine adjuvants

Adjuvants are used in the majority of vaccines, particularly in inactivated vaccines and sub-unit vaccines, to increase the immunogenicity of the vaccine antigen to and direct the immune response towards a specific type of response (Th1- vs. Th2-type T cell response). Adjuvants were classically defined as stimulatory and non-stimulatory adjuvants, with stimulatory adjuvants directly stimulate APCs, and non-stimulatory adjuvants act as delivery vehicles, creating a depot at the site of injection (Pashine et al., 2005). However, as scientists continue to study vaccine adjuvants and their mechanisms of action, it becomes clear that this categorization does not represent the full scope of the adjuvants of each category, and that these mechanisms are not mutually exclusive. Adjuvants may have several mechanisms of action including crossing the epithelial barrier (depot formation in parenteral vaccine), up-regulation of cytokines and chemokines, antigen presentation cell recruitment, increased antigen uptake by APCs, activation

and maturation of APCs and directing of the immune response (Th1, Th2, Th17-type T cell or CTL) and their general mode of action is summarised in Figure 1-5 (Awate et al., 2013). Currently, there are very few adjuvants approved for use in humans and these include aluminum salts (alum), oil in water or water in oil emulsion (MF59 or AS03), or monophosphoryl lipid A (MPL, derived from LPS), which limits opportunities in vaccine design (Di Pasquale et al., 2015). Far more adjuvants have been approved for use in veterinary vaccine including the saponin based Quil-A, including its inclusion as an immune stimulating complex (ISCOM), or the synthetic polymer carbopol (Burakova et al., 2018). These adjuvants allow for more options in developing mucosal vaccines, with the hope that eventually these adjuvants will also be approved for use in humans. The limitations in approved adjuvants are particularly important in the design of an intrauterine vaccine which must be administered with semen where emulsions impede sperm motility and viability. Aluminum salts have not been found to be an effective adjuvant for use in mucosal vaccines and are a poor inducer of mucosal immunity, so they are likely not going to be effective when used as part of an intrauterine vaccine (Rhee et al., 2012). Throughout the remainder of this section, I will evaluate potential vaccine adjuvants that would be considered for use in an intrauterine vaccine.

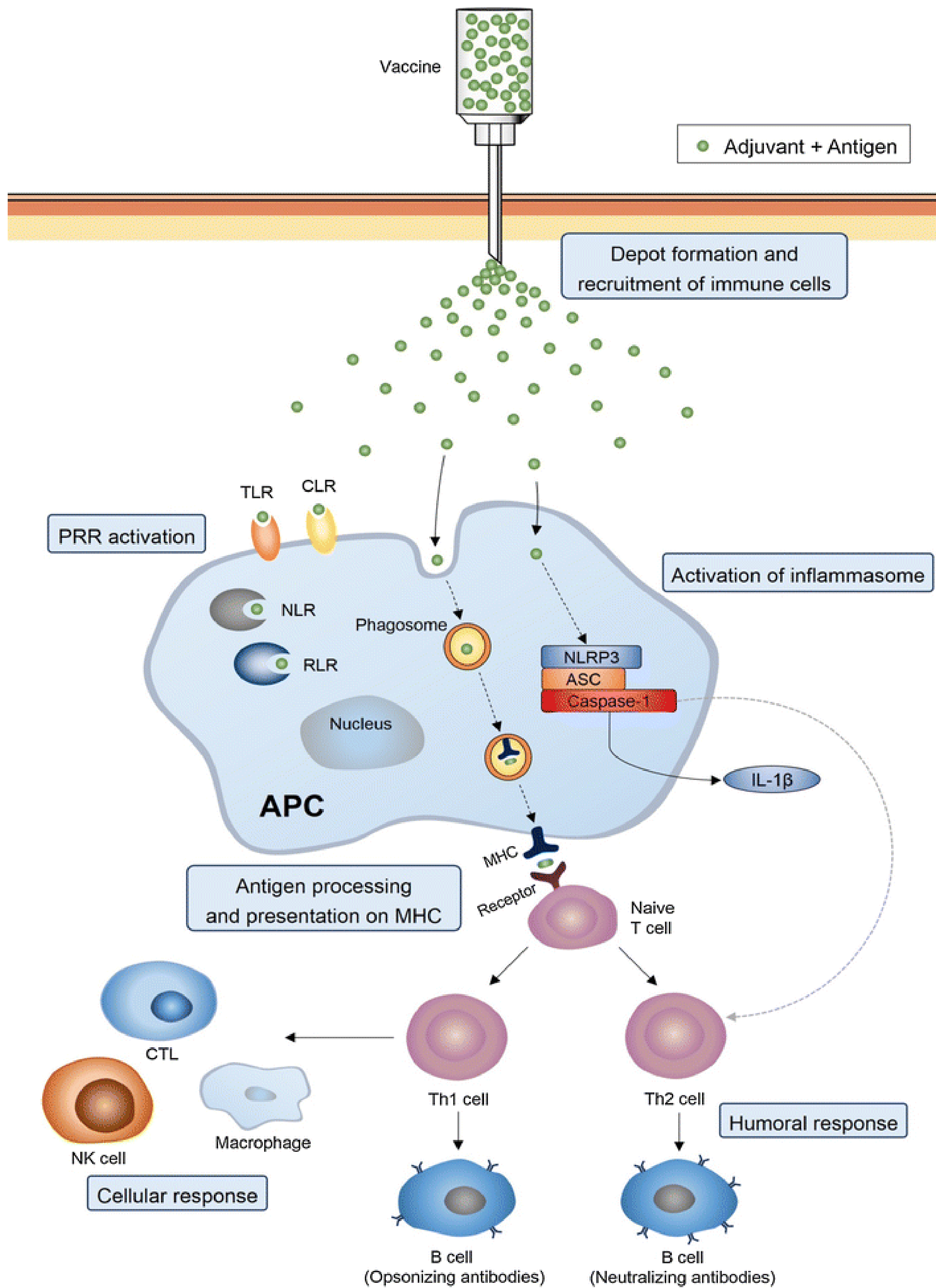

Figure 1-5. Schematic demonstrating the mode of action of adjuvants in generating an immune response taken from (Bastola et al., 2017)

Common adjuvants that could be used in mucosal vaccines include combinations of PRR agonists, particularly TLR agonists as they are potent immunostimulatory molecules that can direct the immune response and they have been implicated in APC activation and maturation (Srivastava et al., 2015). PRRs that have been used experimentally as vaccine adjuvants include monophosphoryl Lipid A (MPL; TLR4 agonist), polyI:C (TLR3 and MDA5 agonist), flagellin (TLR5 agonist), CpG oligodeoxynucleotide (CpG ODN; TLR9 agonist) and MDP (NOD2 agonist). MPL is a component in the oil in water emulsion AS04, and alone it has been effective in inducing a mucosal immune response in mice when delivered IN, directing the response towards a Th1-type T cell response (Baldridge et al., 2000). PolyI:C is a synthetic analog of dsRNA and has been found to induce differential immune responses towards ovalbumin when delivered to the airway of mice, showing a Th2/Th17-type T cell response at low polyI:C concentrations and a Th1-type T cell response to a higher dose of polyI:C (Choi et al., 2010; Jeon et al., 2007). When delivered intraperitoneally in mice, polyI:C was able to induce a strong CTL response against ovalbumin, indicating together with the previous studies that both dose and vaccination route with polyI:C can impact the type of immune response generated by (Wang et al., 2010). Additionally, when polyI:C was formulated as the adjuvant in an IN influenza vaccine evaluated in mice, it induced a strong mixed Th1/Th2-type T cell response that was both protective against the vaccinated strain and a variant strain indicating a degree of cross-protection (Ichinohe et al., 2005). Flagellin is a powerful vaccine adjuvant that is as effective as cholera toxin at generating an immune response, however, it is much safer (Srivastava et al., 2015). When delivered IN, flagellin-induced strong mucosal and systemic immune responses in mice to tetanus toxoid, allowing mice to survive delivery of 200x the lethal dose to tetanus toxoid (Lee et al., 2006). CpG ODN has been used in several experimental mucosal vaccines including both IN and intravaginal administration

in mice with influenza or herpes simplex virus, respectively, and it helped induce mucosal immune responses including secretion of antibodies in the genital tract and induction of a Th1-type T cell response (Moldoveanu et al., 1998; Tengvall et al., 2006). CpG ODN formulated as part of a vaccine against bovine respiratory syncytial virus administered subcutaneously induced a strong Th1-type T cell response in both mice and rabbits, however when combined with emulsigen or alum the response increased significantly in both species (Ioannou et al., 2003; Oumouna et al., 2005). CpG ODN formulated with or without alum in an IM vaccine against bovine herpesvirus-1 showed similar levels of efficacy between CpG ODN alone and in combination, however, CpG ODN alone led to significantly less tissue damage at the site of injection (Rankin et al., 2002). Because the mechanisms of activation for many PRRs have been well-studied in APCs and other immune cells, PRR agonists can be selected to direct the desired immune response.

As an alternative to stimulating the production of cytokines by formulating a vaccine with immunostimulatory adjuvants, cytokines and chemokines themselves have been evaluated as vaccine adjuvants. IL-12 delivered IN with tetanus toxoid resulted in a strong Th2-type T cell immune response in mice, but the inclusion of pro-inflammatory IL-6 with IL-12 had no impact on the immune response (Boyaka et al., 1999). Additionally, IN delivery of an HIV peptide with IL-1α, IL-12 and IL-18 in mice resulted in a strong Th2-type T cell response with large amounts of IgA secreted throughout the gastrointestinal tract and the vagina (Bradney et al., 2002). Although the previous two studies showed cytokines induced immune responses through mucosal delivery, it should be noted that they both required 3-6 doses delivered every seven days, which limits the practicality of these formulations. CCL5 included in a vaccine with ovalbumin and delivered IN in mice induced monocyte recruitment to the site of administration and it was effective in promoting a Th1-type T cell response (Lillard et al., 2001). Cytokines and chemokines

show promise as adjuvants, however, their short half-lives, toxicity in high doses and the associated cost of synthesis and purification have hindered their use/acceptance (Srivastava et al., 2015).

Another common type of adjuvant are carrier molecules or delivery vehicles that aid in trafficking the antigen to APCs, however several of these molecules also possess immunostimulatory capabilities. ISCOMs are small nanoparticles that include saponins, and that carry co-formulated antigen to APCs (Gerdts et al., 2006). ISCOMs induce immune cell recruitment specifically through IL-12 stimulation and have been shown to induce Th1-type, Th2-type T cell and CTL responses to ovalbumin when delivered as mucosal adjuvants in mice (Smith et al., 1999). They have also been successfully used to induce mucosal and systemic immune responses in several experimental mucosal vaccines delivered orally with ovalbumin and IN with influenza in mice (Mowat et al., 1993; Sanders et al., 2009). Although ISCOMs are promising candidates as mucosal adjuvants, not all antigens can be co-formulated with ISCOMs without modifications which have limited their use (Srivastava et al., 2015). Polyphosphazenes are water-soluble polymers that have also been found to be effective carrier molecules with an inherent immunostimulatory capacity and which can be used to form microparticles with antigen relatively easily (Andrianov and Payne, 1998). When delivered IN to mice alone, compared to the same antigens formulated with CpG ODN, a polyphosphazene was found to induce a stronger humoral response to multiple antigens both systemically and mucosally (Shim et al., 2010). Additionally, although its mechanism of action has not been characterized when administered to mucosal surfaces, *in vitro* studies with mice splenic DCs have indicated that polyphosphazenes may induce activation of the inflammasome leading to secretion of IL-1β (Awate et al., 2014). When delivered as a vaccine adjuvant IM in mice, polyphosphazene was shown to upregulate several inflammatory

cytokines and chemokines including CCL2 and IL-1β, in addition to upregulation of the inflammasome receptor NLRP3 (Awate et al., 2012). In pigs, polyphosphazene delivered by the intradermal route similarly showed induction of Il-1β at the site of injection, and when formulated with inactivated swine influenza, the vaccine produced neutralizing antibodies and decreased the viral load in response to infectious challenge (Magiri et al., 2018; Magiri et al., 2019). Taken together, these studies indicate that polyphosphazene may be effective as both a mucosal and parenteral vaccine adjuvant, possibly through activation of the NLRP3 inflammasome. Delivery systems or carrier proteins that increase APC uptake and presentation of antigen are effective adjuvants; however, if the carrier proteins can act in an immunostimulatory capacity as is observed with polyphosphazenes and ISCOMs, this leads to promising adjuvants for use in mucosal vaccines.

Because effective vaccines may require activation and recruitment of several arms of the immune system, this may be difficult to achieve with a single adjuvant. Recently, there has been increasing development of combinatorial adjuvants that carry out a diverse assortment of responses. A combinatorial adjuvant platform composed of a TLR ligand (CpG or polyI:C), a host defence peptide and a polyphosphazene has shown promise as an effective adjuvant combination (triple adjuvant combination (TriAdj)) when evaluated in several parenteral and mucosal vaccine formulations in multiple species. Host defence peptides are antimicrobial molecules that have the capacity to modulate the immune response. *In vitro* studies have found that HDPs increase monocyte recruitment to CCL3 and CCL5 through induction of increased CCR5 expression (Madera and Hancock, 2015). Further *in vitro* studies with a human monocyte cell line showed that the HDPs BMAP-27 and LL-37 suppressed the induction of TNF-α in response to LPS and when characterized in bovine cells was found to induce a similar suppression if inflammation in

response to LPS (Mookherjee et al., 2006). Additionally, select HDPs (BMAP-27 and BMAP-28) were found to decrease the capacity for porcine PBMCs to respond to stimulation (Levast et al., 2014). LL-37 may play an important role in wound healing of the skin, in addition to its antimicrobial activity (Dybvig et al., 2011). Formulation of the HDP with CpG ODN and polyphosphazene with antigen injected into mouse muscle synergistically increased the antigen-specific serum response relative to the response observed in mice injected with vaccines formulated with single or double adjuvants (Kovacs-Nolan et al., 2009a). The synergistic impact of combining these three adjuvants was confirmed in cattle vaccinated with TriAdj and hen egg lysozyme through the subcutaneous route generated a balanced Th1/Th2-type T cell response (Kovacs-Nolan et al., 2009b). The triple combination (using polyI:C in place of CpG) induced a strong Th1/Th2-type T cell balanced response to human parainfluenza virus subunit vaccine when delivered both IN and IM in rats, with IN vaccination also conferring a strong protective mucosal immune response (Garg et al., 2017b). The triple combination administered to murine macrophages *in vitro* significantly induced expression of several pro-inflammatory cytokines and chemokines, in addition to increasing macrophages MHC class II expression and co-stimulatory molecule expression (Sarkar et al., 2018). Use of the TriAdj with respiratory syncytial virus antigen administered IN into mice or administered via the intrauterine route with ovalbumin and a bovine herpesvirus protein in rabbits triggered strong systemic and mucosal immune responses from a single vaccine dose (Garg et al., 2014; Pasternak et al., 2017). Additionally, maternal vaccination with a subunit respiratory syncytial virus vaccine delivered IM in pregnant ewes resulted in the maternal transfer of immunity to newborn lambs which protected them against challenge (Garg et al., 2016). This strong passive immunity transfer was also observed against respiratory syncytial virus and human parainfluenza virus when evaluated in the pregnant ewe

vaccination model (Garg et al., 2015; Garg et al., 2019). The TriAdj has shown to be an effective adjuvant combination in delivery across several species including pigs, cows, koalas (Khan et al., 2014), sheep, rabbits, mice and rats delivered both through parenteral and mucosal routes. As we continue to better understand the mechanism of action of adjuvants, combinatorial adjuvants will allow for the intelligent design of vaccines to optimize the desired response dependant on the route of delivery, mucosal or parenteral.

1.3.3 Delivering intrauterine vaccines in sows

Delivery of vaccines in large animals can be difficult and a potential safety hazard, as is observed by the large portion of swine veterinarians who report needle-stick injuries, with 40% of those injuries occurring when delivering vaccines (Hafer et al., 1996). Because most mucosally-delivered vaccines do not require needles, their use will reduce the number of vaccine-related injuries acquired by veterinarians and swine staff. While oral and IN vaccines may be practical for delivering vaccines to piglets, there is a greater degree of difficulty in delivering these vaccines to full-grown animals such as gilts and sows because their administration would require snaring of the animals. Challenges associated with snaring such large, muscular animals further highlights how an alternative route of immunization such as intrauterine vaccination in swine may be advantageous. When gilts or sows are in estrus and in proximity to a boar or its pheromone androstenone, they undergo a lordosis response where they are rigid in preparation for mounting (Dorries et al., 1997). During lordosis, semen and a vaccine can be combined and easily and safely delivered. Breeding of sows by AI is already an established practice in commercial pig operations, and it takes place multiple times per year which offers a safe and easy means of incorporating a new vaccination regimen into current husbandry practises.

The uterus has a well-known natural inflammatory immune response generated against extended semen which may mean that the extended semen could act as an ‘adjuvant’ for the intrauterine vaccination in sows It has been widely acknowledged that breeding in swine elicits an inflammatory immune response and neutrophil infiltration into the lumen (Katila, 2012; Rozeboom et al., 1999). Other studies indicate that cytokine and chemokine genes are induced following breeding. Notably, GM-CSF was induced in uterine tissue following breeding in pigs with extended semen and a corresponding increase in MHC class II positive cells in both the *lamina propria* and directly basolateral to the epithelial layer (O'Leary et al., 2004). Another study showed that the semen extender Androhep and seminal plasma alone induced IL-10, TGF-β, IL-8, and TNF-α, however when combined with spermatozoa, the level of gene expression of these genes was reduced suggesting that spermatozoa may contribute to a degree of suppression(Taylor et al., 2009). There is significant leukocyte recruitment to the uterine lumen (including APCs) in response to seminal plasma and semen extenders, but the majority of the cells recruited are granulocytes which are not the target cell population for an intrauterine vaccine. Other adjuvants that promote APC recruitment to the lumen or direct stimulation of the uterine epithelial cells lining the uterus may be key to an effective intrauterine vaccine combined with extended semen.

2 HYPOTHESIS AND RESEARCH OBJECTIVES

General hypothesis:

The epithelial layer of the porcine uterus will respond to vaccine adjuvants combined with semen to induce an environment conducive to effective vaccination. Cultured primary pig uterine epithelial cells will provide an efficient model to select adjuvants to be included in an intrauterine vaccine.

Research Objectives:

1. Characterize receptors on primary porcine uterine epithelial cells both *in vivo* and after isolation and culturing using a transwell system.
2. Characterize the innate immune response of uterine epithelial cells to vaccine adjuvants alone and in combination.
3. Characterize the innate immune response and immune cell recruitment following exposure to vaccine adjuvants combined with semen, *in vivo*.
4. Characterize the effect of stimulated epithelial cell secretions on monocytes and monocyte-derived dendritic cells, *in vitro*.

Rational

UECs and their role in the uterine immune response has been well characterised in humans and mice, however few studies have been carried out in other species including large animals such as pigs. In humans and mice UECs have been shown to not only express large numbers of PRRs, respond to stimulation, recruit immune cells, present antigen to T cells and modulate the responses of antigen presenting cells. Because of this there is the potential of porcine UECs to play a significant role in initiating the immune response to an intrauterine vaccine.

3 CHARACTERIZATION OF PORCINE UTERINE EPITHELIAL CELLS AND ESTABLISHMENT OF A POLARIZED CULTURE SYSTEM.

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Author Contributions

GH, JAP and HLW conceived of and designed the experiments. JAP, TK and GH developed the UEC isolation and culturing protocol. GH and NK carried out qPCR experiments, GH and JAP developed and optimized all staining protocols. GH optimized and carried out laser-capture and stimulation experiments. GH analyzed all data and drafted the manuscript. All authors read and approved the manuscript.

Abstract

Preservation of a pathogen-free uterine environment is critical for maintaining healthy swine herds with high reproductive performance. Considering that uterine epithelial cells are the most numerous and thus the most likely point of cellular contact for pathogens in the uterus, we hypothesize that these cells may be critical for activating the immune system to clear uterine infections. Although uterine epithelial cells have not been well characterized in pigs, studies in several other species have shown that these cells express several pattern recognition receptors (PRR) and thus may act as sentinels for the uterine immune response. To characterize PRR expression in the porcine uterine epithelia, we used laser-capture microdissection to isolate epithelial cells lining the porcine uterus to quantify *in vivo* mRNA expression levels for select PRRs. As well, primary uterine epithelial cells (UECs) were isolated, cultured, polarized, and PRR expression was quantified. Immunohistofluorescence and immunofluorescence were used to determine the subcellular localization of TLR3, TLR4, and TLR9 in both uterine tissues and polarized primary UECs. Finally, polarized primary UECs were stimulated with ligands for TLR3, TLR4, TLR9, and NOD2 to determine their functional innate immune response. Uterine epithelial cells (*in vivo* and *in vitro*) were shown to express TLR1-7, TLR9, NOD1, NOD2, NLRP3, NLRP6, NLRX1, RIG1, MDA5 and LGP2. Subcellular localization of *in vivo* and polarized primary UECs exhibited TLR3 and TLR9 localized to the apical cell surface whereas TLR4 was localized to the intracellular space. Polarized primary 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. These results indicate that pig uterine epithelial cells are functional innate immune cells that may act as sentinels to protect against uterine infection

3.1 Introduction

Reproductive health is essential in maintaining litter sizes and piglet health, and biological mechanisms must be in place to ensure pathogens are quickly cleared from the porcine reproductive tract. As in other species, epithelial cells in swine are the first line of defence against invading pathogens at all mucosal surfaces, and they form a physical barrier preventing pathogen invasion. Additional to forming a physical barrier, these cells express several PRRs (Mair et al., 2014) which, upon stimulation, can direct the immune response through secretions of cytokines and chemokines. While the porcine uterine epithelia have not been well characterized, human uterine epithelial cells have been described as sentinels which can initiate the immune response in the uterus upon PRR activation and signalling (Wira et al., 2005b).

PRRs are expressed by numerous cell types in the body, and they bind PAMPs enabling detection of a wide array of pathogens. PRRs are categorized as TLRs, NLRs, RLRs, and CLRs based on their backbone protein structure. Among these PRRs, TLRs are the best characterized and consist of 10 members in most mammalian species, with 12 members identified in mice and rats. TLRs bind a wide variety of PAMPs including dsRNA or polyI:C (TLR3 ligand), LPS (TLR4 ligand), flagella (TLR5 ligand) and unmethylated CpG DNA (TLR9 ligand)(Kawai and Akira, 2010). Broad spectrum PAMP recognition enables TLRs to recognize and bind both viral, bacterial, and fungal pathogens (Kumar et al., 2011). Originally, TLR1, 2, 4, 5 and 6 were thought to be exclusively localized to the outer membrane of the cell and TLR3, 7, 8 and 9 were thought to be localized to the endosomal membranes. However new research, notably in epithelial cells, has identified the non-canonical subcellular localization of TLRs indicating that there are cell and tissue-dependent localization of these receptors (Hamonic et al., 2018a; McClure and Massari, 2014). NLRs encompass a large family of cytosolic receptors that include 22 members in humans

and 34 in mice, and many play an important role in the formation of the inflammasome (Motta et al., 2015). Ligands for NLRs include peptidoglycans (which target NOD1 and NOD2)(Kumar et al., 2011), viral RNA (which targets NLRX1) (Hong et al., 2012) and various as yet undefined molecules that can initiate activation of the inflammasome (through NLRPs) (Lupfer and Kanneganti, 2013). RLRs are a family of 3 cytosolic receptors, including RIG-I, MDA5 (which bind dsRNA) and LGP2 (which influences RIG-I and MDA5 activity) (Yoneyama and Fujita, 2009). Finally, CLRs are a large family of receptors that recognize fungal pathogens through several receptors, including Dectin-1 (gene name CLEC7A), which recognize β-glucans (Hardison and Brown, 2012). Although they were not investigated in this study, some CLRs can recognize both viral pathogens (CLEC5A) and bacterial pathogens (CLECSF8) (Hoving et al., 2014).

The expression, localization, and functionality of each PRR family and their constituents vary greatly across mucosal tissue and species, in a manner indicative of local immune requirements. Human uterine epithelial cells have been shown to express multiple PRRs and behave as sentinels to initiate the immune response when pathogens enter the uterus (Wira et al., 2005b). We hypothesize that porcine uterine epithelial cells also express multiple PRRs that may be necessary to initiate the immune response and combat infection. The following study determines PRR expression in porcine uterine epithelial cells both *in vivo* and *in vitro* and by comparing expression levels to tissues and cellular populations with known differential PRR expression provide context to the levels of expression of uterine epithelial cells. Defining the expression and functionality of select PRRs in porcine uterine epithelial cells will improve our understanding of their immune response and role in the immune response following exposure to pathogens in the uterus.

3.2 Materials and Methods

Sample collection for qPCR analysis:

All experimental procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC) under approval from the Animal Research Ethics Board at the University of Saskatchewan.

Duplicate uterine tissue (UT) was collected from the middle of the left uterine horn of Landrace-cross sows in estrous (n=3). Sows were synchronized to estrus through daily oral progestin treatment with Regu-Mate (Merck Animal Health) for 14 days, followed by an 800 I.U. intramuscular injection of Folligon (equine chorionic gonadotropin, Merck Animal Health) 24 hours after final Regu-Mate dose. Eighty hours after Folligon injection sows were administered a 5 mg intramuscular injection of Lutropin-V (porcine luteinizing hormone, Bioniche Animal Health). Fifty-six hours later, sows were euthanized, and estrus was confirmed by analyzing the follicles on the ovaries. Tissue was collected from the middle of the left uterine horn and flash frozen in dry ice within 20 minutes of humane euthanasia. One duplicate was used for laser-capture microdissection of the uterine epithelia, and the other was used for RNA isolation from whole UT.

Uterine lymph node, testes and peripheral blood mononuclear cells (PBMCs) were collected to provide context to the transcript levels detected in the uterine tissue and uterine epithelia.

Uterine lymph nodes (LN; n=3) were collected from the broad ligament of animals and flash frozen in dry ice within 20 minutes of euthanasia. These cells served as a reference cell with a relatively high level of PRR expression.

Porcine testicular tissue (TES; n=3) was collected during routine castration of male piglets at a local barn and frozen on dry ice until processing. These tissues served as a reference cell type with a relatively low level of PRR expression.

Peripheral blood mononuclear cells (PBMC): Whole blood was collected from sows (n=4) using EDTA Vacutainers (BD Biosciences), and buffy coats were collected by spinning at 1100 x g for 30 minutes. Buffy coats were resuspended in PBS, layered on Ficol-Paque plus (GE Life Sciences) and separated at 400 x g for 40 minutes. PBMCs were collected and washed 3x in PBS with centrifugation at 250 x g for 10 minutes. Finally, pellets were collected in Trizol (Invitrogen) for RNA isolation and cDNA preparation (described below).

Laser-capture microdissection of uterine epithelia for RNA isolation

Frozen UT from each animal was mounted into optimal cutting temperature compound (OCT; ThermoFisher) blocks and cryosectioned at a thickness of 14 µm onto polyethylene naphthalate membrane slides (ThermoFisher). Slides were immediately fixed in 70% ethanol, and OCT was removed by submersion in DEPC treated water (Invitrogen). Slides were stained with cresyl violet (Sigma) for 30 seconds, and the excess stain was removed by submersion in 70% ethanol, followed by 100% ethanol. Slides were then used for laser-capture microdissection (LCM) using a PALM-microbeam system (Zeiss). The basolateral third of the epithelial cells were trimmed with the cutting laser prior to collection to ensure no contamination of underlying stromal cells (Supplemental Figure 3-2). The laser-captured uterine epithelial cells (LC-UE) were collected within 45 minutes of sectioning to maintain RNA integrity. RNA was isolated using the Picopure RNA isolation kit (Thermofisher) following manufacturer instructions with an on-column DNase treatment (Qiagen).

Isolation, Culture, and Treatment of uterine epithelial cells:

Reproductive tracts from post-pubertal gilts and sows were collected from a local abattoir (n=5), and uterine horns were excised then flushed with PBS containing 1X Anti-Anti (Gibco). Horns were cut into 15 cm segments and inverted to expose the epithelial layer to the buffer. Horn segments were ligated at one end with suture material, the interior cavity was filled with PBS containing 1X Anti Anti (Gibco), and then the other end of the segment was closed with a similar ligature to prevent leakage. Inverted horns were then placed in flasks and submerged in an enzyme solution containing 250 units/L of dispase (BD Bioscience) and 12 g/L of pancreatin (laboratory grade, Fisher Scientific) in PBS containing 1X Anti Anti (Gibco) and incubated at 4°C overnight with shaking. H&E staining of the tissue after enzyme digestion shows that the epithelial cells were effectively sloughed off, and the remaining tissue was relatively intact (Supplemental Figure 3-1). The sloughed cells were centrifuged at 400 x g for 10 minutes then treated with GEYS solution [$CaCl_2$ (0.220 g), KCl (0.370 g), KH_2PO_4 (0.03 g), $MgCl_2$ (0.210 g, $MgSO_4$ (0.070 g), NaCl (8.000 g), $NaHCO_3$ (0.227 g), Na_2HPO_4 (0.120 g), D-glucose (1.000 g; all from Sigma) in 1 L distilled water] for 10 minutes at room temperature (RT) to lyse red blood cells and then washed twice in PBS. Washed cells were filtered through a 40 µm cell strainers to remove large debris prior to culture.

Sloughed uterine epithelial cells (UECs) were cultured and polarized on transwell polyethylene terephthalate (PET) membranes with 0.4 µm pore size (Greiner Bio-One) in DMEM:F12 (1:1; GE Life Sciences) with 2.5 mM glutamine (Gibco)**,** 10 mM HEPES (Gibco), 10% FBS (Sigma-Aldrich) and 1x Anti Anti (Gibco) with media changes every second day. To confirm that the cells on the transwell membranes were epithelial cells, representative membranes from each animal's cells were stained with the epithelial cell marker claudin 4 (see

immunofluorescence of cells grown on a transwell membrane below). The transwell membranes were imaged at ten random locations to ensure all cells on the membrane were positive for the epithelial marker (data not shown). Transepithelial electrical resistance (TEER) was measured prior to each media change, and when the TEER measurements were stable and over 1000 ohm, the cells were considered polarized. Polarization took approximately 10-14 days of culture and polarity was confirmed using anti-zona occludens 1 staining (see below), the polarized UECs were collected for RNA isolation, stained for immunofluorescent microscopy or treated with PAMPs for functional analysis. PAMP treatments included polyI:C (50 µg/ml, Invivogen; TLR3 ligand), Lipopolysaccharide (LPS; 50 µg/ml, *Salmonella enterica* serotype Minnesota from Sigma-Aldrich; TLR4 ligand), CpG ODN (CpG 2395; 50 µg/ml, Merial; TLR9 ligand) and muramyl dipeptide (MDP; 50 µg/ml, Sigma-Aldrich; NOD2 ligand) for 24 hours with ligands in both the apical and basolateral compartments of the transwell. Ligand concentrations were determined by an initial dose titration experiment, and as we observed no significant decrease in TEER at the endpoint of the experiment, we determined the selected concentrations were non-toxic. Supernatants were collected from the apical compartment of the transwell at 24 hours for cytokine analysis.

RNA isolation and qPCR analysis

To isolate RNA from the uterine horn, testicles and lymph node, the tissues were frozen to -80°C, were ground with a mortar and pestle, and 100 mg of ground tissue was thoroughly lysed in 1 ml of Trizol (Invitrogen) with vigorous shaking. For RNA isolation from PBMCs and UECs, 500 µl Trizol was added directly to the pelleted cells. RNA was then isolated by the double precipitation method as previously described (Pasternak et al., 2015). The RNA was DNase treated using Turbo DNAse free kit (Ambion) performed following the manufacturer's instruction. The

RNA integrity was determined by denaturing gel electrophoresis (for cell culture experiments) or by Bioanalyzer (Agilent; for LCM experiments), and only high-quality samples were carried forward. RNA was quantified by Nanodrop (ThermoFisher), and quantity was normalized for each experiment to 150-1000 ng/reaction and converted to cDNA using the High-Capacity cDNA Reverse Transcription kit (Ambion) as per manufacturer's instructions. cDNA was diluted, and qPCR was performed using a Step One Plus thermocycler (Applied Biosystems) (4 ng for the LCM experiments and 10ng for the cell culture experiments) per sample in 15 µl reactions using SYBR mastermix (KAPA Biosystems) with primer concentrations at 0.2 mM. Primer pairs and annealing temperatures are described in Table 3-1. Primer amplification efficiency for every primer pair at optimal annealing temperature was measured and found to be greater than 90% in all cases. Where possible, primers were designed using primer3 to span exon-exon junctions identified in relation to Sus scrofa 10.2 genome. Multiple reference genes (HPRT, GAPDH, and RPL19) were analyzed, and those that were stable across all tissue or cell types were included as a geometric mean to normalize expression results (see figure legends).

Due to the low RNA concentrations obtained following laser-capture microdissection and the large number of gene targets being analyzed, laser-captured epithelial cells were collected in two batches. Reverse transcription reactions were performed on one set of cells to analyze TLRs, and a repeat reverse transcription reaction was performed on the second set of isolated cells to determine mRNA expression of the non-TLR gene targets. For each batch, cDNA for identical reference genes were analyzed. The analysis was limited to within batches to prevent any variation associated with the individual reverse transcription assays.

Table 3-1. Primer names, sequences, and annealing temperature for characterization of uterine epithelial cells.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Tm (°C)	Target sequence or reference
HPRT	GGACTTGAATCATGTTTGTG	CAGATGTTTCCAAACTCAAC	60	(Nygard et al., 2007)
GAPDH	CTTCACGACCATGGAGAAGG	CCAAGCAGTTGGTGGTACAG	63	(Bruel et al., 2010)
RPL19	AACTCCCGTCAGCAGATCC	AGTACCCTTCCGCTTACCG	60	(Meurens et al., 2009a)
YWHAZ	TGATGATAAGAAAGGGATTGTGG	GTTCAGCAATGGCTTCATCA	60	(Nygard et al., 2007)
TLR1	TGGAAGAGGTCAGGATCACC	GATGGCAAAATGGAAGATGC	60	NM_001031775.1
TLR2	ACGGACTGTGGTGCATGAAG	GGACACGAAAGCGTCATAGC	62	(Meurens et al., 2009a)
TLR3	GACCTCCCGGCAAATATAAC	GGGAGACTTTGGCACAATTC	60	NM_001097444
TLR4	TGTGCGTGTGAACACCAGAC	AGGTGGCGTTCCTGAAACTC	60	(Meurens et al., 2009a)
TLR5	GGACTTGACAACCTCCAGATTCT	AGGAACCTGAATGTTTGGTCCT	60	NM_001123202.1
TLR6	GGGGAACCCTAATCCAGTTC	AGCTGCGAGAGAAAGCTGAT	60	NM_213760.1
TLR7	CGGTGTTTGTGATGACAGAC	AACTCCCACAGAGCCTCTTC	61	NM_001097434
TLR8	CACATTTGCCCGGTATCAAG	TGTGTCACTCCTGCTATTCG	60	NM_214187
TLR9	GGCCTTCAGCTTCACCTTGG	GGTCAGCGGCACAAACTGAG	64	(Dobrescu et al., 2014)
TLR10	GCCCAAGGATAGGCGTAAAT	CGAGACCCTTCATTCAGCTC	60	NM_001030534.1
NOD1	GCCTCACGGTCATCAGACTC	ACGTACCTGGCTCCGACATC	62	(Käser et al., 2015)
NOD2	GAGCGCATCCTCTTAACTTTC	ACGCTCGTGATCCGTGAAC	60	(Tohno et al., 2008)
NLRP3	GCAACCTGGCTGTAACATTC	GATCCAGTTCCACCAACTTC	60	JQ219660
NLRP6	CTGCAAACCCTCAGGCTAAC	TCGTGTGTGATGACCAGTCC	60	XM_003124236.3
NLRX1	ACTCCAGCCAGCAGATGAAC	ATCACTGTCCGCTGAGCTTC	62	NM_001204769.1
RIG1	CGACATTGCTCAGTGCAATC	TCAGCGTTAGCAGTCAGAAG	60	(Dobrescu et al., 2014)
MDA5	AGCCCACCATCTGATTGGAG	TTCTTCTGCCACCGTGGTAG	62	NM_001100194
LPG2	AGAGGGACCAGCAAGAAGTG	ATTGGTCAGGAGCCCATAGC	61	NM_001199132
CLEC7A	TGTGGTCTCCGAGAAAGGA	CCCAGTTGAGAGCATTGTCTT	60	NM_001145866.1

Immunohistofluorescence

Immunohistofluorescence (IHF) was performed on UT to evaluate TLR subcellular localization. Reproductive tracts from post-pubertal gilts and sows were collected from a local

slaughterhouse (n=4), and tissue sections from the middle of the uterine horn were obtained. Tissue sections were cut to 5 mm by 5 mm segments and were either flash frozen and embedded in Shandon Cryomatrix (ThermoFisher) for cryosectioning or tissues were fixed in formalin for 36 hours followed by paraffin-embedding in a Tissue-Tek VIP 6 (Sakura) tissue processor.

Paraffin-embedded tissue blocks were cut at a thickness of 4 µm, and sections were floated onto superfrost plus slides (Thermofisher). Slides were baked for 20 minutes at 60°C, deparaffinized in xylene (10 min x2), and then rehydrated by incubations in decreasing concentrations (v/v) of ethanol. De-paraffinized slides underwent heat-mediated antigen retrieval at 90°C for 60 minutes in TRIS-HCl pH 9.0 before blocking for one hour in 5% skim milk in TBST (TBS containing 0.05% Tween 20). Slides were washed three times in water and incubated overnight at 4°C in primary antibody diluted in dilution buffer (PBS with 1% BSA, 1% horse serum, 0.3% Triton-X and 0.01% sodium azide). For all antibodies used in IHF concentrations and information detailed in Table 3-2. Slides were washed three times in TBST and incubated in secondary antibody for two hours at RT (Table 3-2), and stained slides were again washed 3x in TBST, and the nuclei were counterstained with 4', 6-diamidino-2-phenylindole, dilactate (DAPI, Invitrogen) for 10 minutes at RT. Slides were rinsed in methanol, cover-slipped and imaged on a TCS SP5 scanning confocal microscope (Leica Microsystems), equipped with a 63X oil immersion lens. All antibodies used were validated to be specific through the use of the relevant isotype control antibodies carried out in parallel during staining.

For frozen IHF, tissue was embedded in Shandon Cryomatrix and was cryosectioned at a thickness of 7 µm onto superfrost plus slides (ThermoFisher). Slides were dried after sectioning for 30 minutes and then stained by the same method described above.

Table 3-2 Antibody targets and concentrations used for IHF and immunofluorescence assays.

Target	Species (clone name)	Isotype (flourochrome)	Staining concentration	Supplier (catalog#)
Primary antibodies				
Claudin 3	Rabbit (polyclonal)	IgG	0.4 µg/ml	Abcam (ab15102)
Claudin 4	Rabbit (polyclonal)	IgG	4 µg/ml	Abcam (ab53156)
Claudin 7	Rabbit (polyclonal)	IgG	2 µg/ml	Abcam (ab27487)
E-Cadherin	Rabbit (polyclonal)	IgG	4 µg/ml	Abcam (ab15148)
ZO-1	Rat (R40.76)	IgG_{2a}	2 µg/ml	Santa Cruz Biotechnology (sc-33725)
TLR3	Mouse (TLR3.7)	IgG_1	10 µg/ml	Abcam (ab12085)
TLR4	Rabbit (polyclonal)	IgG	10 µg/ml	ThermoFisher (PAS-23284)
TLR9	Mouse (26C593)	IgG_1	10 µg/ml	BioCarta (IMG-305)
Secondary antibodies				
mIgG	Donkey (polyclonal)	IgG (Alexa647)	4 µg/ml	Abcam (ab150107)
rbIgG	Goat (polyclonal)	IgG (Alexa555)	4 µg/ml	Abcam (ab150082)
ratIgG	Donkey (polyclonal)	IgG (Alexa488)	4 µg/ml	Abcam (ab150153)

Immunofluorescence of cells grown on transwell membranes

When UECs cultured on transwell membranes (n=4) achieved stable TEERs and polarized, cells were washed in PBS and fixed in ice-cold methanol for 10 minutes. Membranes were washed 3x with PBS+2% FBS and stained with the primary antibodies (described in Table 3-2) for 45

minutes at RT. Membranes were again washed 3x in PBS plus 2% FBS and incubated in the appropriate secondary antibody for 30 minutes at RT (Table 3-2). Finally, membranes were washed 3x in dH_2O, stained with DAPI for 10 minutes at RT and rinsed in methanol. Membranes were trimmed, placed on slides, the coverslip was mounted, and the slides were imaged using a TCS SP5 scanning confocal microscope (Leica Microsystems), equipped with a 63X oil immersion lens. Z-stack composite images were produced using LS ASF software using default settings (Leica Microsystems). All antibodies used were validated to be specific through the use of the relevant isotype control antibodies carried out in parallel during staining.

Bioplex ELISA of stimulated UECs

Bioplex bead coupling was performed as per manufacturer's instructions (BioRad). Capture antibodies conjugated to beadsets (listed in Table 3-3) were diluted to a concentration of 1200 beads per well in diluent (PBS + 1% New Zealand Pig Serum (Sigma-Aldrich) plus 0.05 sodium azide) and incubated with recombinant standards (listed in Table 3-3) and samples in duplicate. Standards at 5000 pg/ml were serially diluted two-fold across 11 wells, and samples were plated neat. The plate was incubated for 1 hour at RT with agitation at 800 rpm. Plates were washed three times with PBS plus 0.05% Tween 20 (Sigma-Aldrich). Biotinylated detection antibodies (listed in Table 3-3) were added to each well at a concentration of 0.5 µg/ml in diluent and incubated for 30 minutes at RT with agitation at 800 rpm. Plates were washed three times with PBS plus 0.05% Tween 20 before Streptavidin RPE (Prozyme) was diluted to 5 µg/ml in diluent and incubated for 30 minutes at RT with agitation at 800 rpm. Plates were washed three times with PBS plus 0.05% Tween 20 before beads were resuspended in Tris-EDTA buffer. Plates were read on the BioRad Bio-Plex 200 instrument following the manufacturer's instructions, reading a minimum of 60 events per beadset. For all samples, mean fluorescent intensity data were corrected

by subtracting the background levels. For all cytokines, the intra-assay coefficient of variation fell between 2.2-4.3% and the inter-assay coefficient of variation fell between 4.0-6.3%.

Table 3-3 Antibodies and recombinant standards used by Bioplex ELISA.

Cytokine	Capture antibody	Detection antibody	Recombinant Standard	Bead region
	Antibody; Supplier (catalog #)	Antibody; Supplier (catalog #)	Standard name; Supplier (catalog #)	
IL-1β	MAb anti porc IL-1β/IF2; R&D (MAB6811)	Goat anti porc IL-1β/IF2 biotin; R&D (BAF681)	porcine IL-1β/IF2; R&D (681-PI-010)	26
IL-6	Goat anti porcine IL-6; R&D (AF686)	Goat anti porcine IL-6 biotin; R&D (BAF686)	porcine IL-6; R&D (686-PI-025)	65
IL-10	MAb anti swine IL-10; Invitrogen (ASC0104)	MAb anti swine IL-10 biotin; Invitrogen (ASC9109)	porcine IL-10; Invitrogen (PSC0104)	28
IL-13	Goat anti-swine IL-13; Kingfisher (PB0094S-100)	Goat anti-swine IL-13 biotin; Kingfisher (PBB0096S-050)	porcine IL-13; Kingfisher (RP0007S-005)	52

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc.). mRNA expression analysis on laser captured epithelium and tissue samples was analyzed by one-way ANOVA and the Holm-Sidak multiple comparisons test between all samples. mRNA expression analysis between cultured UECs and PBMCs was analyzed by unpaired t-test with Welch's correction. Cytokine secretion analysis was analyzed by Kruskal-Wallis tests, and multiple comparisons between mock and treated samples were analyzed by Dunn's multiple comparisons test. Significant differences were determined by $p<0.05$.

3.3 Results

Validation of uterine epithelial cell culture

Using IHF on UT, we determined that several tight junction-associated markers were solely expressed by epithelial cells in the porcine endometrium. For instance, claudin 3 (Figure 3-1A),

claudin 4 (Figure 3-1C), claudin 7 (Figure 3-1E) and E-cadherin (Figure 3-1G) were all expressed across the pericellular region of the uterine epithelia. Zona occludens-1 (ZO-1; Figure 3-1I) was also identified in the pericellular region of the uterine epithelial however the localization was restricted to the apical tip of the pericellular region. Additionally, ZO-1 staining was observed within the endometrium in endothelial cells (data not shown) whereas all the claudin markers and E-cadherin were expressed exclusively on the epithelial cells. Immunofluorescence analysis of the polarized primary UECs displayed claudin 3 (Figure 3-1B), claudin 4 (Figure 3-1D), claudin 7 (Figure 3-1F) and ZO-1 (Figure 3-1J) in the pericellular region between UECs. E-cadherin was not expressed in the polarized primary UECs (Figure 3-1H) The z-stack images of ZO-1 staining in the primary cultured UECs confirmed the cells were correctly polarized with the staining being present at the apical tips of the pericellular region showing conservation with the IHF stained tissues (data not shown). Additionally, claudin 3 (Figure 3-1B) and claudin 7 (Figure 3-1F) displayed minor levels of nuclear staining in cultured polarized UECs.

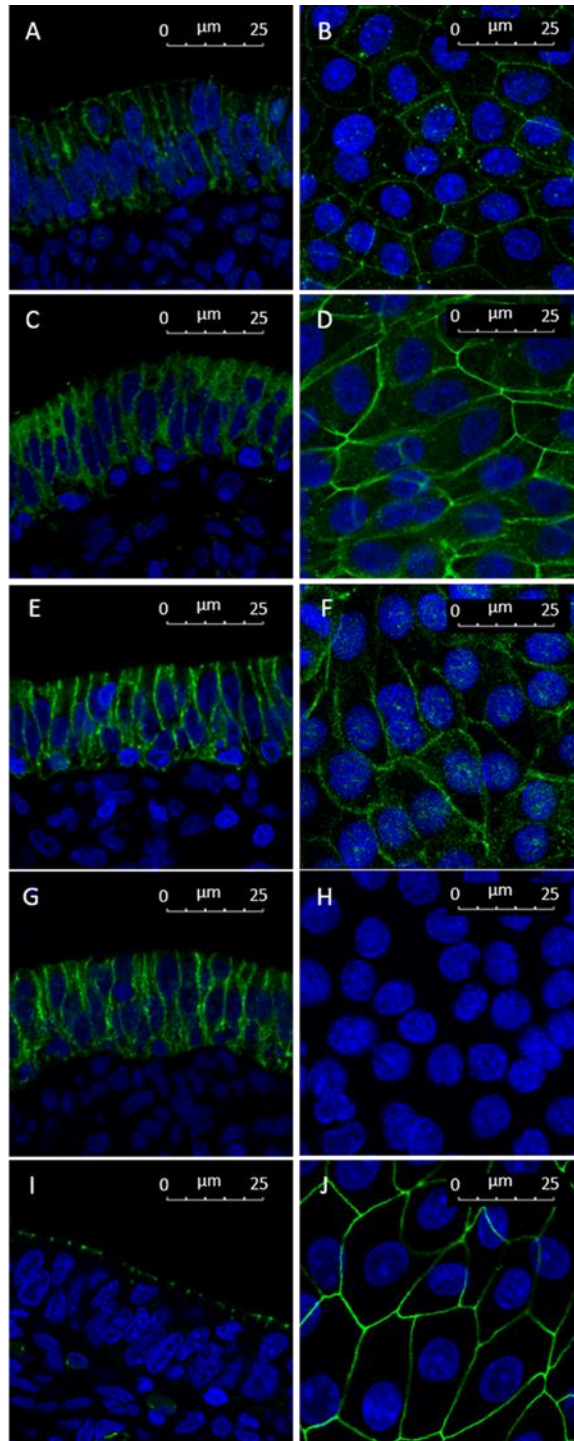


Figure 3-1 Representative IHF of UT and IF of UECs for tight junction proteins. IHF of UT (A, C, E, G and I) and immunofluorescence of primary uterine epithelial cells (B, D, F, H and J) showing staining for claudin 3 (A and B), claudin 4 (C and D), claudin 7 (E and F), E-cadherin (G and H) and ZO-1 (I and J) with protein staining in green and nuclear staining in blue with DAPI. Isotype controls for all antibodies were also imaged (data not shown) and slides were imaged at 63x. Staining was performed on tissue collected from 4 separate animals.

Expression of PRRs in the uterine epithelia and primary uterine epithelial cells

The mRNA expression profile for PRRs was performed on LC-UEs and UT. Also, TES and LN were included to provide context to the relative abundance of transcripts. Figure 3-2 shows the mRNA expression profiles in a heat-map format with the LN acting as the reference tissue (Log_2 fold change values are in Table 3-4). Results from each of the three biological replicates are shown as separate rectangles within each gene and across the tissues. Relative to the uterine LN transcript abundance, genes in the LC-UE, UT or TES that were upregulated are shown as orange to red and downregulated transcripts are shown as green. The transcripts for each gene were detected in every tissue except TLR8, TLR10, and CLEC7A which were undetectable in the LC-UE (see hatch lines). mRNA expression for TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10 was expressed at significantly lower levels (range of 1.8 - 5.7 mean fold down-regulation) in the LC-UE, UT, and TES relative to the LN. TLR3 showed significantly higher mRNA expression (1.2 fold increase, p=0.028) in the LC-UE relative to the LNs and TLR3 mRNA expression in the TES had significantly reduced expression (1.0 fold decrease, p=0.043) relative to the LNs. TLR5 showed a similar level of expression across all tissues. TLR1, TLR2, TLR4, and TLR7 mRNA expression was significantly lower (TLR1: 1.3 fold lower, p=0.0211; TLR2: 2.1 fold lower, p=0.0114; TLR4: 2.33 fold lower, p=0.0122; TLR7: 2.1 fold lower, p=0.0186) in LC-UE relative to the corresponding transcript in the UT.

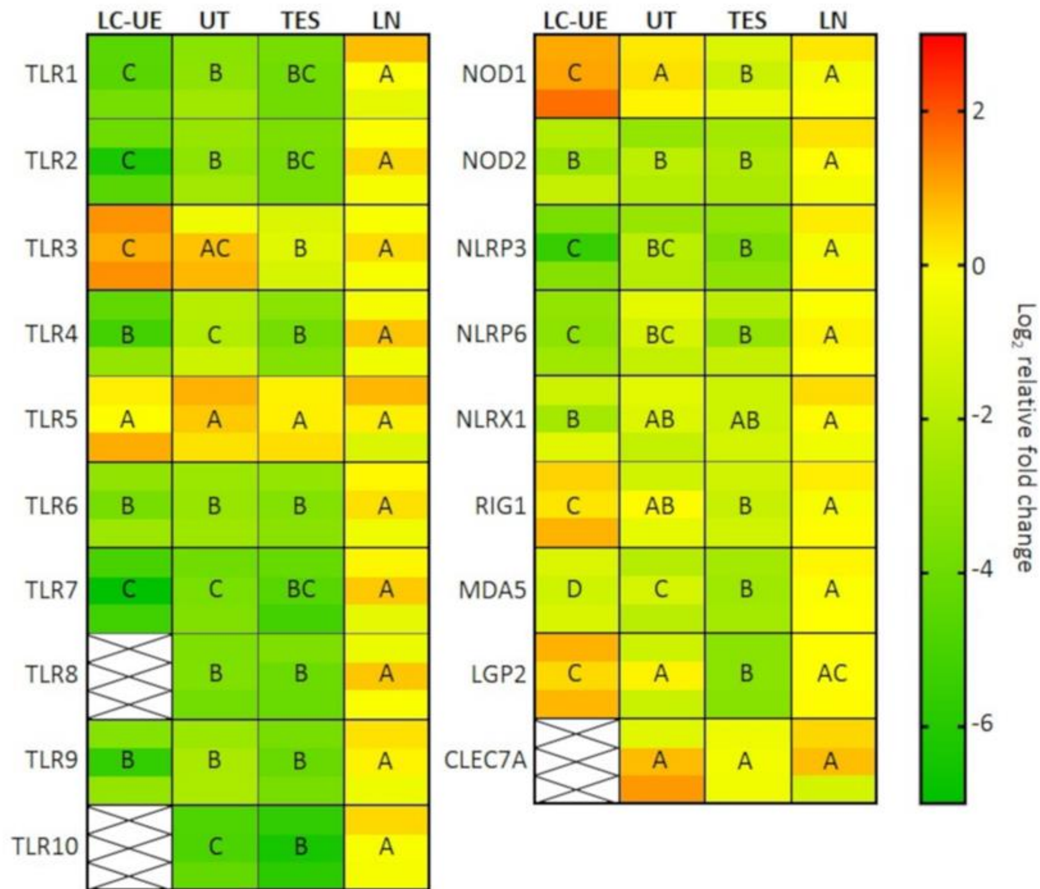


Figure 3-2 Relative expression of several PRRs by LC-UE, UT and TES compared to LN mRNA expression (n=3 per tissue). Relative mRNA expression was determined by qPCR analysis normalized to the stable reference gene YWHAZ and presented relative expressions were calculated using Log_2 2^-(Δ ΔCT). Downregulation is denoted by green colour, upregulation is denoted by orange to red colour, and no detectable expression is denoted by hatched lines. Significant differences in each gene were determined by one-way ANOVA and Holm-Sidak multiple comparisons test with significant differences between tissues (P<0.05) denoted by a differing letter (A, B, C, or D).

Table 3-4 Mean fold changes and standard deviations of fold changes relative to LN expression presented in Figure 3-2.

	Tissue mean Log_2 fold change			
	LC-UE	UT	TES	LN
TLR1	- 4.29 ± 0.45	- 2.96 ± 0.33	- 3.85 ± 0.09	0 ± 0.71
TLR2	- 4.95 ± 1.20	- 2.84 ± 0.26	- 3.68 ± 0.09	0 ± 0.38
TLR3	1.16 ± 0.19	0.38 ± 0.68	- 1.01 ± 0.15	0 ± 0.34
TLR4	-4.14 ± 1.13	- 1.81 ± 0.40	- 3.47 ± 0.31	0 ± 0.58
TLR5	0.38 ± 0.50	0.61 ± 0.28	0.23 ± 0.13	0 ± 0.94
TLR6	- 3.13 ± 0.53	- 2.74 ± 0.09	- 3.18 ± 0.21	0 ± 0.39
TLR7	- 5.73 ± 1.06	- 3.68 ± 0.24	- 4.66 ± 0.49	0 ± 0.66
TLR8	ND*	- 3.63 ± 0.16	- 3.90 ± 0.32	0 ± 0.60
TLR9	- 3.95 ± 1.47	- 2.48 ± 0.23	- 3.87 ± 0.25	0 ± 0.40
TLR10	ND*	- 4.67 ± 0.32	- 5.95 ± 0.36	0 ± 0.39
NOD1	1.24 ± 0.38	0.24 ± 0.11	- 1.01 ± 0.44	0 ± 0.28
NOD2	- 2.14 ± 0.59	- 2.31 ± 0.57	- 2.37 ± 0.14	0 ± 0.32
NLRP3	- 4.17 ± 1.17	- 2.24 ± 0.53	- 3.25 ± 0.26	0 ± 0.24
NLRP6	- 2.91 ± 0.26	- 1.17 ± 0.47	- 2.13 ± 0.72	0 ± 0.12
NLRX1	- 1.54 ± 0.84	- 1.14 ± 0.46	- 1.34 ± 0.12	0 ± 0.41
RIG1	0.56 ± 0.30	- 0.65 ± 0.67	- 1.39 ± 0.15	0 ± 0.21
MDA5	- 1.13 ± 0.17	- 1.69 ± 0.45	- 2.57 ± 0.06	0 ± 0.10
LGP2	0.72 ± 0.25	- 0.93 ± 0.91	- 3.24 ± 0.04	0 ± 0.04
CLEC7A	ND*	0.38 ± 1.07	- 0.38 ± 0.08	0 ± 1.06

*ND denotes no detectable expression.

When non-TLR mRNA expression was examined across sample type, NOD1 had a significantly higher expression (1 fold higher) in LC-UE compared to all other tissues. RIG1 and LGP2 mRNA expression were significantly higher in the LC-UE relative to the UT increasing by 1.2 fold and 1.6 fold respectively, but no significant increase between the LC-UE and the LNs. NOD2, NLRP3, NLR6 and to a lesser extent NLRX1 and MDA5 showed significantly lower mRNA expression (NOD2: 2.1 fold lower, p=0.0015; NLRP3: 4.2 fold lower, p=0.0004; NLRP6: 2.9 fold lower, p=0.0003; NLRX1: 1.5 fold lower, p=0.0417; MDA5: 1.1 fold lower, p=0.0016)

in the LC-UE relative to the LNs. CLEC7A was not significantly different across LN, UT, TES, and the transcript for CLEC7A was not detected in the LC-UE.

To determine if the expression of PRRs in primary UECs was consistent with what was observed *in vivo,* we compared the expression profiles of PRRs between polarized primary uterine epithelial cells and PBMCs (Figure 3-3). Relative to cultured UECs, PBMCs had significantly higher expression for TLR1 (6.3 fold higher, p=0.0018), TLR2 (5.2 fold higher, p=0.0052), TLR5 (2.0 fold higher, p=0.0384), TLR6 (6 fold higher, p=0.011), TLR7 (8.3 fold higher, p=0.0002) and TLR9 (6.7 fold higher, p=0.0023). TLR3 and TLR4 genes did not show differences in expression between the two cell populations, and UECs had undetectable levels of TLR8 and TLR10 transcripts, which is consistent with the results from the LC-UE in Figure 3-2. When the mRNA expression for non-TLR genes was examined, it was determined that PBMCs expressed significantly higher NOD1 (1.2 fold higher, p=0.0028), NOD2 (5.0 fold higher, p=0.0062), NLRP3 (11.3 fold higher, p=0.0024), NLRP6 (1.3 fold higher, p=0.0304), NLRX1 (2.5 fold higher, p=0.039), RIG1 (4.9 fold higher, p<0.0001), MD5A (5.8 fold higher, p=0.0013), and LPG2 (3.6 fold higher, p=0.0002) genes relative to the UECs. Consistent with the *in vivo* results, CLEC7A was not expressed in cultured UECs.

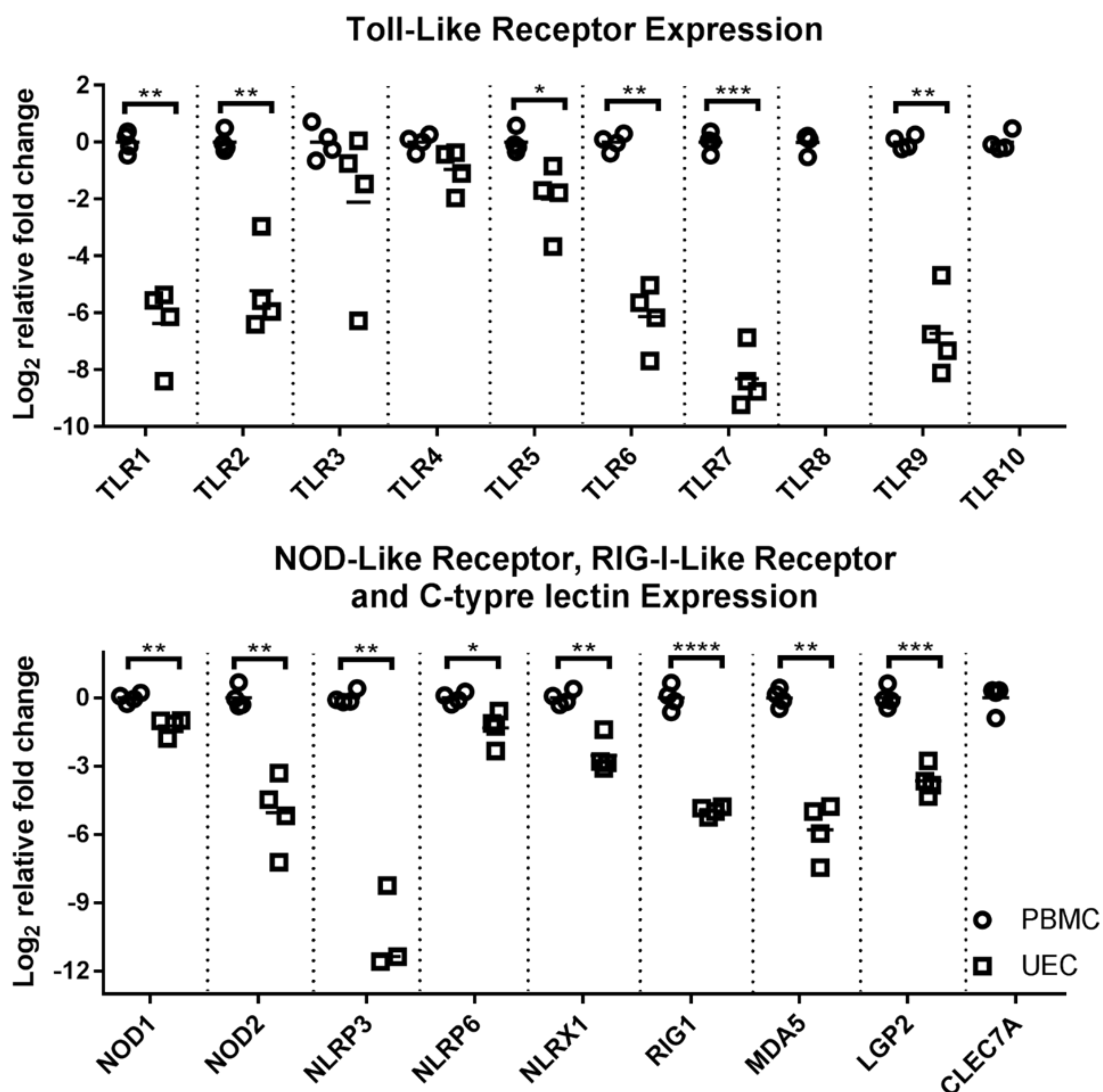


Figure 3-3 Relative expression of several PRRs by primary UECs compared to PBMCs (n=4 per cell type). Relative mRNA expression was determined by qPCR analysis normalized to the geometric mean of stable reference genes HPRT and GAPDH and presented relative expression were calculated using Log_2 2^-(ΔΔCT). Samples that were undetectable by PCR are denoted by ND. Significant differences in each gene were determined by unpaired t-test with Welch's correction with significant differences between cell types ($p<0.05$ = *; $p<0.01$ = **; $p<0.001$=***; $P<0.0001$=****).

Protein expression and localization of TLR3, 4 and 9 in uterine tissues and in cultured primary UECs

Next, we performed IHF to establish subcellular localization of TLR3, 4 and 9 in UT and primary UECs as these proteins have previously been shown to display non-canonical localization. IHF analysis of UT determined that TLR3 (yellow in Figure 3-4A) and TLR9 proteins (yellow in Figure 3-4C) were localized to the apical cell surface of the uterine epithelia whereas TLR4 protein was confined to the cytoplasm of the uterine epithelia (yellow in Figure 3-4B). The expression of each protein target was detected at levels significantly above that of the relevant isotype control (Figure 3-4D). Despite low transcript levels, results from IHF demonstrate that TLR4 and TLR9 proteins were expressed in the epithelial cells from UT, suggesting that low transcript levels are not necessarily indicative of negligible protein expression (Figure 3-4).

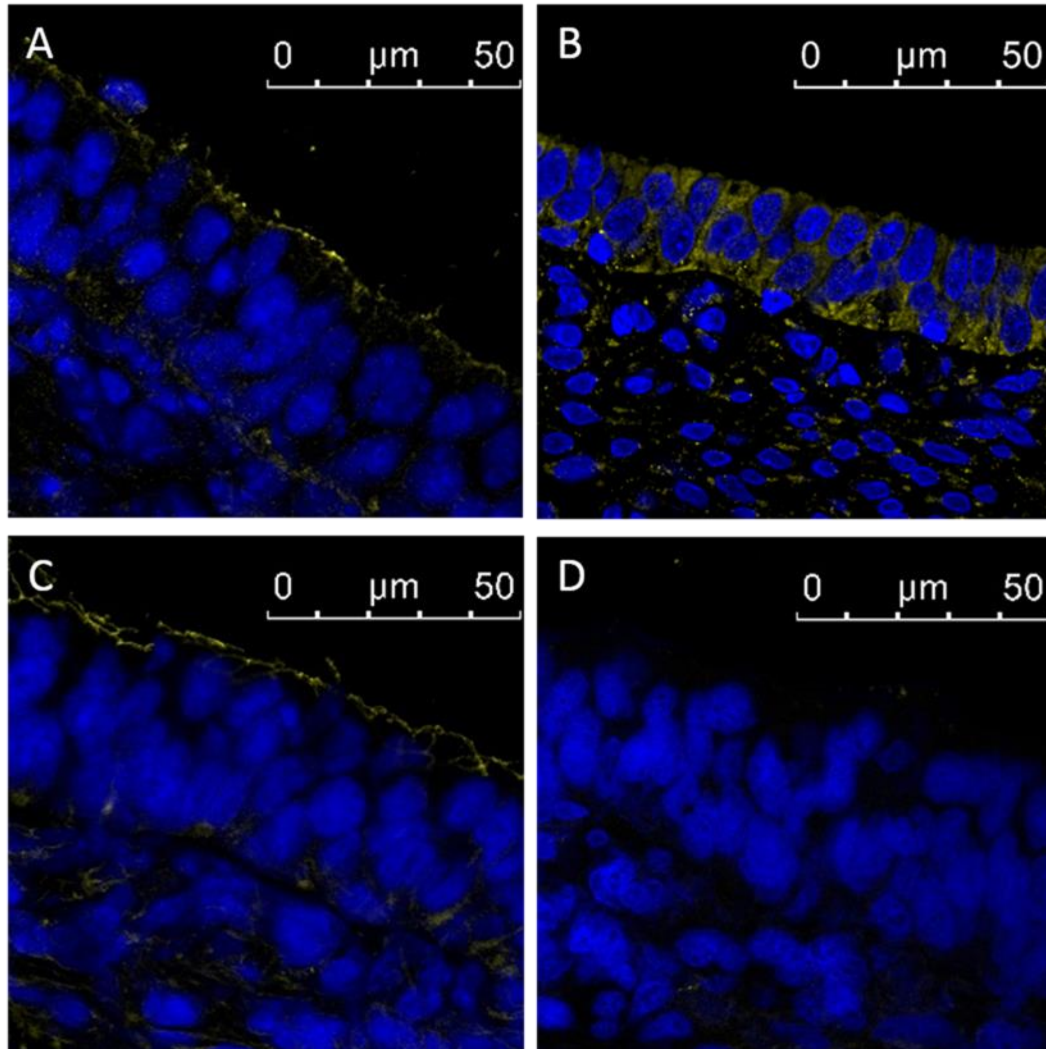


Figure 3-4 Representative IHF of UT for select TLRs. Staining for TLR3 (A), TLR4 (B) and TLR9(C) with TLR staining in yellow and nuclear staining in blue with DAPI. Isotype controls for all antibodies were also imaged (D) with isotype control staining in yellow and slides were imaged at 63x. Staining was performed on tissue collected from 4 separate animals.

To establish whether polarized primary UECs maintained the subcellular localization of TLR3, TLR4 and TLR9 pattern observed in the tissue, immunofluorescence was performed, and ZO-1 was included to act as a representative surface marker. TLR4 remained localized to the cytosol in polarized cultured primary cells (yellow in Figure 3-5Bi and merged in 3-5biii) and TLR3 (Figure 3-5Ai and merged in 3-5Aiii) and TLR9 (Figure 3-5Ci and merged in 3-5Ciii)

remained localized to the cell surface which is conserved with the IHF in Figure 3-4. Z-stacks of the polarized cells also clearly indicates that the TLR3 (yellow in Figure 3-5D) and TLR9 (yellow in Figure 3-5F) proteins are localized to the apical surface and TLR4 (yellow in Figure 3-5E) remained localized to the cytoplasm. Thus, TLR3, 4, and 9 were localized to non-canonical sites of the UT and localization was maintained in polarized primary UECs.

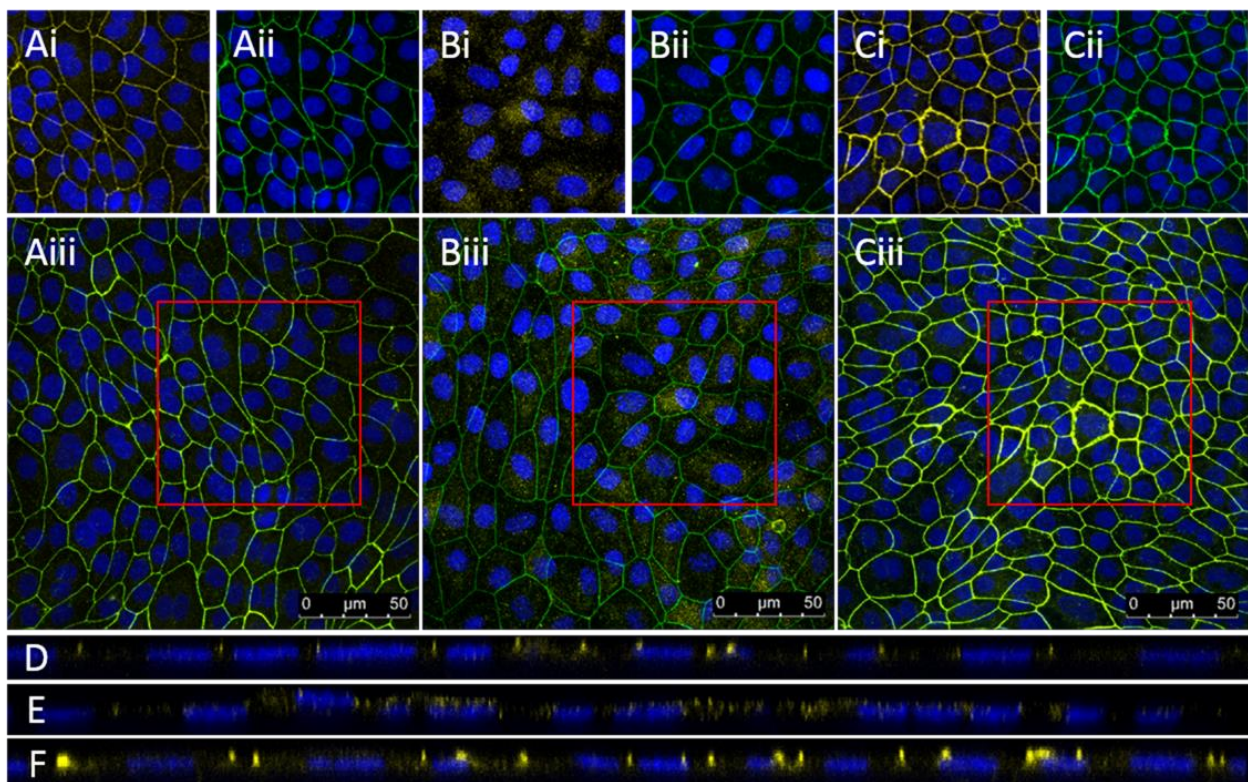


Figure 3-5 Representative IF of primary UECs on transwell membranes for select TLRs. Staining for ZO-1 (Aii, Aiii, Bii, Biii, Cii, and Ciii) in green, TLR3 (Ai and Aiii), TLR4 (Bi and Biii) and TLR9 (Ci and Ciii) staining in yellow and nuclear staining in blue with DAPI. For images A, B and C, I denotes TLR staining, ii denotes ZO-1 staining and iii denotes a merged image. Z-stacks of the cells were imaged and cross sections for TLR3 (D), TLR4 (E) and TLR9 (F) staining were produced with the apical surface of the cells up. Slides were imaged at 63x with an Isotype control which showed no nonspecific staining (not shown). Staining was performed on cells isolated from 4 different animals.

Cytokine secretion by primary uterine epithelial cells in response to PRR ligands

Polarized primary UECs were stimulated for 24 hours with the corresponding PRR ligands for TLR3, TLR4, TLR9, NOD2 or media alone (mock treated) and then a selection of classically defined pro-inflammatory (IL-1β and IL-6) and classically defined anti-inflammatory (IL-10 and IL-13) cytokine production was measured to confirm that the receptors were functional. PolyI:C (ligand for TLR3) induced significant secretion of IL-6 ($p=0.0238$) relative to the mock-treated cells (Figure 3-6B) but failed to induce secretion of the other examined cytokines (IL-1β, IL-10 or IL-13). LPS (ligand for TLR4) induced significant secretion of IL-13 ($p=0.0038$) relative to the mock-treated cells (Figure 3-6D), but it induced no significant secretion of IL-1β, IL-6, or IL-10. CpG (ligand for TLR9) significantly increased secretion of IL-10 ($p=0.0308$; Figure 3-6C) with no significant effect on the secretion of IL-1β, IL-6 or IL-13. NOD2 ligand MDP failed to significantly induce production of IL-1β, IL-6, IL-10, or IL-13 at 24hr compared to a mock treatment at 24 hr (Figure 3-6A-D). These results indicate that at least TLR3, TLR4, and TLR9 are functionally expressed in primary UECs polarized on transwells.

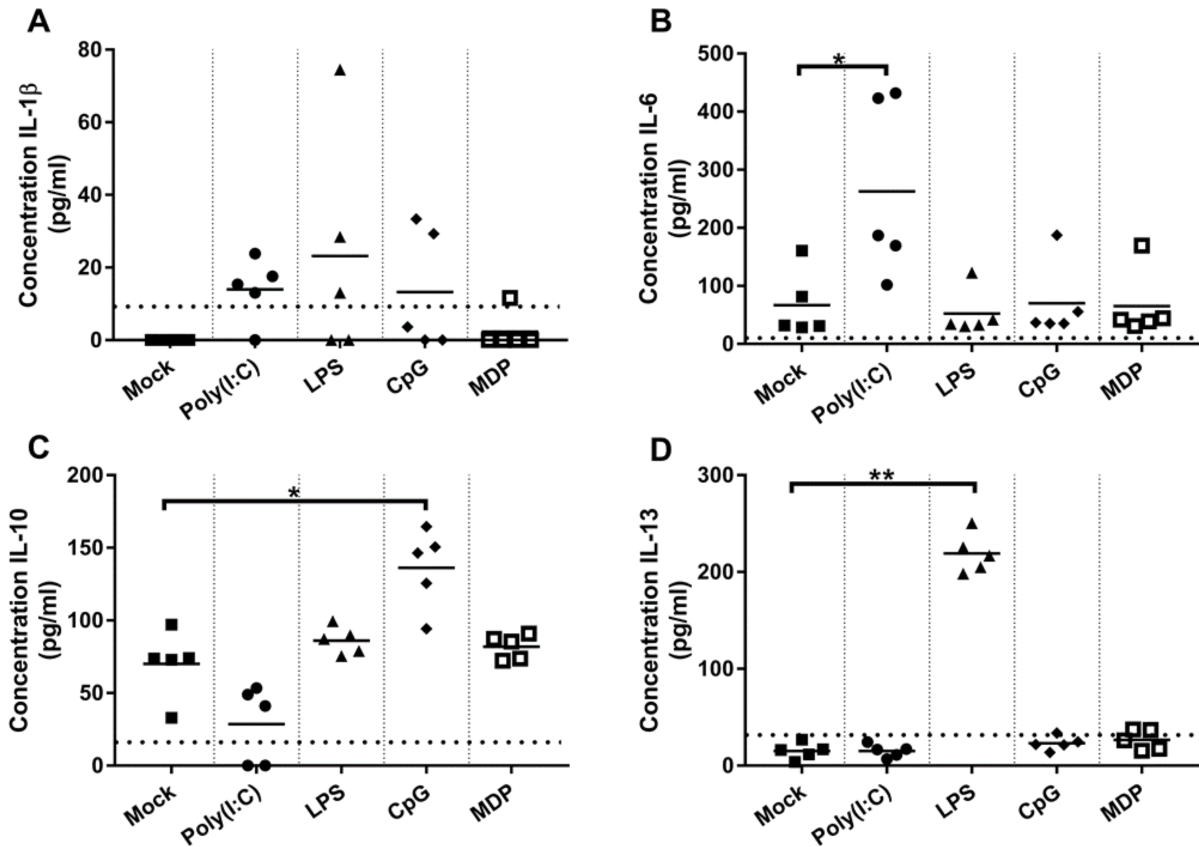


Figure 3-6 Cytokine secretion of primary UECs in response to stimulation with polyI:C, LPS, CpG, and MDP. Cells were cultured on transwell membranes until TEER readings increased 10X and stabilized and were then the apical and basolateral surfaces were exposed to the indicated stimuli for 24 hours (n=5 biological replicates). Cytokine quantification of IL-1β (A), IL-6 (B), IL-10(C) and IL-13 (D) was performed by BioPlex ELISA on apical supernatants, and experimental limit of detection for each cytokine is denoted by a dotted horizontal line. Significant differences were determined by Kruskal-Wallis tests and treatment differences compared to the mock stimulation were determined by Dunn's multiple comparisons ($p<0.05$ = *; $P<0.01$ = **).

3.4 Discussion

The porcine uterus is susceptible to several infections that affect reproductive performance such as porcine respiratory and reproductive virus and porcine parvovirus. The immune response in the uterus is not limited to pathogens that enter the uterus or those that are introduced during insemination, but also the uterus has an active immune response to semen and seminal plasma

during natural breeding or AI (Bischof et al., 1994b). This uterine immune response allows for clearance of bacteria or viruses that are sourced from the semen, but also clearance of excess sperm (Katila, 2012). Immune mechanisms such as large influxes of polymorphonuclear cells (Rozeboom et al., 1998) into the uterus combat both bacterial and viral pathogens, but the mechanisms by which these cells are recruited and/or innate immune cell activation have not been well characterized. The role of uterine epithelial cells as sentinels for the uterine immune response has been much more widely studied in other species including humans (Wira et al., 2005b) where UECs responded to immunostimulation with cytokine and chemokine secretion and subsequent immune cell recruitment (Schaefer et al., 2004; Soboll et al., 2006a).

Consistent with what has been observed in the other regions of the porcine upper reproductive tract in the oviduct and ovaries (Marantidis et al., 2015), we observed a full array of PRRs expressed on epithelial cells collected from the uterine horns. These results show good consistency with other species where mRNA expression of TLR1-6 is highly conserved in primary uterine epithelial cell cultures in mouse (Soboll et al., 2006a), human (Young et al., 2004a) and bovine species (Davies et al., 2008a). Interspecies variation in TLR expression is limited to TLR7-9 where mouse expression varied between samples (Soboll et al., 2006a), primary human UECs lacked TLR7, 8, and 10 (Young et al., 2004a) and bovine UECs matched expression of the porcine UECs lacking TLR8 and 10 (Davies et al., 2008a). Interestingly no UEC in any species currently characterized expresses TLR10.

Current research on non-TLR PRR expression by the uterine epithelia is limited, however, NOD1 and 2 are also expressed in both human and bovine UECs (Hart et al., 2009; King et al., 2009; Silva et al., 2012) and the trend of expression shows conservation with what was observed in the pig. Previous studies have focused on presence or absence of the PRR transcripts, however,

this data provides an insight into which receptors are more highly expressed in UECs and may correlate with families of pathogens the epithelia is primed to respond towards. The majority of PRRs are found at relatively low levels in the uterus, and when compared to the uterine epithelial layer, they are found at lower expression levels. In the case of PRRs which are expressed at low levels in the uterus and lower in the epithelial layer, the primary source of PRR expression in the tissue is likely associated with other cells within the endometrium. However, TLR3, NOD1, RIG1, MDA5, and LGP2 mRNA transcripts were all transcribed at a higher or equivalent level in LC-UE compared to UT, indicating the epithelial cells are the primary source for transcription of these receptors. Interestingly the majority of these enriched receptors recognize viral pathogens suggesting the epithelial layer may be predisposed to initiate the immune response to viral pathogens.

We opted to use primary UECs rather than cell lines as a model to characterize the uterine innate immune response to incoming pathogens because immortalized uterine epithelial cell lines have been shown to display conflicting expression of TLR transcripts compared to each other and when compared to the results from primary human UECs (Aboussahoud et al., 2010; Schaefer et al., 2004; Young et al., 2004a). Although more representative of *in vivo* cells then immortalized cell lines, caution must still be used in interpreting results from primary cells as they have been removed from neighbouring cells and from the hormonal environment which may impact their gene expression profiles. Additionally isolated epithelial cells often revert to a mesenchymal phenotype through an epithelial to mesenchymal transition (Gonzalez and Medici, 2014). Therefore verification of maintenance of an epithelial phenotype should be performed. In order to further validate that the primary UECs maintained an epithelial phenotype in culture, we characterized several tight junction proteins known to be expressed on epithelial cells. The

majority of these cells maintained expression and subcellular localization of these select tight junction markers, but the loss of E-cadherin expression may indicate the initiation of an epithelial to mesenchymal transition (van Roy and Berx, 2008). With this limitation in mind, we initially characterized mRNA expression of uterine epithelial cells collected by laser capture microdissection, thereby eliminating any effect on transcript expression that may have been induced by enzymatic digest, culturing and/or removal from their native environment. The transcriptomic data and subcellular localization of PRRs showed excellent conservation between the uterine epithelial layer *in vivo* and the results collected from cultured primary uterine epithelial cells. This conservation suggests that polarized primary porcine UECs may be an effective model to characterize the response of the uterine epithelia to both stimulants and pathogens using *in vitro* experiments.

Although we observed cytoplasmic expression of TLR4 and surface localization of both TLR3 and TLR9 which are considered non-canonical patterns of TLR localization, surface-localization of TLR3 in pig uterine epithelial cells shows agreement with uterine epithelial cells in humans (Schaefer et al., 2005) and rabbit (Pasternak et al., 2017). Culturing primary human polarized uterine epithelial cells with anti-TLR3 antibodies prior to stimulation with PolyI:C resulted in reduced secretion of cytokines and chemokines which suggests that TLR3 was indeed expressed on the cell surface (Schaefer et al., 2005). Continued research in this area shows that the 'canonical' localization patterns of TLRs may not be conserved across cell and/or tissue types, and may also vary in response to stimulation, age, disease or cellular environment (reviewed in (Hamonic et al., 2018a)). Hyaluronic acid has been shown to activate DCs through TLR4 signalling (Termeer et al., 2002) and because the uterine lumen and semen contain hyaluronic acid, cytoplasmic TLR4 localization on uterine epithelial cells could be necessary to avoid triggering a

non-productive inflammatory response. Alternatively, altered localization of TLRs may mediate activation by damage-associated molecular patterns, For instance, TLR9 surface localization may be necessary for detection of mitochondrial DNA sourced from damaged epithelial cells, luminal cell populations or from sperm cells during insemination (Zhang et al., 2010). Surface localization of TLR3 may allow uterine epithelial cells to be responsive to viral pathogens and/or dsRNA released from necrotic luminal cells or neighbouring epithelial cells.

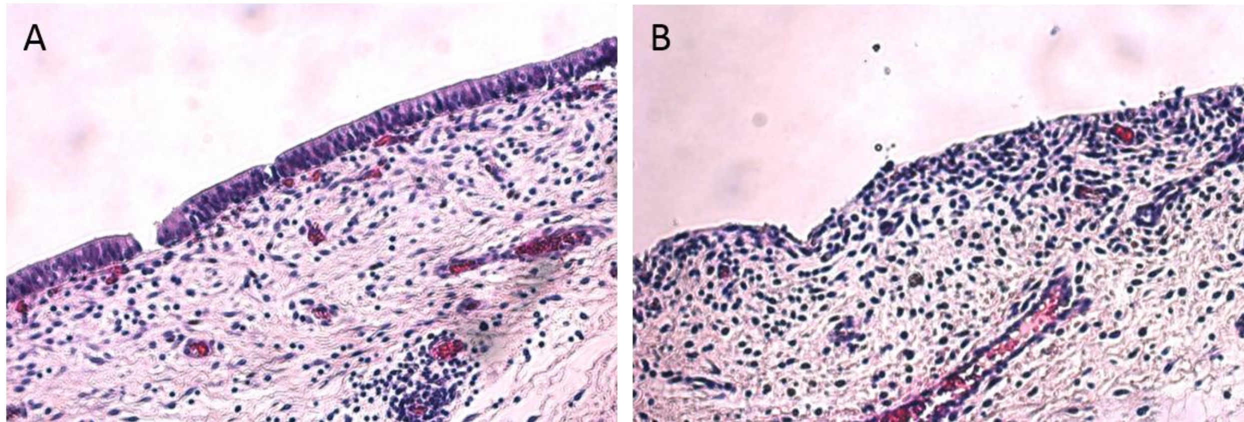
Porcine UECs stimulated with polyI:C and CpG showed induction of IL-6 and IL-10, respectively which shows conservation of cytokine expression profiles with studies in mice and humans uterine epithelial cells whereof the two only polyI:C induced pro-inflammatory cytokines (Fahey et al., 2008; Soboll et al., 2006a). In contrast, mouse and human uterine epithelial cells showed increased secretion of IL-6 in response to LPS whereas pig UECs stimulated with LPS triggered significant expression of IL-13 and poor expression of IL-6 (Schaefer et al., 2004; Soboll et al., 2006a). Because human corneal epithelial cells also failed to respond to LPS with IL-6 secretion and display intracellular localization of TLR4, these results may indicate a link between the activity of TLR4 and its subcellular localization (Ueta et al., 2004). As there is no data on the subcellular localization of TLR4 in both human and mouse UECs, it is difficult to draw conclusions regarding a link between intracellular TLR4 and its corresponding response upon stimulation. Finally, no response to NOD2 stimulation by MDP may indicate that NOD2 is not functional in pig UECs, or it may be that MDP stimulation triggers a non-canonical response to inflammasome activation.

The female reproductive tract in sexually mature animals is a dynamic system that undergoes several modifications in response to the hormonal status of the animals throughout the estrous cycle. Characterization of cells or responses in the reproductive tract should be carried out with a

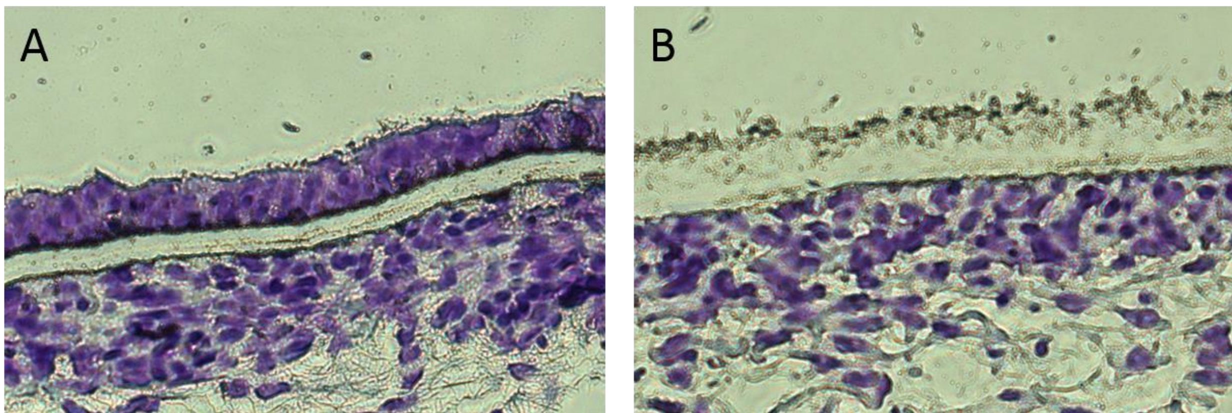
recognition of the possible effect of hormones. During estrous, the cervix is more permissive to allow for breeding to occur, which allows for pathogens to pass through to the uterus or are delivered to the uterus with semen. Because of the susceptibility of pathogen entry into the uterus during estrous, *in vivo* results of this study were carried out during estrus providing results on the expression and localization of PRRs in a high estrogen environment. Although the primary UECs were cultured in a neutral hormonal environment (no hormones added), the subcellular localization of PRRs was conserved, which suggests that estrogen will not alter the localization of TLR3, 4 and 9 in pig uterine epithelia. However, studies in cultured human uterine epithelial cells showed that estradiol impacted the amount of IL-6 secreted in response to polyI:C stimulation (Fahey et al., 2008). The effect of progesterone on uterine epithelial immune function requires further research.

This work supports the theory that porcine uterine epithelial cells maintain the capacity to act as sentinels for the detection of incoming pathogens. This capacity is manifest in the conservation of PRR expression and localization patterns with other species uterine epithelial cells which have been suggested to also act as sentinels for the uterine immune response. Additionally, the level of expression of PRRs that recognize viral pathogens and the pro-inflammatory response observed in response to dsRNA indicates that the uterine epithelia may be primed to detect incoming viral pathogens.

3.5 Supplemental Figures



Supplemental Figure 3-1 H&E staining of UT pre (A) and post (B) enzymatic digestion with pancreatin and dispase at 4°C overnight. In both images, the uterine lumen is on the top of the figure. Post enzymatic digestion, the epithelial cells are removed while the remaining tissue remains intact.



Supplemental Figure 3-2 Frozen UT slices were cut to a thickness of 14µm and stained with cresyl violet. Laser-capture microdissection was performed using a PALM-microbeam system (Zeiss). To avoid contamination of non-epithelial cells, the bottom third of uterine epithelial cells were initially trimmed off with the cutting laser (A) before the cells were launched from the slide and captured (B).

3.6 Transition statement

Identification of PRRs is essential for the identification of receptors that can be targeted by vaccine adjuvants. In this chapter, we established that primary UECs maintain their phenotype and polarize to represent the uterine epithelia *in vivo*. In the next chapter, we characterize the innate immune response triggered in polarized pig primary uterine epithelial cells in response to adjuvants. The establishment of a primary uterine epithelial cell culture system will enable a cost-effective method of determining which adjuvant or combinations of adjuvants are most likely to induce the desired response, *in vivo*.

4 THE INNATE IMMUNE RESPONSE IN POLARIZED UTERINE EPITHELIAL CELLS TO VACCINE ADJUVANTS ALONE AND IN COMBINATION.

(In preparation)

Authors

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Author contributions

GH, JAP, and HLW conceived of and designed the experiments. GH carried out all *in vitro* assays, laser-capture microdissection, and qPCR analysis. GH, JAP, and NF carried out all *in vivo* sampling and flow cytometry staining. GH analyzed all data and drafted the manuscript. All authors read and approved the manuscript

Abstract

Significant investments are directed towards the development of vaccines to protect the reproductive health of sows and gilts in farrowing barns. Intrauterine vaccination, with an appropriate adjuvant combination, delivered during artificial insemination, may induce strong mucosal immunity in the reproductive tract. An *in vitro* culture of uterine epithelial cells was used to select an adjuvant combination capable of recruiting antigen-presenting cells into the uterus. The selected triple adjuvant combination of polyI:C, a host defence peptide and a polyphosphazene was shown to induce CCL2 gene expression significantly and was subsequently used during breeding of sows to evaluate localized gene expression and cellular recruitment, *in vivo*. Sows bred with semen plus adjuvant had decreased γδ T cells and monocytes in the blood, however, no corresponding increase in the number of monocytes and macrophages was detected in the endometrium. Compared to sows bred with semen alone, sows bred with semen plus adjuvants showed increased CCL2 gene expression in the epithelial layer. The inclusion of a triple adjuvant combination with an extended semen dose initiated an immune response distinct from extended semen alone suggesting that the adjuvants may further augment a local immune response and, therefore, may be suitable for use in an intrauterine vaccine.

4.1 Introduction

Mucosal vaccination has the potential for several benefits over classical parenteral vaccinations, including the initiation of a strong mucosal and systemic immune response (Gerdts et al., 2006; Srivastava et al., 2015) while potentially reducing the incidence of common needlestick injuries by swine veterinarians (Hafer et al., 1996). However, several challenges need to be overcome to generate a successful mucosal immune response including vaccine elimination by the flow of mucosal fluids across mucosal surfaces and establishing a suitable dose and volume for the vaccine to traverse the epithelial barrier (Woodrow et al., 2012). Under normal circumstances, mucosal surfaces are primed to induce a tolerogenic response towards antigens, thereby limiting the reaction to microflora, food, and environmental particles (Czerkinsky et al., 1999). Currently, no studies have identified a commensal flora in the upper reproductive tract of pigs, which may mean that the porcine uterus may be less predisposed to a tolerogenic bias to antigens encountered at its surface. In fact, studies in rats and rabbits have shown that the uterus may be a suitable immunization site as vaccines delivered to the uterus triggered a measurable antigen-specific systemic and local humoral immunity (Pasternak et al., 2017; Pasternak et al., 2018; Wira and Sandoe, 1989). Because many economically important diseases in pigs impact reproduction such as porcine parvovirus, porcine reproductive and respiratory syndrome virus, and porcine epidemic diarrhea virus, it may be beneficial to have a mode of vaccine delivery that triggers a strong mucosal immune response in the uterus to protect growing fetuses against the numerous pathogens that impact reproductive success (Pozzi and Alborali, 2012). For livestock systems that use natural breeding, the uterus is not readily accessible for immunization. However, the majority of commercial pigs are bred by AI which means that commercial husbandry practices

(Knox, 2016) allowing routine access to the uterus during each reproductive cycle in which vaccines could be potentially delivered.

Adjuvants used in mucosal vaccine formulations facilitate uptake of the antigen across the epithelial barrier, recruitment of APCs, activation of APCs, and protecting the antigen from degradation (Srivastava et al., 2015). One or several of these mechanisms of action may be required to generate a successful mucosal vaccine response, and therefore, the inclusion of multiple adjuvants may be necessary for the final vaccine formulation (Garg et al., 2017a). The mechanism of action of adjuvants can be as both delivery systems protect the antigen from degradation, promote uptake or transport across the epithelial barrier, and stimulation of cytokine and chemokine secretion to recruit and activate the necessary immune cells (Srivastava et al., 2015). Certain mucosal surfaces have specialized epithelial cells such as M cells which are efficient at sampling antigens in the lumen and delivering them to the underlying immune cells (Woodrow et al., 2012). These specialized epithelial cells can be targeted with the vaccine adjuvants, such as the targeting of M cells with FimH or several other ligands being currently evaluated (Kim and Jang, 2014). However, the uterine epithelial has no known specialized epithelial cells that can be targeted for vaccine delivery, and therefore the vaccine may need to be directed towards normal epithelial cells or at immune cells recruited to the uterine lumen. Despite the lack of M cells in the uterus, an intrauterine vaccine administered to rabbits which contained OVA and recombinant protein TgD formulated with polyI:C, a HDP, and a polyphosphazene triggered a strong humoral mucosal response in the uterus and distal mucosal sites such as the vagina and nasal tissues (Pasternak et al., 2017).

The following study aims to determine which adjuvant components and combinations can generate an immune response in porcine uterine epithelial cells (UECs). Additionally, we seek to

determine if the inclusion of adjuvants in a semen dose modulates the uterine immune response to breeding and what role, if any, the uterine epithelial cells play in this response.

4.2 Materials and Methods

Isolation, culture, and stimulation of primary uterine epithelial cells

Primary UECs were isolated from uterine tissue of gilts/sows collected from a local abattoir (n=4) as described in detail in chapter 3 (Hamonic et al., 2018a). In brief, uterine tissue was inverted and digested in a buffer consisting of PBS containing 250 units/L of dispase (BD Bioscience), 12 g/L of pancreatin (laboratory grade, Fisher Scientific) and 1x Anti-Anti (Gibco) at 4°C overnight with shaking. Sloughed cells were washed in PBS, filtered through a 40 µm cell strainer to remove cell debris and plated on transwell PET membranes with a 0.4 µm pore size (Greiner Bio-One) at a concentration $2.5x10^5$ cells per well in DMEM:F12 (1:1; Gibco) containing 2.5 mM glutamine, 15 mM HEPES, 10% FBS (Sigma-Aldrich) and 1x Anti-Anti (Gibco). Cells were polarized for 7-10 days as determined by stable 10x increase in TEER with media changes taking place every second day. After cells achieved stable TEER, they were stimulated with 50 µg/ml polyI:C (Invivogen), 50 µg/ml lipopolysaccharide (LPS; *Salmonella enterica* serovar Minnesota from Sigma-Aldrich), 50 µg/ml muramyl dipeptide (MDP; Sigma-Aldrich), 100 µg/ml host defence peptide 1002 (HDP; Genscript), 50 µg/ml polyphosphazene (PCEP; Idaho National Laboratory) or combined together in various combinations at the stated concentrations including as the triple combination adjuvant (TriAdj, polyI:C, HDP, PCEP). Six hours post-stimulation, cells were collected in Trizol (Invitrogen) and frozen at -80°C for later RNA extraction (described below).

Animal ethics, breeding and sample collection

All experimental procedures were conducted in accordance with the guidelines of the CCAC under approval from the Animal Research Ethics Board at the University of Saskatchewan.

Seven first parity Landrace-cross sows were synchronized following an established fixed time AI schedule(Degenstein et al., 2008) summarized in Figure 4-1. Sows were synchronized by oral progestin (Regu-mate; Merck) for 14 days with blood collected using EDTA Vacutainers (BD Biosciences) 12 days into Regu-Mate (d-2 on Figure 4-1) as a pre-bleed to obtain peripheral blood mononuclear cells (PBMC) for isolation and immunotyping. Twenty-four hours after the final dose of oral-progestin, sows received 800 I.U. of pregnant mare serum gonadotrophin (Folligon; Merck) by intramuscular injection to induce follicular maturation. Eighty hours later, sows were given 5 mg porcine pituitary luteinizing hormone (Lutropin-V; Bioniche Animal Health) by intramuscular injection to induce ovulation. Sows were bred by post-cervical AI 32 hours post-Lutropin-V injection by either a standard semen dose containing 3.2 ml of PBS (mock control sows, n=3) or a standard semen dose containing 4 mg polyI:C, 8 mg HDP and 4 mg PCEP in 3.2 ml of PBS (TriAdj sows, n=4). Twenty four hours after breeding, blood was collected, and sows were then humanely euthanized. PBMCs were isolated from blood collected, by initial centrifugation at 1100 x *g* for 30 min, buffy coats were collected and layered onto Ficol-Paque plus (GE life sciences) and centrifuged at 400 x *g* for 40 min. The PBMC layer was collected, washed in PBS 3 times with centrifugation at 250 x *g* for 10 minutes and stained for immunotyping by flow cytometry. The uterine horns were removed from the sows and flushed with 25 ml PBS + 1% BSA per horn to collect luminal cell populations, which were counted and stained for immunotyping by flow cytometry analysis and to quantify CCL2. Small sections of tissue were collected from the cervix, lower uterine horn, mid uterine horn, upper uterine horn, ampulla, isthmus and ovaries and fixed

in formalin for 36 hours. Duplicate sections were also collected from the lower, middle and upper uterine horn such that one duplicate was flash frozen in liquid N for RNA isolation and the other duplicate was frozen in Shandon cryomatrix (Thermofisher) for laser-capture microdissection collection.

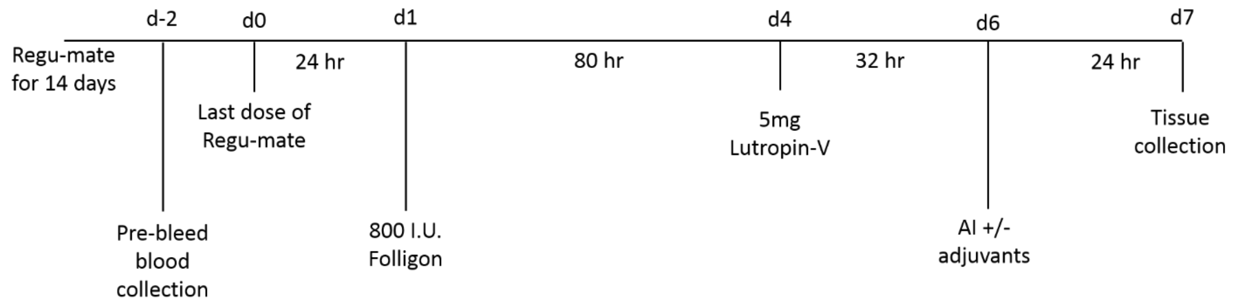


Figure 4-1. Schematic timeline of hormonal synchronization method for fixed time artificial insemination of sows.

RNA isolation and gene expression analysis

RNA analysis was carried on both *in vitro* UECs and *in vivo* uterine tissue as previously described in chapter 3 (Hamonic et al., 2018a). Uterine tissue collected from the animal trial were ground at -80 °C by mortar and pestle until the entire tissue section was reduced to a fine powder. Up to 100 mg of tissue was dissolved in 1 ml of Trizol (Invitrogen) for RNA extraction by vigorous shaking for 5 minutes. Both *in vitro* and *in vivo* samples were treated in an identical fashion from this point onwards. A 200 µl volume of chloroform (Sigma-Aldrich) was added to the samples in Trizol, they were mixed by shaking for 2 min and then centrifuged at 10,000 x *g* for 10 min at 4°C. The top aqueous layer was collected, and RNA was precipitated at 0 °C by isopropanol, following by washing of the pellet with 100% ethanol. RNA was resuspended in water and again precipitated in isopropanol at -20 °C overnight pelleted at 14,000 x *g* and pellets were washed with 100%

ethanol. DNAse treatment was carried using the Turbo DNAse kit (Thermofisher) following the manufacturer's specifications and the inclusion of 10 units RNase inhibitor (Thermofisher). RNA quantity was determined by Nanodrop (Thermofisher), and RNA quality was validated by denaturing agarose gel. cDNA was generated from 2 μg of RNA using the high capacity cDNA kit (Thermofisher) following the manufacturer's specifications. Gene expression analysis was carried out on a StepOne Plus (Thermofisher) using KAPA SYBR mix (Sigma-Aldrich), containing 0.2 mM primer concentrations (primer sequences and annealing temperature used in Table 4-1) and 10 ng/sample cDNA in 15 μl reactions run in duplicate. Primer amplification efficiency was measured at the optimal annealing temperature and in all instances, was found to be greater than 90%. Gene expression was normalized to the geometric mean of multiple stable reference genes, RPL19, YWHAZ, and GAPDH for the *in vitro* analysis, and GAPDH and ActB for the *in vivo* analysis (Table 4-1).

Table 4-1. Primer names, sequences, annealing temperature, and target sequence used in all qPCR experiments.

Gene Name	Forward primer (5'-3')	Reverse Primer (5'-3')	Tm (°C)	Target sequence or reference
RPL19	AACTCCCGTCAGCAGATCC	AGTACCCTTCCGCTTACCG	60	(Meurens et al., 2009a)
YWHAZ	TGATGATAAGAAAGGGATTGTGG	GTTCAGCAATGGCTTCATCA	60	(Nygard et al., 2007)
GAPDH	CTTCACGACCATGGAGAAGG	CCAAGCAGTTGGTGGTACAG	63	(Bruel et al., 2010)
ActB	CACGCCATCCTGCGTCTGGA	AGCACCGTGTTGGCGTAGAG	63	(Nygard et al., 2007)
IFNβ	AGTTGCCTGGGACTCCTCAA	CCTCAGGGACCTCAAAGTTCAT	60	(Razzuoli et al., 2011)
TNFα	CCAATGGCAGAGTGGGTATG	TGAAGAGGACCTGGGAGTAG	60	(Meurens et al., 2009b)
GMCSF	GAAACCGTAGACGTCGTCTG	GTGCTGCTCATAGTGCTTGG	62	(Meurens et al., 2009b)
IL6	ATCAGGAGACCTGCTTGATG	TGGTGGCTTTGTCTGGATTC	60	(Meurens et al., 2009b)
IL8	TCCTGCTTTCTGCAGCTCTC	GGGTGGAAAGGTGTGGAATG	62	(Meurens et al., 2009b)
CCL2	AGTCACCTGCTGCTATACAC	GCGATGGTCTTGAAGATCAC	60	NM_214214
CCL3	GCCTGCTGCTTCTCCTATAC	TCAGCTCCAGGTCAGAGATG	60	AY643423
CCL4	AACCTCTCCTCCAGCAAGAC	GTCACGAAGTTGCGAGGAAG	60	NM_213779
CCL20	GCTCCTGGCTGCTTTGATGTC	CATTGGCGAGCTGCTGTGTG	66	(Meurens et al., 2009b)
CCL28	GCTGCTGCACTGAGGTTTC	TGAGGGCTGACACAGATTC	62	(Meurens et al., 2009b)
SLA-DRA	ATCTCCCCTTCATGCCCTCA	AGCTTCAAACTCCCAGTGCT	60	NM_001113706

Laser-capture microdissection sample collection

Laser-capture microdissection was carried out on cryoblocks from only the middle of the uterine horn as we observed no significant differences in gene expression between lower, middle, and upper uterine horn UTE. Cryoblocks were sectioned at 14 µm thickness onto polyethylene naphthalate membrane slides and immediately fixed in 70% ethanol. Residual cryomatrix was removed by submersion in DEPC treated water (Invitrogen), and slides were stained in cresyl violet (Sigma-Aldrich) for 30 s. Excess stain was removed by submersion in 70% and then 100% ethanol. Epithelial cells were captured within 45 minutes of staining using a PALM-Microbeam System (Zeiss), removing the basolateral third of the epithelial cell prior to capture to eliminate contamination of samples from sup-epithelial lymphocytes. Epithelial cell material was collected into ultrapure water (Invitrogen) and RNA was isolated using the Picopure RNA isolation kit (Thermofisher) following the manufacturer's specifications, including an on-column DNase treatment (Qiagen). RNA quantity and integrity was confirmed using the Bioanalyzer (Agilent), and 200 ng RNA per sample was converted to cDNA using the High-Capacity cDNA Reverse transcription kit as described above. Gene expression analysis was carried out as described above using 4 ng/sample in each reaction.

Immunotyping of PBMCs and cells obtained by uterine flush

Cells collected from uterine flush were washed 2x in PBS + 0.1% EDTA at 400 x *g* for 15 min and counted by a coulter counter (Beckman Coulter). Both PBMCs and cells flushed from the uterine tissues were stained for flow cytometry (FCM) analysis in 96 well plates with 1 x 10^6cells/wells. All FCM stains were incubated in stains diluted in PBS + 2% FBS for 10 min at

RT followed by 3x washes in PBS + 2% FBS centrifuging at 500 x *g* for 3 min. All antibody concentrations and details are available in Table 4-2. PBMC and flushed T cells were stained in a four-step stain procedure beginning with anti-CD4, anti-CD8α and anti-TCRγδ, followed by the secondary antibodies anti-IgG2b-FITC, anti-IgG2a-Alexa 647, and anti-IgG1-biotin. Next, IgG and Streptavidin (SA)-PerCP-Cy5.5 was added, followed by the directly labelled anti-CD3-PE antibody. PBMCs and flushed B cells were stained with anti-CD21, followed by anti-IgG1-APC. PBMC monocytes were stained with anti-CD172 and anti-CD14, followed by anti-IgG1-PE and anti-IgG2b-APC. Flushed myeloid cells were stained with anti-CD172, anti-MHCII, anti-SWC9, and anti-CD16, followed by anti-IgG2b-FITC, anti-IgG2a-PE, and anti-SA-PerCP-Cy5.5. FCM samples had 60,000 events for PBMCs and 250,000 events for flushed cells, all of which were immediately collected on a FacsCalibur (BD) with appropriate fluorescence minus one (FMO), single stains and isotype stains. FCM analysis was carried out using FlowJo (FlowJo LLC). A representative flow cytometry gating scheme for blood analysis is shown in supplemental Figure 4-1, such that $CD3^{-}CD8\alpha^{+}$ represent natural killer (NK) cells, $CD3^{+}TCR\gamma\delta^{-}CD4^{+}CD8\alpha^{-}$ represent $CD4^{+}$ T cells, $CD3^{+}TCR\gamma\delta^{-}CD4^{-}CD8\alpha^{+}$ represent $CD8^{+}$ T cells, $CD3^{+}TCR\gamma\delta^{-}CD4^{+}CD8\alpha^{+}$ represent $CD4^{+}CD8^{+}$ T cells, and $CD21^{+}$ represent B cells. A representative gating scheme for the flushed myeloid cells is shown in supplemental Figure 4-2, such that $CD172^{+}MHCII^{-}CD16^{+}$ cells represent neutrophils, and $CD172^{+}MHCII^{+}SWC9^{-}$ cells represent APCs. Markers and their associated cellular identification have previously been validated for immunotyping by FCM in swine (Piriou-Guzylack and Salmon, 2008).

Table 4-2. Antibodies used in FCM analysis, final concentrations, and suppliers.

Target	Clone (Isotype)	Fluorochrome	Final concentration	Supplier
Primary Antibody targets				
Anti-CD4	74-12-4 (mIgG2b)	N/A	10 µg/ml	Monoclonal Antibody Center
Anti-CD8α	76-2-11 (mIgG2a)	N/A	10 µg/ml	Monoclonal Antibody Center
Anti-TCRγδ	PGBL22A (mIgG1)	N/A	5 µg/ml	Kingfisher Biotech
Anti-CD3	PPT3 (mIgG1)	PE	10 µg/ml	Southern Biotech
Anti-CD21	BB6-11C9.6 (mIgG1)	N/A	5 µg/ml	Southern Biotech
Anti-CD14	Cam36A (mIgG1)	N/A	5 µg/ml	Monoclonal Antibody Center
Anti-CD172	74-22-15A (mIgG2b)	N/A	2 µg/ml	Monoclonal Antibody Center
Anti-MHCII	MSA3 (mIgG2a)	N/A	2 µg/ml	Kingfisher Biotech
Anti-SWC9	PM18-7(mIgG1)	Biotin	2 µg/ml	Bio-Rad Antibodies
Anti-CD16	G7 (mIgG1)	Cye5	5 µg/ml	Bio-Rad Antibodies
Secondary Antibody targets				
N/A	IgG	N/A	10 µg/ml	Chrompure
Anti-mIgG1	N/A	Biotin	1.25 µg/ml	Southern Biotech
Anti-mIgG1	N/A	APC	1.25 µg/ml	Southern Biotech
Anti-mIgG1	N/A	PE	1.25 µg/ml	Southern Biotech
Anti-mIgG2a	N/A	Alexa647	2.5 µg/ml	Southern Biotech
Anti-mIgG2a	N/A	PE	1.25 µg/ml	Southern Biotech
Anti-mIgG2b	N/A	FITC	1.25 µg/ml	Southern Biotech
Anti-mIgG2b	N/A	APC	1.25 µg/ml	Southern Biotech
Streptavidin	N/A	PerCP-Cy5.5	0.5 µg/ml	eBioscience

Histology and Immunohistofluoresence

Formalin-fixed tissue was processed and embedded into paraffin blocks. Blocks were sectioned at 4 µm and floated onto superfrost plus slide (Thermofisher). Cervix, lower, middle and upper uterine tissue, isthmus, ampulla, and ovaries were deparaffinized by xylene and rehydrated by decreasing concentrations of ethanol prior to Haemotoxylin and Eosin (H&E) staining. Duplicate slides were deparaffinized and rehydrated from the middle uterine tissue for anti-CD163 immunohistofluoresence (IHF). IHF slides underwent heat-mediated antigen retrieval in 10 mM Na-Citrate, pH 6 for 30 min at 90°C before being blocked in 5% skim milk in TBS for 1 hr at RT. Primary antibody staining with mouse anti-human CD163 (EdHu-1; Biorad antibodies) at 10 µg/ml in dilution buffer (PBS with 1% BSA, 1% horse serum, 0.3% triton-X and 0.01% sodium azide) overnight at 4°C. Slides were washed 3x in TBS + 0.05% Tween 20 and incubated in 5 µg/ml donkey anti-mouse IgG Al555 (Invitrogen) for 90 min at RT. Slides were again washed 3x in TBS + 0.05% Tween 20 and then stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) in methanol for 10 min before being coverslipped and imaged on Axiovert 200M (Zeiss) at 20x magnification with appropriate isotype controls. CD163 positive cells were counted in ImageJ by analyze particles, selecting particles between 100-1000 pixels and circularity of 0.2-1.0. Identified cells were confirmed manually.

CCL2 ELISA

Uterine horn luminal CCL2 was quantified by sandwich ELISA against porcine CCL2 (Kingfisher Biotech) following the manufacturer's instructions. Briefly, 96 well high binding plates (Immulon 2, VWR) were coated with a polyclonal anti-swine CCL2 (Kingfisher Biotech) at 1 µg/ml in PBS overnight at RT. Plates were then blocked by 4% BSA in PBS for 2 hours at RT

prior to a 1 hr RT incubation with CCL2 standard (1in 2 dilutions from 10 ng/ml to 10 pg/ml) and undiluted flush samples. Plates were washed with TBST, and biotinylated anti-swine CCL2 antibody was incubated at 0.5 µg/ml in PBS + 4% BSA for 1 hour at RT followed by washing and a 30 min RT incubation with streptavidin-HRP. Plates were developed with TBS for approximately 30 minutes in the dark before stopping with a 2 N sulfuric acid and absorbance was read at 450 nm.

Statistical analysis

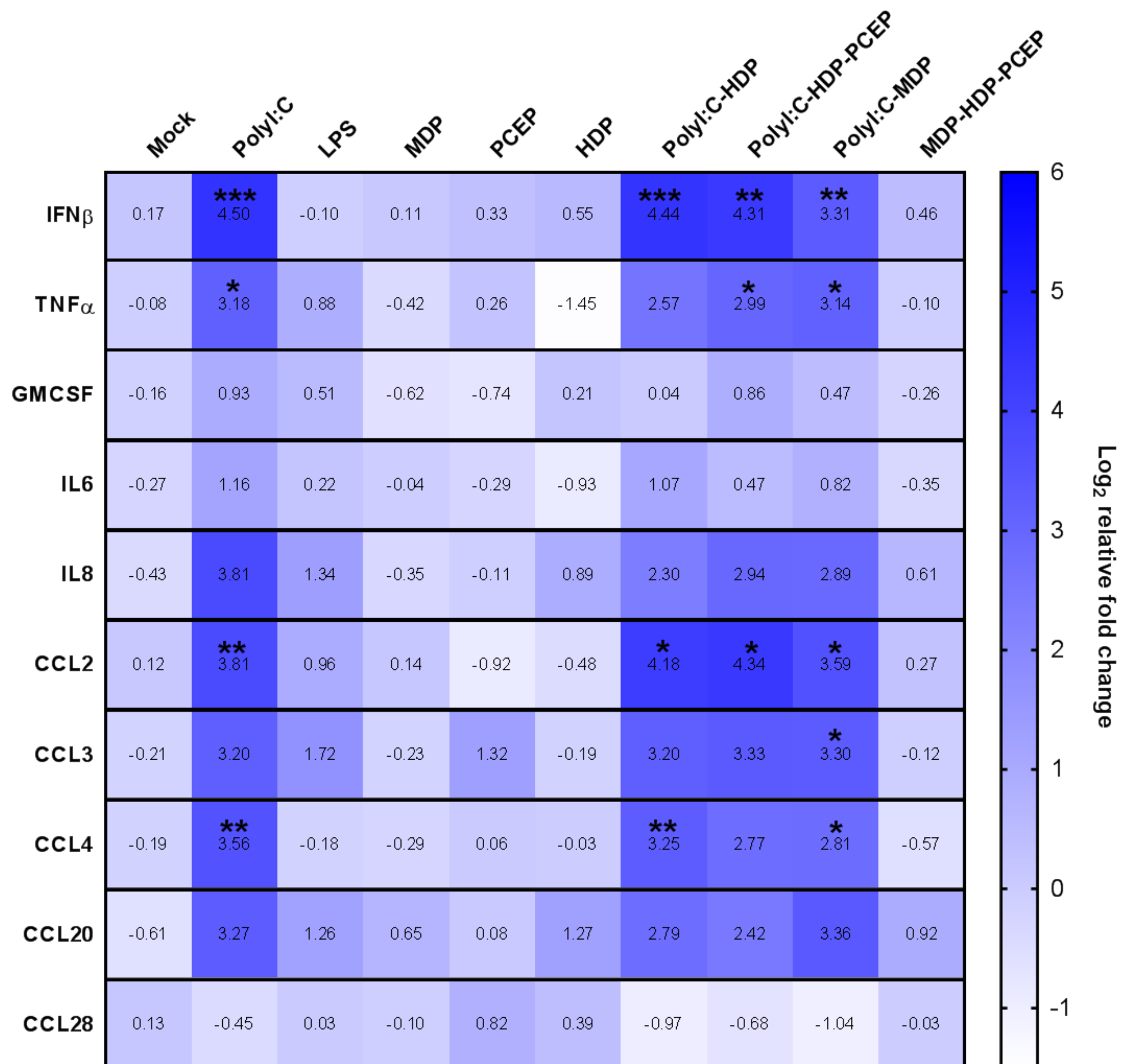
All statistical analysis was carried out using GraphPad Prism 7 (GraphPad Software). Gene expression analysis of *in vitro* UEC stimulation was evaluated by one-way ANOVA, and significant differences between mock-treated cells and individual treatments were determined by Hol-Sidak's multiple comparisons test. Gene expression and blood immunotyping from *in vivo* experiments were evaluated by unpaired t-test with Welch's correction. Uterine flush immunotyping was evaluated by Mann Whitney test. CD163 recruitment analysis was evaluated by unpaired t-test with Welch's correction. In all cases, significant differences were reported by $p<0.05$=*, $p<0.01$=**, $p<0.001$=***.

4.3 Results

Cytokine and chemokine gene expression changes in response to stimulation with adjuvant components alone and combination.

Cytokines and chemokine gene expression changes in UECs were measured after a 6 hr incubation with single adjuvants or multiple combinations of adjuvants (Figure 4-2). Compared to mock-stimulated UECs, polyI:C stimulation significantly increased UEC expression of IFNβ (4.5

fold increase), TNFα (3.18 fold increase), CCL2 (3.81 fold increase) and CCL4 (3.56 fold increase). LPS, MDP, PCEP, HDP alone, and MDP-HDP-PCEP combination did not impact the expression of the evaluated genes. When polyI:C was co-incubated with other adjuvants, there was a significant change in gene expression relative to the mock-stimulated cells of equivalent scale with the response that was observed when polyI:C stimulated the cells alone. No adjuvants or combinations were able to induce the expression of GM-CSF, IL6, and CCL28 when compared to the mock stimulation. SLA-DRA gene expression was not detected in any UEC stimulation sample (data not shown) indicating porcine UECs do not express MHC class II.



	Mock	Poly I:C	LPS	MDP	PCEP	HDP	Poly I:C-HDP	Poly I:C-HDP-PCEP	Poly I:C-MDP	MDP-HDP-PCEP
IFNβ	0.17	*** 4.50	-0.10	0.11	0.33	0.55	*** 4.44	** 4.31	** 3.31	0.46
TNFα	-0.08	* 3.18	0.88	-0.42	0.26	-1.45	2.57	* 2.99	* 3.14	-0.10
GMCSF	-0.16	0.93	0.51	-0.62	-0.74	0.21	0.04	0.86	0.47	-0.26
IL6	-0.27	1.16	0.22	-0.04	-0.29	-0.93	1.07	0.47	0.82	-0.35
IL8	-0.43	3.81	1.34	-0.35	-0.11	0.89	2.30	2.94	2.89	0.61
CCL2	0.12	** 3.81	0.96	0.14	-0.92	-0.48	* 4.18	* 4.34	* 3.59	0.27
CCL3	-0.21	3.20	1.72	-0.23	1.32	-0.19	3.20	3.33	* 3.30	-0.12
CCL4	-0.19	** 3.56	-0.18	-0.29	0.06	-0.03	** 3.25	2.77	* 2.81	-0.57
CCL20	-0.61	3.27	1.26	0.65	0.08	1.27	2.79	2.42	3.36	0.92
CCL28	0.13	-0.45	0.03	-0.10	0.82	0.39	-0.97	-0.68	-1.04	-0.03

Figure 4-2. Gene expression heat map of polarized uterine epithelial cells (UECs) stimulated with multiple adjuvant components alone and in combination. UECs were cultured until polarized and stimulated by adjuvant components (horizontal axis) for 6 hours before cells were collected, RNA isolated, and gene expression analyzed by qPCR. Median $\log_2$ increases are presented in the heat map with significant differences were evaluated by one-way ANOVA and significant differences between mock-treated cells and individual treatments were determined by Holm-Sidak's multiple comparisons tests ($p<0.05$=*, $p<0.01$=**, $p<0.001$=***).

Following stimulation of UECs for 6 hours with polyI:C-HDP and polyI:C-HDP-PCEP, TEER values dropped significantly (Figure 4-3A). However, values returned to initial levels by 24 hours post stimulation (Figure 4-3B) which suggest that these combinations of adjuvants may transiently impact tight-junction integrity and/or formation.

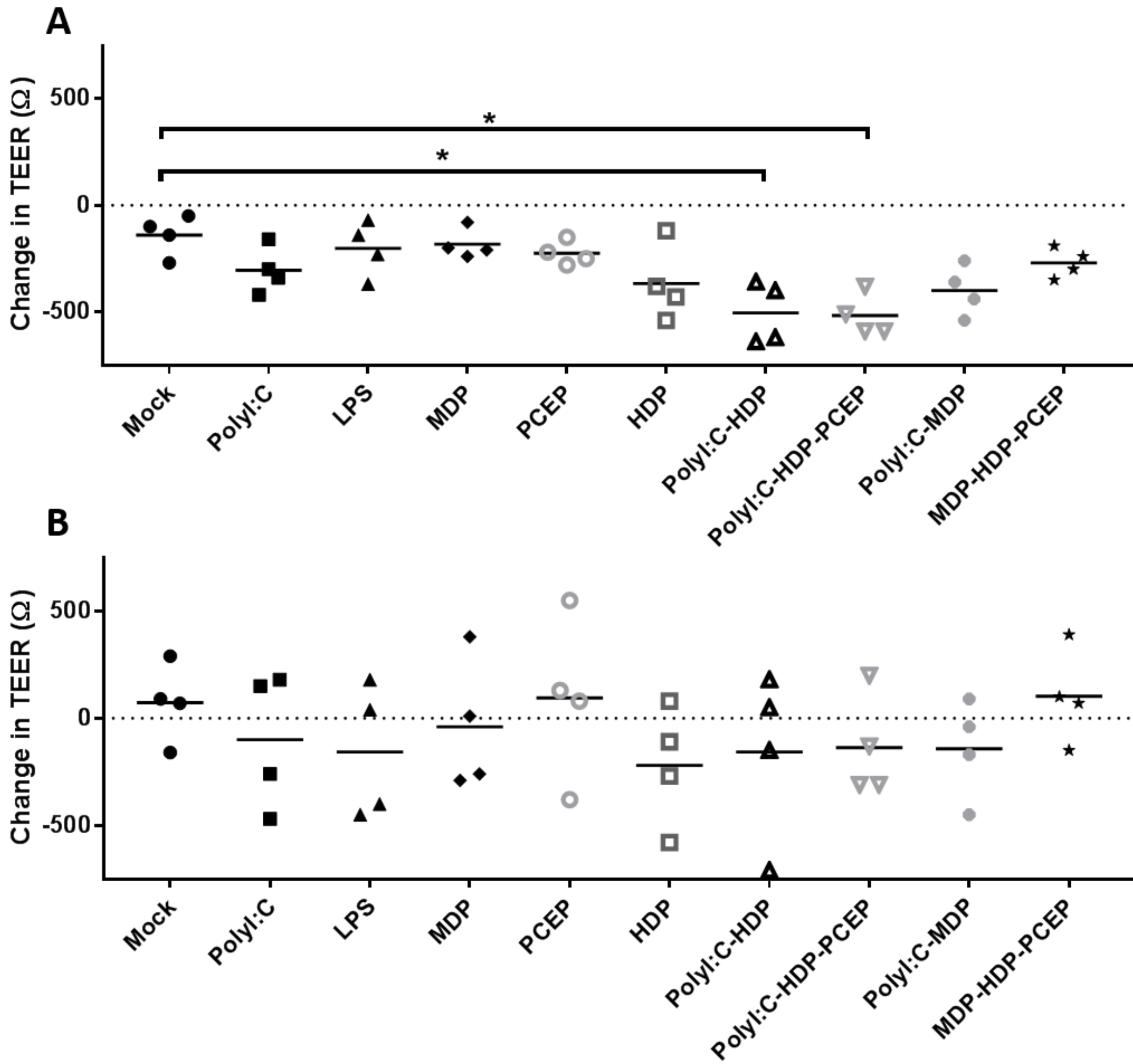


Figure 4-3. Changes in the primary uterine epithelial cell (UEC) transepithelial electrical resistance TEER stimulated with multiple adjuvant components alone and in combination. UECs were cultured until polarized and stimulated by adjuvant components (horizontal axis) and had the TEER measured prior to the addition of stimulants at 6 hours (A) and again at 24 hours (B). Statistical analysis was done by Kruskal-Wallis test, and significant differences between mock and individual stimulations were determined by Dunn's multiple comparison tests ($p<0.05=*$). Each circle, square, etc. represents a unique biological replicate.

To better understand how adjuvants impact the immune response of the pig uterus during AI, our next steps were to measure changes in gene expression of uterine tissue (UTE) 24 hours after breeding with semen only (SO) or with semen spiked with TriAdj (STA; includes polyI:C, PCEP, and HDP). Relative to the UTE exposed to SO, the inclusion of TriAdj in semen did not result in significant differences in expression of TNFα, IFNβ, GM-CSF, IL-6, IL-8, CCL2, CCL3, CCL4 or CCL28 (Figure 4-4). No significant differences were observed between lower, middle, and upper uterine horn whole tissue gene expression (data not shown).

We speculated that because the UTE is comprised of multiple cell populations, we could not discern whether gene expression profiles of the uterine epithelial cells were being influenced by the presence of TriAdj in the semen. Therefore, we performed LCM such that we captured only the uterine epithelial cells (LC-UEs). LC-UE cells from animals bred with SO or STA also showed no changes in expression of TNFα, IFNβ, GM-CSF, IL-6, IL-8, or CCL4 (Figure 4-4). However, STA significantly induced expression of CCL2 (2.4 fold increase; $p<0.027$) relative to the expression profile observed in LC-UE stimulated with SO. Additionally, in the LC-UE, the STA showed a trend toward significance in CCL3 gene expression (1.18 fold increase, $p=0.052$) and CCL28 gene expression (1.21 fold decrease, $p=0.093$) when compared to SO. Lastly, SLA-DRA gene expression was not detected in the LC-UE samples and had no significant differences when observed in tissue (data not shown). These data suggest that TriAdj had an impact on select UEC chemokine expression during breeding.

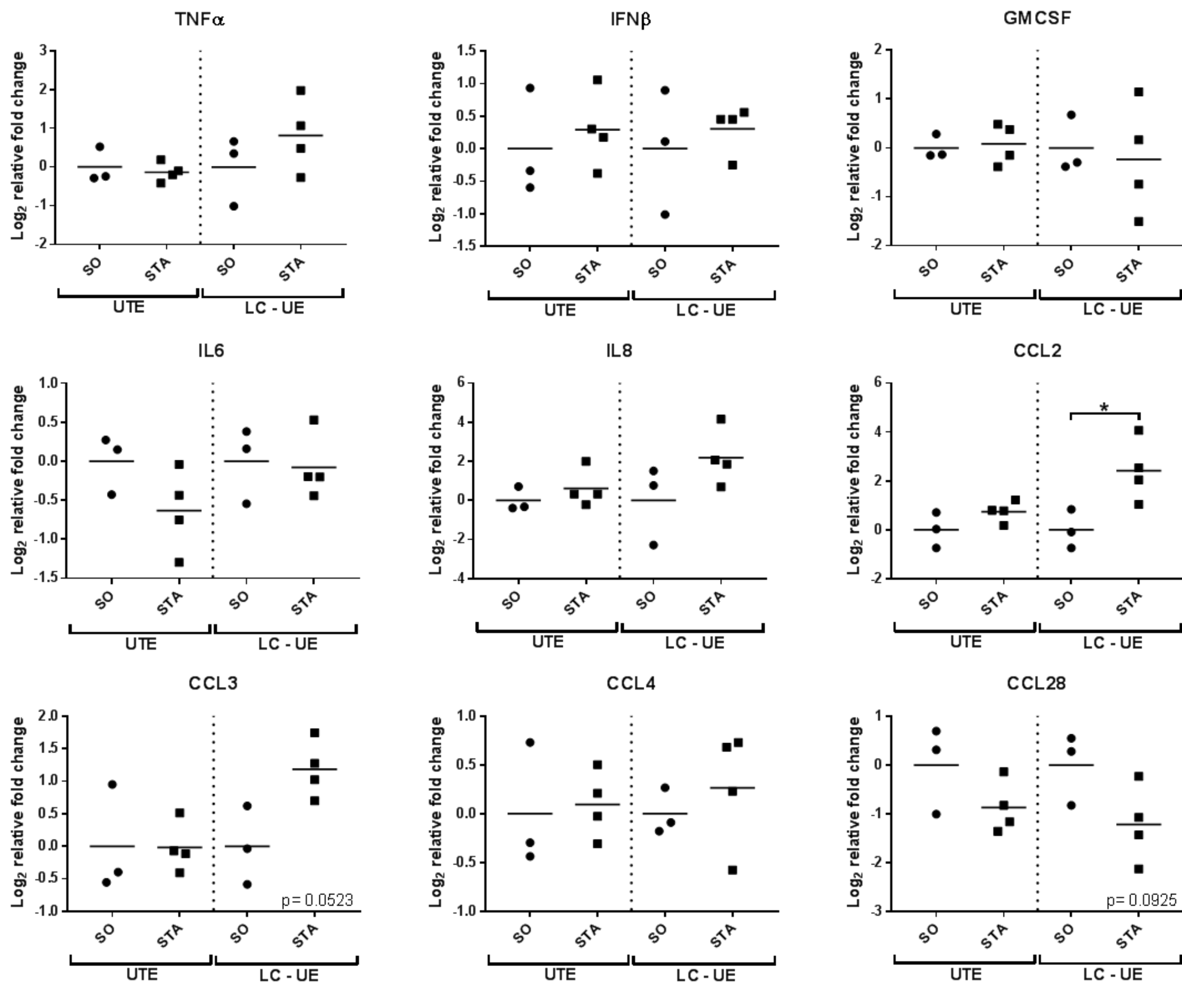


Figure 4-4. Gene expression of uterine tissue (UTE) and laser captured uterine epithelia (LC-UE) of sows 24 hours following breeding with semen only (SO) or semen containing a triple adjuvant combination (STA). UTE expression is average gene expression across the lower, middle, and upper uterine horn, and LC-UE samples were collected from samples in the middle of the uterine horn. Significant differences within sample types were determined by unpaired t-test with Welch's correction (p<0.05=*). Each circle or square represents a unique biological replicate.

Immunotyping of cells in the uterine lumen and blood in response to breeding with semen alone or with TriAdj.

To determine whether the inclusion of TriAdj with the semen dose impacted cell recruitment, we enumerated total cells collected from the uterine lumen (Figure 4-5A). STA triggered a non-significant trend toward increased luminal cells ($p=0.0571$) compared to SO. The most predominant cell populations in the uterine lumen following breeding were neutrophils with mean population percentages at 45% total events in response to SO and 53% of total events in response to STA (Figure 4-5B). All other cell populations were below 1% of total events, regardless of treatment with the exception of one animal bred with STA which had higher total events for NK (3.0%), TCRγδ T cells (8.4%) and $CD8^+$ T cells (6.98%). Additionally, when luminal CCL2 was measured, there were no significant differences observed between animals bred with SO and STA (Figure 4-5C). The inclusion of TriAdj in semen does not appear to significantly impact the proportions of immune cell populations in the uterine lumen, although there is a non-significant increase in total cells compared to animals bred with SO.

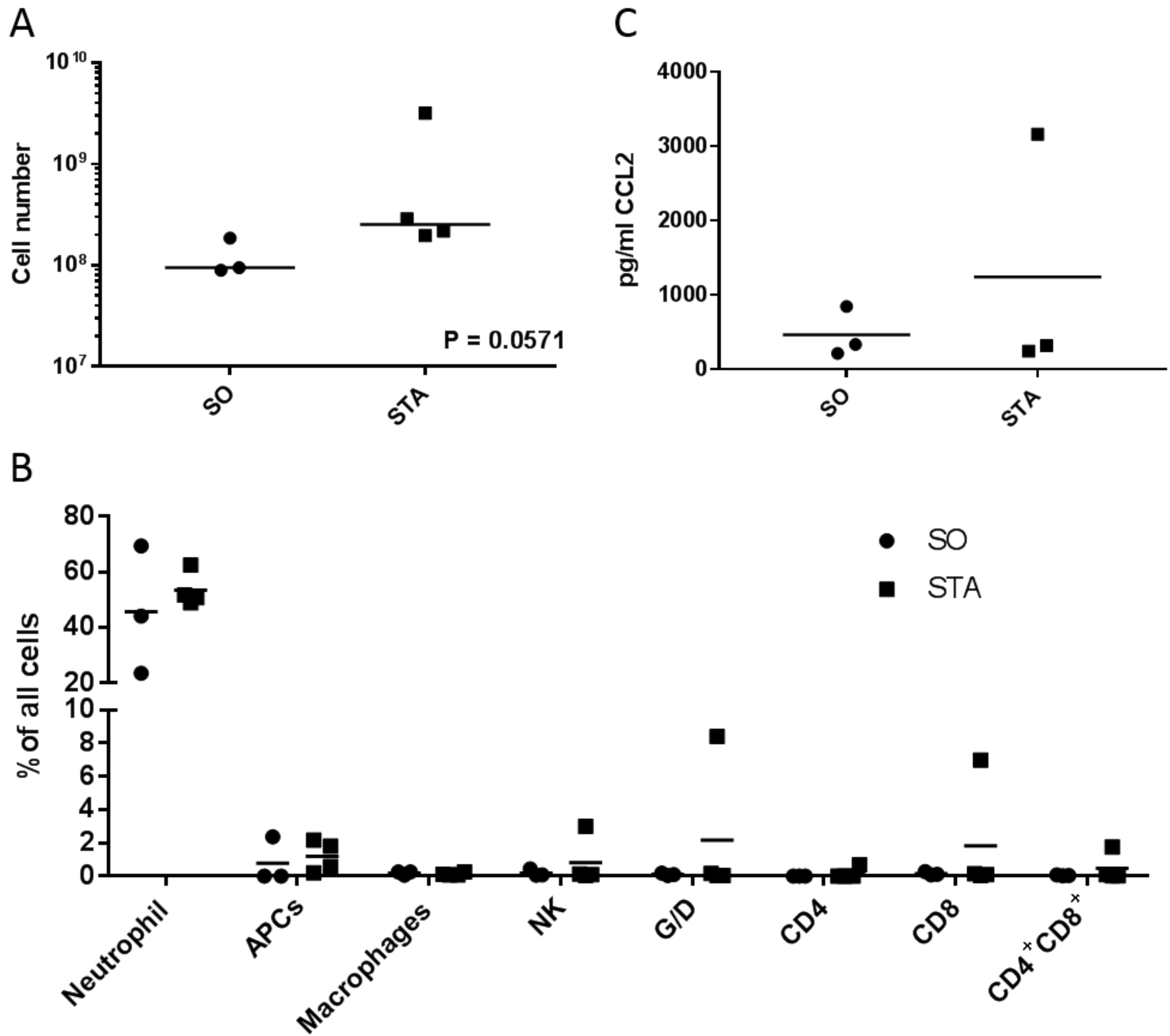


Figure 4-5. Uterine flush cell counts and immunotyping of luminal cell populations of sows 24 hours after breeding with semen only (SO) or semen containing a triple adjuvant combination (STA). Flushed cells were counted by coulter counter (A), and immunotyped cells were stained with CD3, CD4, CD8α, TCRγδ, CD172, MHCII, SWC9, and CD16 (B). Stained cells were analyzed on a FACScalibur and significant differences between treatments determined by Mann Whitney test. Luminal CCL2 was quantified by sandwich ELISA (C) and significant differences between treatments determined by Mann Whitney test. Each circle or square represents a unique biological replicate.

To investigate whether semen +/- TriAdj impacted the systemic immune response, we performed immunotyping on PBMCs to discern whether the number of T cell subsets, B cells, and

monocytes were impacted by either breeding (i.e., pre-SO vs. post-SO; pre-STA vs. post-STA) or by the adjuvants (SO vs. STA). There was no significant change in the percentages of the blood cell population of $CD3^{-}CD8^{+}$ NK cells, CD4 T cells, CD8 T cells, $CD4^{+}CD8^{+}$ co-positive T cells, or $CD21^{+}$ B cells before and after breeding with SO or STA (Figure 4-6). After animals were bred with STA, there was a significant drop in the percentage of TCRγδ T cells (10.5% decrease) and monocytes (4.7% decrease) in the PBMC mixed cell populations relative to the percentages present in PBMCs prior to STA immunization suggesting that the TriAdj may have impacted blood cell composition. However, when we compared the blood cell populations in sows bred with SO vs. sows bred with STA, we did not observe significant differences in any of the population percentages.

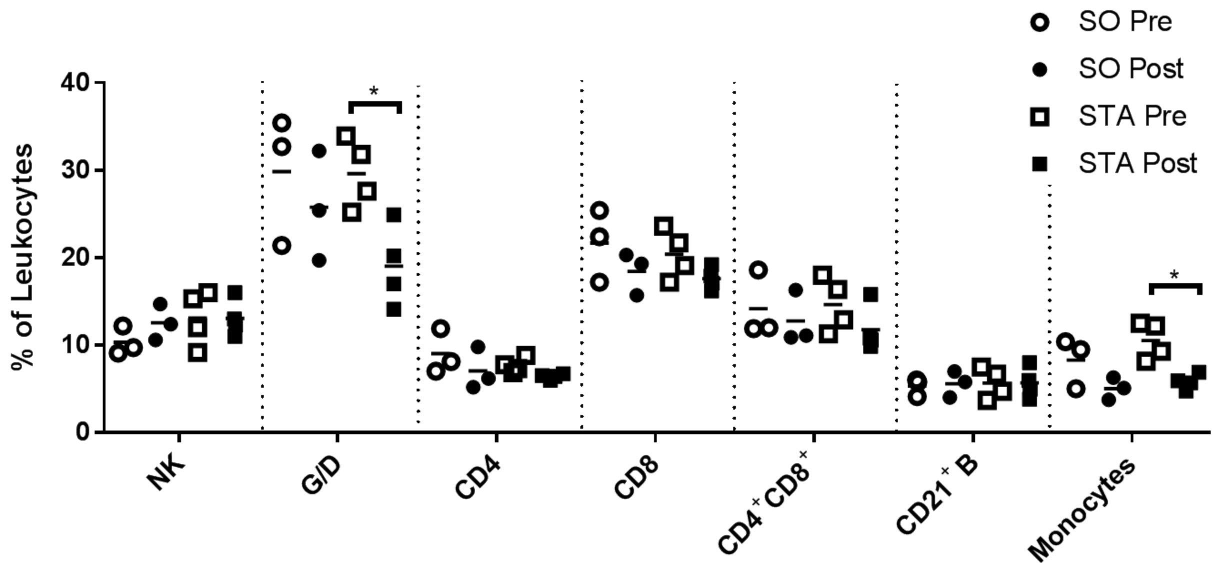


Figure 4-6. Blood immunotyping of T cells, B cells and monocytes of sows before and 24 hours after breeding with semen only (SO) or semen containing a triple adjuvant combination (STA). PBMCs were isolated from blood and stained for CD3, CD4, CD8α, TCRγδ, CD21, CD172 and CD14 before being analyzed on a FACScalibur. Significant differences were determined between pre and post cell percentages by unpaired t-test with Welch's correction ($p<0.05$=*). Each circle or square represents a unique biological replicate.

CD163 positive cell recruitment to uterine tissue following breeding.

To determine if the decreasing monocytes in blood in response to STA (shown in Figure 4-6) shows a correspondent influx of $CD163^+$ cells into uterine tissue, immunohistofluorescence was carried out on sections from the middle of the uterine horn (representative staining Figure 4-7A). $CD163^+$ cells were enumerated per 100 μm^2 section. No significant differences in the number of $CD163^+$ cells were found in the uterine tissue from sows bred with SO (1.23 cells per 100 μm^2) or sows bred with STA (2.03 cells per 100 μm^2) (Figure 4-7B). Additionally, sections of tissue spanning the entire length of the upper reproductive tract were stained with H&E to determine if the inclusion of TriAdj in semen caused any microscopic damage in the tissue. There were no significant microscopic lesions observed on slides taken from the anterior cervix, lower, middle and upper uterine tissue, ampulla or isthmus on either group of sows (representative images in Supplemental Figure 4-3) suggesting that the adjuvants did not promote tissue injury.

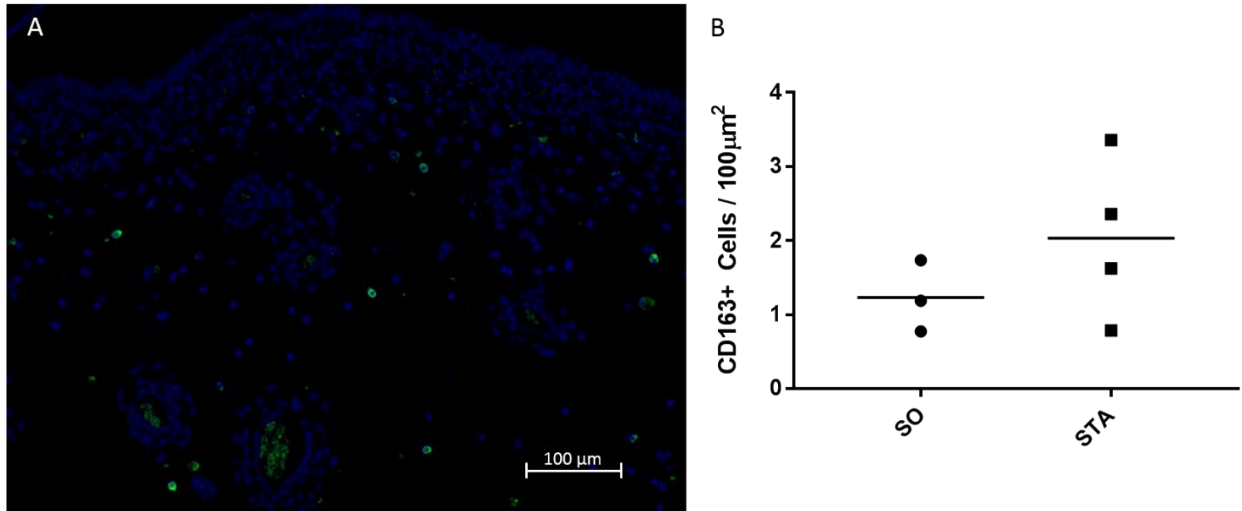


Figure 4-7. Representative immunohistofluorescence of CD163$^+$ cells (A) in uterine tissue 24 hours after breeding with semen alone or semen containing a triple adjuvant combination (Semen+TriAdj). Stained slides were imaged in 10 random fields of view, and CD163 positive cells were counted by image J (B). Significant differences were determined by unpaired t-test with Welch's correction. Each circle or square represents a unique biological replicate.

4.4 Discussion

Initiating a strong mucosal immune response to inactivated or subunit vaccines require potent adjuvants that overcome the mucosal barriers and initiate recruitment of APCs to the mucosal surface. Defining the mechanism of action for adjuvants is essential for identifying the optimal adjuvant components and combinations to be used in vaccines. As the uterine epithelial layer is the first cellular contact for an intrauterine vaccine, generating a strong chemoattractive response that leads to APC recruitment to the uterine tissue or the uterine lumen may increase intrauterine vaccine efficacy.

Immunostimulatory vaccine adjuvants frequently considered for use in mucosal vaccines are TLR agonists and to a lesser extent other PRR agonists that act through the inflammasome. Although porcine UECs express the necessary receptors for all the ligands evaluated

(TLR3:polyI:C, TLR4:LPS, TLR9:CpG, NOD2:MDP (Hamonic et al., 2018a)), our study showed that these cells only induced expression of the pro-inflammatory cytokine and chemokine genes in response to polyI:C. Previous studies have shown that the presence of the receptors alone does not ensure the expression of pro-inflammatory cytokines and chemokines upon stimulation with the receptors corresponding ligands. *In vitro* experiments have shown polyI:C and LPS stimulation of murine UECs significantly induced secretion of CCL2, while CpG stimulation was unable to induce CCL2 expression (Soboll et al., 2006b). Human UECs, in contrast, have shown that LPS stimulation suppressed CCL2 expression whereas polyI:C induced secretion of TNF-α, GM-CSF, IL-6, G-CSF, CCL2 and CCL4 (Schaefer et al., 2004; Schaefer et al., 2005). Lastly, although porcine UECs showed induced expression of pro-inflammatory cytokines and chemokines genes in response to polyI:C, LPS stimulation had no observable impact on the observed genes. The notable discrepancies of responses between species supports the concept that although TLR expression in uterine epithelial cells is relatively conserved across species, the response upon TLR ligand stimulation between species can vary significantly and caution should be taken in attempting to extrapolate results across species.

The non-TLR ligands are less regularly evaluated as adjuvants, however, porcine UECs expressed the receptors for several potential adjuvants, including NOD2 the receptor for MDP (Hamonic et al., 2018a). Although there are no studies showing significant *in vitro* stimulation of UECs with MDP, *in vitro* studies with mouse APCs showed minimal NF-κβ activation unless MDP was combined with other ligands such as CpG (Shafique et al., 2012). Our results show that pig UECs did not induce expression of any observed genes in response to MDP alone nor did MDP amplify the response generated towards polyI:C. Therefore we do not anticipate that it will be an effective adjuvant in inducing APC recruitment or activation in an intrauterine vaccine. HDP,

which has no known receptor, has been implicated in modulating the immune response in several cell types including monocytes where *in vitro* stimulation resulted in increased CCR5 expression and enhanced recruitment to CCL3 and CCL5 (Madera and Hancock, 2015). Although they have shown HDP modulated activity in other cells, both when alone and combined with other adjuvant components, HDP showed no significant impact on the capacity for porcine UECs to respond to adjuvants. Lastly, although PCEP has limited studies in inducing responses *in vitro*, there have been numerous studies evaluating it in both mucosal and parenteral vaccine formulations where PCEP alone induced protective immune responses (Shim et al., 2010). Intramuscular injection of mice with PCEP triggered local production of CCL2 and pro-inflammatory cytokines as IL-1beta, and IL-18 cytokines and when injected intradermally into pigs, PCEP induced the expression of chemokine CCL2 and pro-inflammatory cytokine IL-6 suggesting that it has immunostimulatory potential (Awate et al., 2012; Magiri et al., 2016). These observations suggest that PCEP can act as an immunostimulatory adjuvant and it may potentiate immune responses to antigens. Despite these results in mice and pigs after parenteral injection/vaccination, porcine UECs stimulated with PCEP did not induce expression of cytokine or chemokine genes and may not be an effective intrauterine vaccine adjuvant alone.

Use of TriAdj as a vaccine adjuvant has been evaluated in multiple vaccine formulations, in multiple species, and delivered via several routes. Primarily it has been evaluated for use as an intramuscular vaccine adjuvant where it has been used in mice, rats, cows, sheep and pigs generating strong systemic immunity against human parainfluenza type 3 (in mice and rats), bovine viral diarrhea virus (in cows and sheep) and porcine epidemic diarrhea virus (in pigs) (Garg et al., 2017b; Makadiya et al., 2016; Snider et al., 2014). TriAdj has also been used to generate a strong single dose humoral and cell-mediated immune response when delivered subcutaneously in

koalas as a subunit chlamydia vaccine (Khan et al., 2014). When TriAdj was used in conjunction with mucosal vaccine studies have observed mucosal immunity and protection generated to an IN vaccine to respiratory syncytial virus in mice (Garg et al., 2014). A promising use for the TriAdj as a mucosal adjuvant was shown when it was administered as part of a subunit vaccine in the rabbit uterus as it induced strong systemic and mucosal humoral immune responses (Pasternak et al., 2017). Although there have been limited studies on the initial innate immune response generated to TriAdj, an *in vitro* study with mouse macrophages found that they induced significant expression of several chemokines including CCL2, CCL3, and CCL4 in addition to upregulation of the co-stimulatory molecules CD80/86 and MHC class II (Sarkar et al., 2018) in the presence of TriAdj. Similarly, although to a lesser extent, our study showed the induction of chemokine gene expression response by the UECs in response to the polyI:C combined as the TriAdj *in vitro* and *in vivo*. Studies that established effective adjuvant combinations, determined that inclusion of a TLR ligand (CpG in these studies), PCEP and HDP acted synergistically together resulting in the strongest immune response (Kovacs-Nolan et al., 2009a; Kovacs-Nolan et al., 2009b). Although we were only successful in inducing chemokine expression with polyI:C and observed no synergistic effect including other vaccine components, the inclusion of polyI:C does provide an immunostimulatory adjuvant component that can be included in vaccine formulations and interact with the first contact point of an intrauterine vaccine.

Because intrauterine vaccination in sows would only be used during AI, it is important to take into account the immune response generated during breeding. It has been widely acknowledged that breeding in swine elicits an inflammatory immune response and neutrophil infiltration into the lumen (Katila, 2012; Rozeboom et al., 1999). However, with the exception of a widely accepted IL-8 induction and corresponding polymorphonuclear cell recruitment to the lumen, there are

limited studies examining the exact cytokine and chemokine genes induced following breeding. Interestingly one previous study showed that the semen extender Androhep and seminal plasma alone induced IL-10, TGF-β, IL-8, and TNF-α, however when combined with spermatozoa, these values returned to baseline expression levels (Taylor et al., 2009). The possible suppression of cytokine and chemokine expression by spermatozoa may contribute to the discrepancy in the magnitude of expression observed *in vivo* that was lower then what was observed in the *in vitro* experiments. However, studies evaluating immune cell recruitment into the endometrium following breeding remain somewhat unclear whether spermatozoa, seminal plasma, or semen extender is the primary inducer of this response (Kaeoket et al., 2003). We speculate that this inflammatory response may reduce the requirement of an intrauterine vaccine to induce an inflammatory response itself, and may instead require the adjuvants to modulate the inflammatory response towards a higher proportion of recruited APCs in the uterine mucosa, possibly through the induction of chemokines that will preferentially recruit APCs, such as CCL2 and CCL3.

Previous studies have described that the lumen of the porcine uterus in a native state has a relatively low-level complement of T cells (Käser et al., 2017), which is consistent with our observations. Further, our data show that not only does STA not impact T cell recruitment to the uterine lumen, but we also show that breeding appeared to have minimal effect on luminal T cell numbers. It remains to be clarified why blood γδ T cells were reduced after animals bred with STA but not in animals bred with SO and why there is no evidence that the γδ T cells were recruited to the uterine lumen. Current data indicate that circulating porcine γδ T cells are primarily pro-inflammatory (Wen et al., 2012) and therefore further research should be carried out to determine if the inflammatory response induced by TriAdj plus semen is specifically recruiting these cells.

Based on the limited data available for γδ T cells and their subtypes in pigs, we currently do not know the impact these cells may have in mounting a response to the intrauterine vaccination.

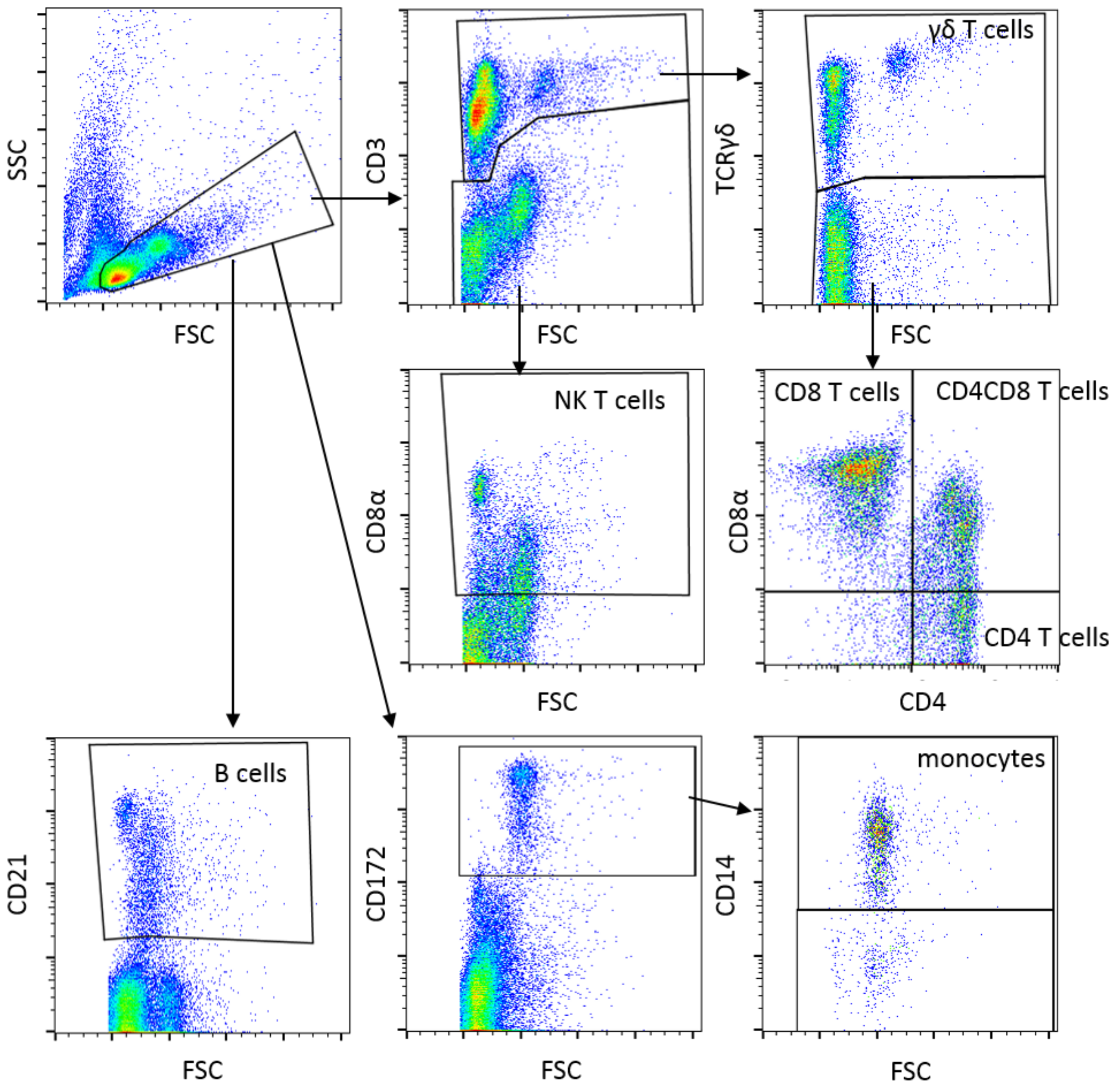
Epithelial cells of several species including humans, mice and dogs have been shown to express MHC class II indicating that the epithelial cells themselves may be able to directly present antigen to T cells (Mair et al., 2014) or that IFNγ pre-treatment may be required to induce MHC class II expression (Wallace et al., 2001). For example, human UECs have been shown to express MHC class II to respond to tetanus toxoid with induced T cell proliferation (Wallace et al., 2001). However, we saw no evidence of MHC class II expression in the porcine UECs, which is supported by the only other published study that reports a lack of MHC class II expression by porcine intestinal epithelial cells (Wilson et al., 1996). Stromal cells have been implicated in further modulating the capacity of UECs within the female reproductive tract to present antigen to naïve and primed T cells (Wira et al., 2005c). The present evidence suggests that unlike other species, porcine UECs are unable to present antigen directly to T cells and therefore their role in an intrauterine vaccine response will be in the transport of vaccine across the epithelial barrier or promoting cellular recruitment of APCs into the endometrium or lumen.

The primary role of the uterine epithelia in the generation of a strong immune response to an intrauterine vaccine may be to initiate recruitment of the APCs into the uterine tissue to increase APC contact with the vaccine. Because the impact of TriAdj was most clearly observed in Laser-captured uterine epithelial cells, it will be difficult to isolate enough cells to quantify chemokine secretion profiles. However, with no detectable increase in luminal CCL2, the increase in uterine epithelial cell gene expression does not appear sufficient to alter the protein levels in the lumen. While it is possible that CCL2 is secreted by the uterine epithelia basolaterally, we would anticipate observing a greater degree of APC recruitment into the endometrium if this were the case. We

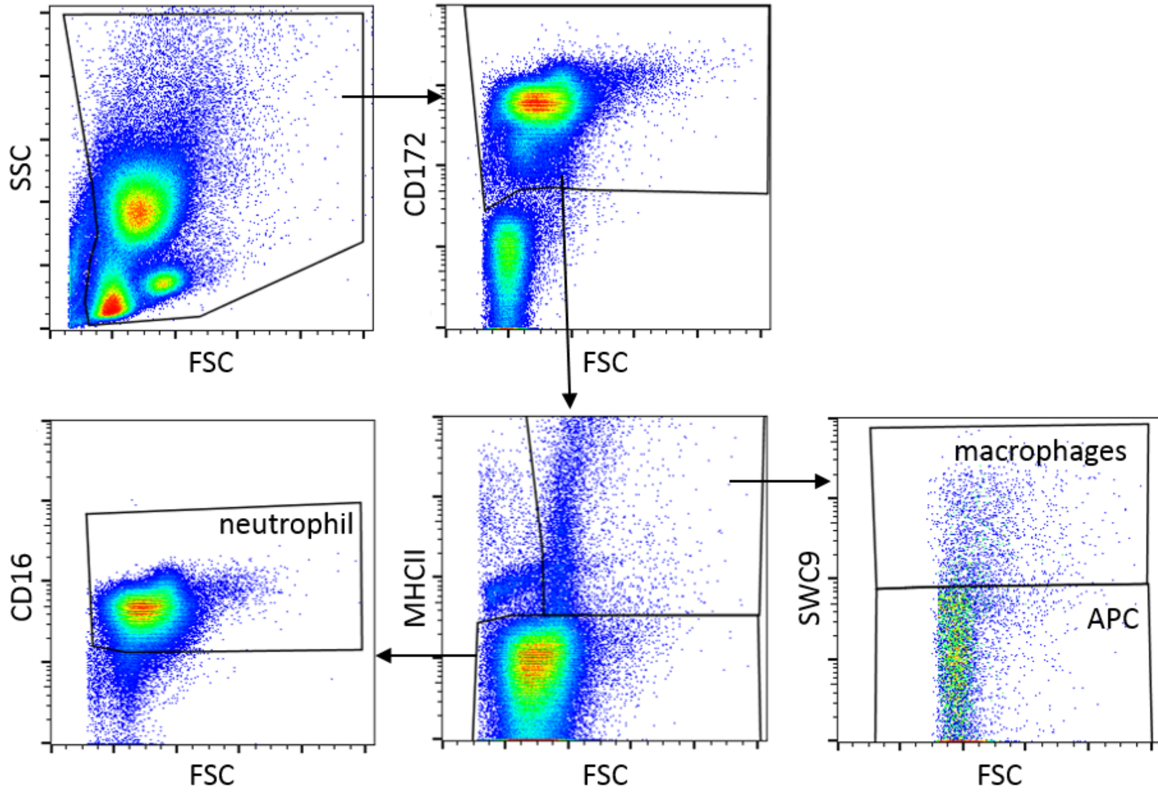
observed both increased expression of chemokines by the uterine epithelia known to promote monocyte and macrophage recruitment chemokines as well as decreased levels of monocytes in the blood. We did not observe a significant increase in the numbers of monocytes/macrophages (CD163 positive cells) in the uterine tissue when compared to the response to extended semen. Although there are numerous studies characterizing the polymorphonuclear cell recruitment into the lumen following breeding and the inflammatory response following breeding with extended semen (Katila, 2012; Rozeboom et al., 1999; Taylor et al., 2009), data on APC recruitment in swine is limited. However, a single study observed increased MHCII expression on uterine macrophages and DCs following breeding, indicative of APC maturation (Bischof et al., 1994b). These data and the non-significant decrease of blood monocytes after breeding in our study may be indicative of a certain degree of APC engagement to extended semen alone and inclusion of TriAdj in semen although more research is required to understand this. Additionally, if extended semen alone is able to recruit APCs to the uterus, other adjuvants may be unnecessary to trigger a strong vaccine-induced immune response to intrauterine vaccination.

This study is the first step in elucidating how the porcine uterus responds to vaccine adjuvants for the development of an intrauterine vaccine. The uterine epithelium is primed and capable of responding to select vaccine adjuvants by inducing expression of both pro-inflammatory cytokines and chemokines. Additionally, the inclusion of vaccine adjuvants in an extended semen dose did not significantly increase the luminal cell populations of immune cells or the frequency of CD163 positive cells within the endometrium. As a large number of immune cells are present throughout the uterus following breeding, the inclusion of adjuvants that induce immune cell recruitment through the uterine epithelial may have greater importance in inducing immune cell recruitment to vaccination for intrauterine vaccines that are not included in a semen dose.

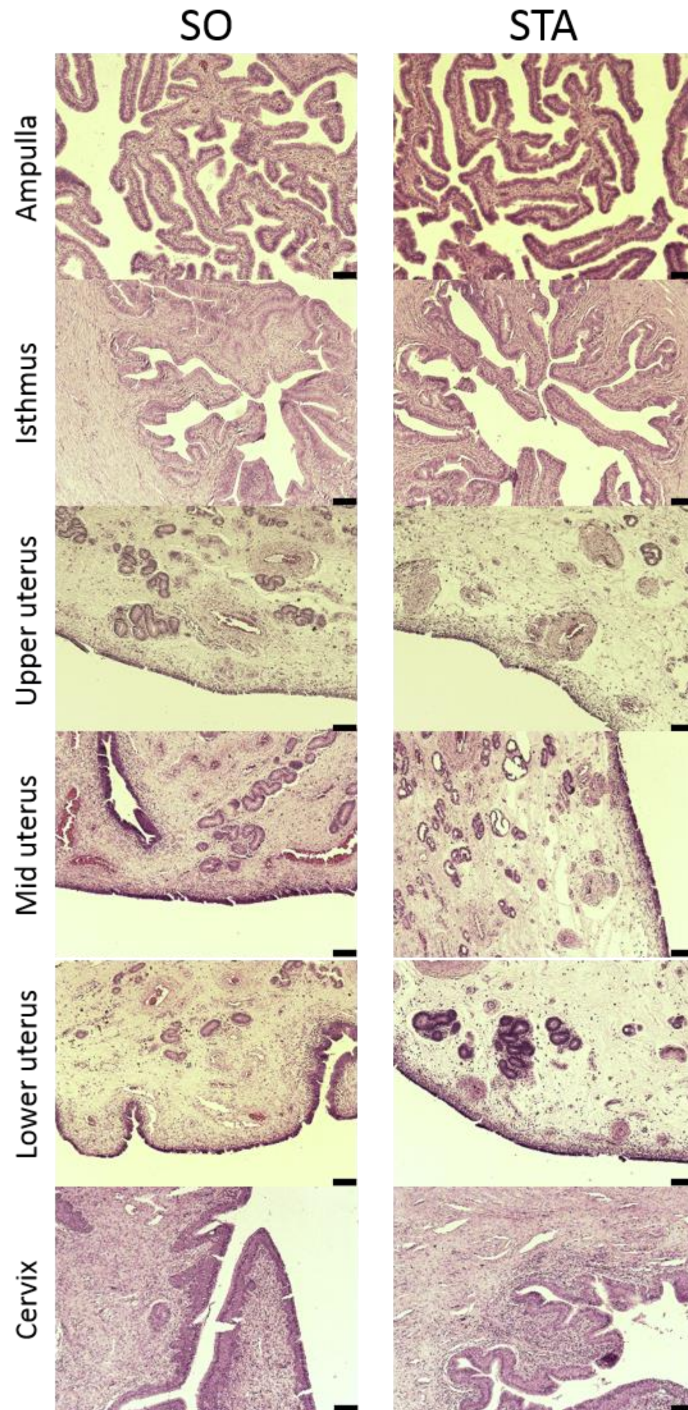
4.5 Supplemental Figures



Supplemental Figure 4-1. Gating strategy used for T cell, B cell, and monocyte immunotyping stains from the blood.



Supplemental Figure 4-2. Gating strategy used for myeloid cell immunotyping stain used for luminal cell populations.



Supplemental Figure 4-3. Haemotoxylin and Eosin stained tissue following breeding with semen only (SO) or semen containing a triple adjuvant combination (STA).

4.6 Transition statement

In vitro and *in vivo* stimulation of pig uterine epithelial cells shows that they respond to the tri-adjuvant combination composed of polyI:C, a host defence peptide and a polyphosphazene. However, there was no clear data to indicate that the secretions produced by the uterine epithelial cells were effective in inducing APC recruitment and activation within the endometrium. Therefore, in the next chapter, we expand the *in vitro* uterine epithelial cell model to determine the modulation of APCs and their activity.

5 MONOCYTE RECRUITMENT AND DIFFERENTIATION IN RESPONSE TO UTERINE EPITHELIAL CELL SECRETIONS *IN VITRO*.

(In preparation)

Authors

Hamonic, G., and Wilson H.L.

Author contributions

GH and HLW conceived of and designed the experiments. GH carried out all assays, analyzed all data and drafted the manuscript. Both authors read and approved the manuscript

Abstract

The uterine environment is a mucosal surface in which the presence of a commensal flora is currently poorly defined, and the generation of immune tolerance in the porcine uterus to antigens from pathogens, seminal contents, and environmental antigens has not been investigated. The epithelial cells that line this surface express several receptors have been classified as sentinels of the uterine immune response in other species. Unspecified secretions from these cells in a natural state in humans modulated the activity of dendritic cells and their capacity to respond to activation and maturation. To investigate the role of uterine epithelial cells in the modulation of the porcine uterine immune response, uterine epithelial cells (UECs) were stimulated with a TriAdj composed of polyI:C, a host defence peptide, and a polyphosphazene. The potential for recruitment of monocytes in response to stimulated UECs was investigated. UEC secretions did not generate a chemotaxis response in monocytes *in vitro,* and upon quantification of stimulated UEC supernatants, there was no increase in CCL2 secretion in response to the TriAdj. Secretions were collected and included in media used for differentiation of monocyte-derived dendritic cells to determine whether unspecified secretions impacted monocyte differentiation and/or moDC activation. Differentiation of monocytes in unstimulated uterine epithelial secretions resulted in increased CD16 expression after six days of differentiation, however, no differences between groups were observed by day 8. moDCs differentiated in mock-stimulated, and TriAdj-stimulated UEC supernatants showed no significant change in moDC maturation, endocytosis of FITC-dextran, or the ability to stimulate proliferation of naïve T cells. These results suggest that in contrast to human endometrium, pig UCEs do not modulate DC function within the endometrium.

5.1 Introduction

DCs are the professional APCs of the immune system, and they are the main bridge from the innate immune system to the adaptive immune system. They are responsible for the uptake, processing, and presentation of foreign antigen to cognate T cells. Immature DCs possess strong endocytic and phagocytic activity. Upon uptake of antigen or upon stimulation with PAMPs, APC's undergo maturation resulting in reduced endocytic/phagocytic activity and increased APC activity (Banchereau et al., 2000). Maturation includes increased expression of MHC class II and the costimulatory molecules CD40, CD80, and CD86 on the surface of the DCs (Auray et al., 2010). Mature DCs express several cytokines and present antigen to naïve T cells through the MHC class II, in addition to the required second signal from the costimulatory molecule and its receptor on the T cell (Banchereau et al., 2000).

Maintaining an effective immune system in the upper reproductive tract requires a delicate balance between suppression and activation as the uterus must remain primed to eliminate pathogens at its surface while maintaining several semi-allogenic fetuses during pregnancy. This delicate balance has not been widely studied in swine, although immune cell populations have been characterized to show the presence of lymphocytes as well as MHC class II^{+} dendritic-like cells present throughout the endometrium in prepubertal gilts as well as in sexually mature animals (Bischof et al., 1994a). During pregnancy when the gilt/sow maintain the semi-allogeneic fetus, MHC class II^{+} macrophages and DCs have been observed at high levels all through the duration of gestation and they are the predominant myeloid cell in the endometrium during mid to late gestation (Bischof et al., 1995). Although it has not been well-characterized, the uterine immune system likely maintains mechanisms to suppress the initiation of an immune response against the developing fetus. In humans where studies have been more widely carried out, UECs secretions

have been determined to suppress DC differentiation and maturation when characterized *in vitro* (Ochiel et al., 2010).

UECs form a barrier that separates the body from any potential pathogen in the lumen and may act as the sentinels of the uterine immune response (Wira et al., 2005b). Porcine UECs express several PRRs (Hamonic et al., 2018a) and respond to stimulation through the induction of several cytokine and chemokine genes (Chapter 4). In rabbits, injection of vaccines formulated with TriAdj into the uterine lumen resulted in induction of a robust humoral local and distal immune response but a poor recall response (Pasternak et al., 2017; Pasternak et al., 2018). These results suggest that in response to an intrauterine vaccine, the uterine epithelia may modulate the initial response to the vaccine.

This study aims to determine what effect UEC secretions from native state epithelial cells have on monocyte-derived dendritic cell differentiation and maturation upon stimulation with TLR ligands LPS, Pam3CSK4, and polyI:C. Additionally, we aim to determine if secretions from stimulated UECs modulate the function of moDCs.

5.2 Materials and Methods

Isolation, culture, and stimulation of primary uterine epithelial cells

Upper reproductive tracts were collected from a local abattoir for primary uterine epithelial cell (UEC) isolation (n=5) as previously described in chapter 3 (Hamonic et al., 2018a). Briefly, segments of uterine horns were inverted and incubated in an enzyme cocktail of 12 g/L pancreatin (Fisher Scientific) and 250 units/L dispase (BD Bioscience) in PBSA at 4°C overnight with shaking. Sloughed UECs were washed and filtered through a 40 µm cell filter and cultured on a

PET membrane with 0.4 µm pore size (Greiner Bio-One) in DMEM:F12 (1:1; Gibco) with 10% FBS (Sigma-Aldrich), 2.5 mM glutamine (Gibco), 10 mM HEPES (Gibco) and 1X Anti-Anti (Gibco). Media was changed every two days, and trans-epithelial electrical resistance was measured until cells stabilized at 10X increased TEER (approximately ten days, indicative as pure epithelial cells as determined in (Hamonic et al., 2018a)). Following stabilization, cells were treated apically with TriAdj (50 µg/ml polyI:C (Invivogen), 100 µg/ml HDP (Genscript), 50 µg/ml PCEP (Idaho National Laboratory)) in DMEM:F12 culture media or media alone for 24 hours, after which the basolateral supernatant was collected, and these are referred to as TriAjd-stimulated UEC secretions and unstimulated UEC secretions, respectively.

Isolation of monocytes

All experimental procedures were conducted in accordance with the guidelines of the CCAC under approval from the Animal Research Ethics Board at the University of Saskatchewan.

Whole blood was collected from pigs (n=5) in EDTA vacutainers (BD Biosciences) and centrifuged at 1100 x g for 20 minutes for collection of the buffy coat containing PBMCs. The buffy coat enriched for PBMCs by layering on Ficoll-Paque Plus (GE Life Sciences) and centrifuged at 400 x g for 40 minutes. PBMCs were then washed 3X in PBSA and then resuspended in MACS buffer (PBS plus 2 mM EDTA, 0.5% BSA) for monocyte isolation. PBMCs were incubated with CD14 Microbeads (Milteny Biotec) for 15 minutes at 4°C. Labelled PBMCs were washed with MACS buffer and loaded onto LS columns (Milteny Biotec) for magnetic separation of monocytes. Columns were washed 3X with MACS buffer to remove CD14 negative lymphocytes, and CD14 positive monocytes were collected.

Chemotaxis of monocytes

Chemotaxis assays were carried out using a 48 well reusable multiwall chemotaxis chamber (Neuro Probe Inc) with eight replicates per treatment and one membrane used per animal. The bottom well contained 30 µl of unstimulated UEC secretions, TriAdj-stimulated UEC secretions, 120 ng/ml CCL2 (Kingfisher Biotech) or media. A polycarbonate membrane with 5 µm pores (Neuro Probe Inc) was carefully placed over bottom wells then the rubber gasket and top plate were then placed over the membrane. Monocytes were plated into the top well carefully to avoid bubble formation on the membrane at a concentration of 1.5×10^4 cells per well, and the chemotaxis chamber was incubated at 37°C for 1 hour. Following incubation, unmigrated cells were carefully washed and scrapped off the membrane, and the membrane was fixed in methanol for 60 seconds. Membranes were then stained with DAPI (Invitrogen) for 10 minutes at RT before being placed on a slide, and a coverslip was applied. DAPI-labelled cells were imaged on an Axiovert 200M (Zeiss) at 20X magnification. Images were analyzed by image J, and cells were counted with the analyze particles function.

CCL2 ELISA

Uterine horn luminal secretion of CCL2 was quantified by sandwich ELISA against porcine CCL2 (Kingfisher Biotech) following manufacturer's instructions. In short, 96 well high binding plates (Immulon 2, VWR) were coated with a polyclonal anti-swine CCL2 (Kingfisher Biotech) at 1 µg/ml in PBS overnight at RT. Plates were then blocked by 4% BSA in PBS for 2 hours at RT prior to a 1 hr RT incubation with CCL2 standard (1in 2 dilutions from 10 ng/ml to 10 pg/ml) and undiluted flush samples. Plates were washed with TBST, and biotinylated anti-swine CCL2 antibody was incubated at 0.5 µg/ml in PBS + 4% BSA for 1 hour at RT followed by

washing and a 30 min RT incubation with streptavidin-HRP. Plates were developed with TBS for approximately 30 minutes in the dark before stopping with a 2N sulfuric acid and absorbance was read at 450 nm.

Generation of moDCs and stimulations of moDCs

In order to generate moDCs, monocytes were cultured in 50% RPMI complete (10% FBS, 1x Anti-Anti, 50 μM β-mercaptoethanol, 2 mM glutamine (Gibco), 10 mM HEPES (Gibco), and 1 x non-essential amino acids (Gibco)) and either 50% DMEM: F12 (Media control), unstimulated UEC secretions, or TriAdj-stimulated UECs secretions supplemented with 100 ng/ml IL4 (KingFisher) and 20 ng/ml GM-CSF (Biosource). After three days in culture, 1/3 of the media was replaced with RPMI complete containing 300 ng/ml IL4 and 60 ng/ml GM-CSF. moDC differentiation and stimulation experiments are summarized in Figure 5-1. At day 6 of culture, cells were considered moDCs, and differentiation was analyzed by FCM (detailed below). To assess moDC maturation, moDCs were stimulated for 48 hours with 25 μg/ml polyI:C (Invivogen), 100 ng/ml lipopolysaccharide (LPS; from *Escherichia coli* O55:B5, Sigma-Aldrich) or 500 ng/ml Pam3CSK4 (Invivogen) and were then analyzed by FCM (detailed below). A selection of moDCs were stimulated with media or 500 ng/ml Pam3CSK4 for 24 hours and then co-cultured with T cells (see below). moDC endocytic capacity was measured by incubation with 0.1 mg/ml or 1 mg/ml FITC dextran (Sigma-Aldrich) for 1 hour at 37°C followed by washing with PBSA + 2% FBS to remove free FITC dextran prior to analysis by FCM.

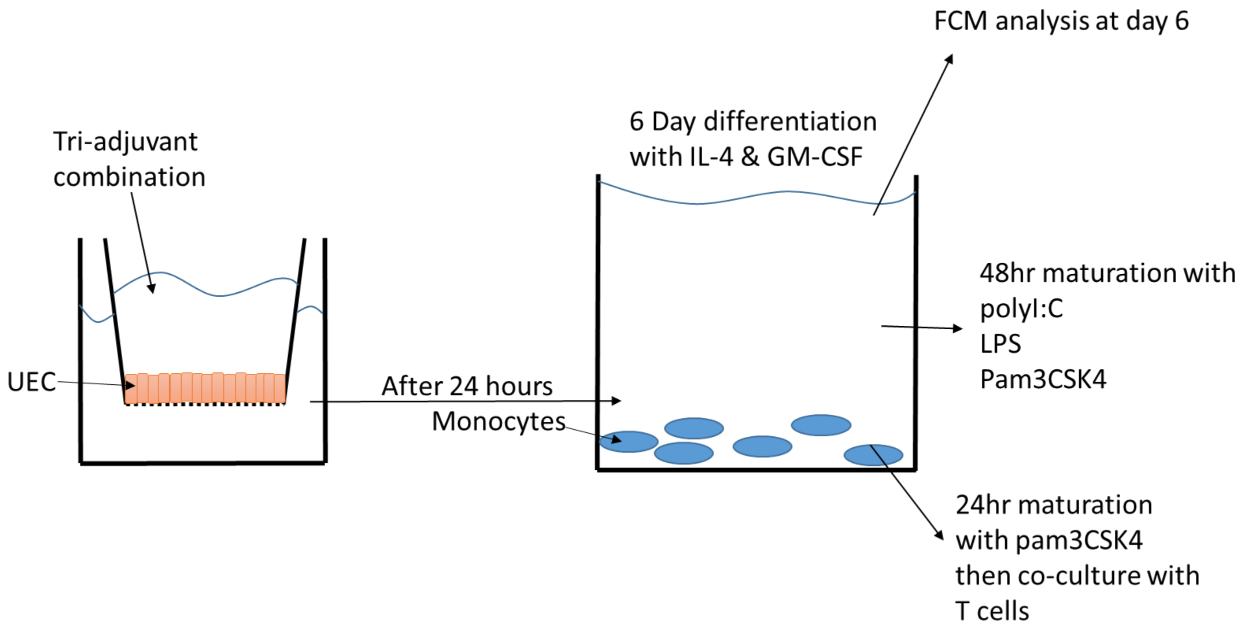


Figure 5-1. Flowchart for moDC differentiation and subsequent experiments and analysis.

Isolation of T cells and T cell proliferation

PBMCs were resuspended in MACS buffer with a mouse anti-CD21 antibody (Clone and supplier) and a mouse anti-CD172 antibody (clone and supplier) for 15 min at 4°C with shaking. Cells were then washed and incubated with anti-mIgG MACS beads (Milteny Biotec) for 15 min at 4°C with shaking. Cells were washed and run through LS columns collecting the negative fraction (T cells). Isolated T cells were resuspended in PBS and 2.5 μM Carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) for 10 min at 37°C. Staining was halted with the addition of FBS and incubation for 15 min at RT. Stained T cells were washed and plated with media, 5 μM concanavalin A (ConA; Sigma-Aldrich), unstimulated moDCs or Pam3CSK4-stimulated DCs at concentrations of 1:5, 1:10 or 1:100 moDCs to T cells. Cells were co-cultured for four days before they were subjected to proliferation analysis by FCM.

Flow cytometry

For all FCM analysis, cells were resuspended in PBS + 2 % FBS, and staining was carried out in 96 well round bottom plates. All antibodies are summarized in Table 5-1, and all stains were in a 10 µl volume for 10 minutes at RT. Additionally, between all stains, cells were washed 3X with PBS + 2% FBS. For characterization of DCs, cells were collected and washed prior to staining with the CTLA4-mIgG2a fusion and anti-CD16. Cells were washed then stained with anti-mIgG1-PE and anti-mIgG2a-APC. Cells were again washed, then mIgG was added for 10 minutes at RT to block secondary antibodies. Cells were washed then stained with anti-MHCII and anti-CD1-FITC, followed by washing and staining with SA-PERcpCy5.5. Proliferated T cells were stained with anti-CD4, anti-CD8β and anti-TCRγδ then washed and staining with anti-mIgG2b-Biotin, anti-mIgG2a-Al647 and anti-mIgG1-PE, and lastly SA-PERcpCy5.5. All cells were run on a FacsCalibur (BD) collecting 100,000 events and analyzed in FlowJo (FlowJo LLC).

Table 5-1. Antibodies, final concentrations, and suppliers of all flow cytometry antibodies used.

Target	Clone (Isotype)	Fluorochrome	Final concentration	Supplier
Primary Antibody				
Anti-CD1	76-7-4 (mIgG2a)	FITC	100 µg/ml	Bio-rad Antibodies
Anti-CD16	G7 (mIgG1)	N/A	5 µg/ml	Bio-rad Antibodies
Anti-MHCII	MSA3 (mIgG2a)	Biotin	5 µg/ml	Kingfisher Biotech
CD80/86	CTLA4-mIgG2a fusion	N/A	1 µg/ml	Sigma-Aldrich
Anti-CD4	74-12-4 (mIgG2b)	N/A	10 µg/ml	Monoclonal Antibody Center
Anti-CD8β	PG164A (mIgG2A)	N/A	10 µg/ml	Monoclonal Antibody Center
Anti-TCRγδ	PGBL22A (mIgG1)	N/A	5 µg/ml	VMRD Inc
Secondary Antibodies				
N/A	(mIgG)	N/A	10 µg/ml	Chrompure
Anti-mIgG1	N/A	PE	1.25 µg/ml	Southern Biotech
Anti-mIgG2a	N/A	Al647	2.5 µg/ml	Southern Biotech
Anti-mIgG2b	N/A	Biotin	1.25 µg/ml	Southern Biotech
Streptavidin (SA)	N/A	PERcpCy5.5	0.5 µg/ml	eBioscience

Statistical analysis

All statistical analyses were carried out in GraphPad Prism 7 (GraphPad Software). Statistical analysis of cell counts of monocytes recruited across membranes was done by ordinary one way ANOVA with Dunnett's multiple comparisons test for determining differences between media and treatment groups. All other analyses were carried out evaluating any effect on differentiating conditions of the monocytes into moDCs by ordinary one way ANOVA with Dunnett's multiple comparisons test.

5.3 Results

Recruitment of monocytes to UEC supernatant.

To determine if pig UECs respond to TriAdj stimulation with chemokine production, we measured monocyte recruitment to UEC secretions. Polarized UECs were stimulated with TriAdj or media (mock stimulation) in the apical chamber of the transwell. After 24 hours, the supernatant in the basolateral compartment was collected [referred to as TriAdj-stimulated UEC secretions (UEC Tri) and unstimulated UEC cells alone (CA) secretions (UEC CA), respectively]. Monocytes purified from PBMCs were plated into the upper chamber of the chemotaxis chamber with the supernatants from stimulated and unstimulated cells added into the bottom chamber. Monocytes that were recruited across the membrane during the 1-hour incubation were stained and quantified, as demonstrated by the representative image in Figure 5-2A. No significant differences in numbers of recruited monocytes could be determined in response to media, unstimulated UECs, and TriAdj-stimulated UECs (Figure 5-2B). CCL2 alone at 120 µg/ml was included as a positive control and was able to significantly induce recruitment of monocytes when compared to the media alone treatment ($p=0.0251$). Next, we quantified CCL2 expression in supernatants from unstimulated and TriAdj-stimulated UECs. There was no significant increase in CCL2 protein levels in response to TriAdj stimulation (Figure 5-2C). These results indicate that UECs did not respond to TriAdj-stimulation with increased CCL2 secretions.

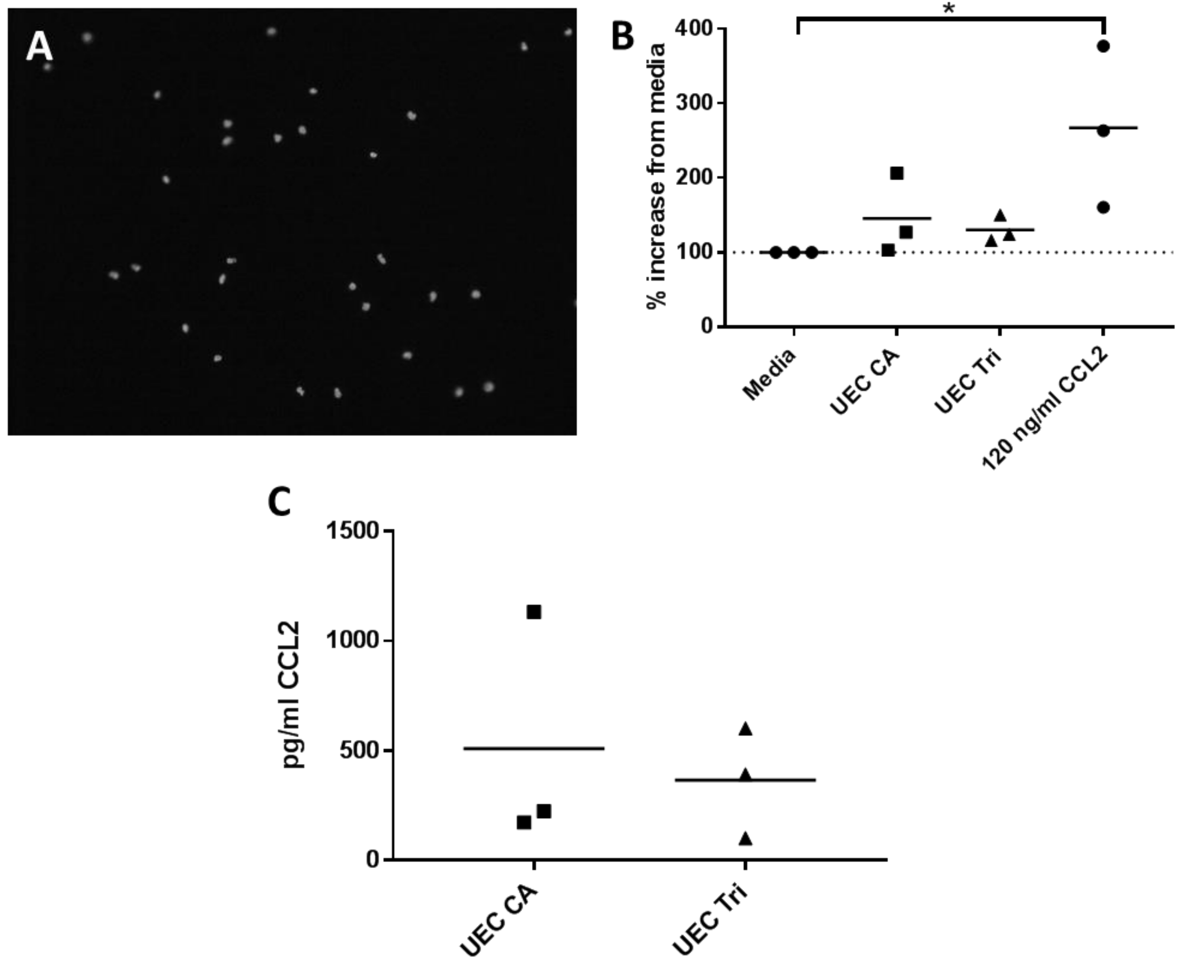


Figure 5-2. Recruitment of monocytes across a membrane in a micro chemotaxis chamber in response to supernatants from unstimulated uterine epithelial cells (UECs) and triple adjuvant combination stimulated UECs. Monocytes were recruited across a polycarbonate membrane with 5 µm pores for 1 hour at 37°C with media, unstimulated UEC supernatant (UEC Media) and stimulated UEC supernatant (UEC Tri). Following recruitment, membranes were stained with DAPI, and recruited monocytes were imaged at 20X on an axiovert 200M microscope. Representative staining in A and cell counts enumerated by ImageJ are presented in B. CCL2 quantities in the UEC supernatants were determined by sandwich ELISA presented in C. Statistical analysis was done by ordinary one way ANOVA and differences between groups determined by Dunnett's multiple comparison tests ($p<0.05=*$). Each circle, square, or triangle indicates a unique biological replicate.

Differentiation and maturation of moDCs in UEC supernatant.

Differentiation of monocytes into moDCs using IL-4 and GM-CSF in pigs has been widely used and accepted as a method to generate large numbers of DCs for use, *in vitro*. We aimed to determine whether supernatants from unstimulated (UEC CA) and stimulated (UEC Tri) UECs modulated moDC differentiation. After six days of differentiation in the presence of IL-4, GM-CSF, and supernatants UEC Tri or UEC CA, monocytes showed no significant differences in the expression levels of moDC maturation markers including CD1 (Figure 5-3A), MHCII (Figure 5-3C), or CD80/86 (Figure 5-3D) across groups. There was a significant increase in the expression of CD16 in the unstimulated UEC supernatant treatment (Figure 5-3B, 32% increase, p=0.0151) compared to the media control that was not observed in the UEC Tri treatment. After 8 days of differentiation, there were no longer any significant differences between expression of CD16 (Figure 5-3F) regardless of whether the monocyte differentiation took place in the presence of secretions from UEC stimulated with TriAdj or not; CD1 (Figure 5-3E), MHCII (Figure 5-3G) and CD80/86 (Figure 5-3H) remained unchanged across treatments.

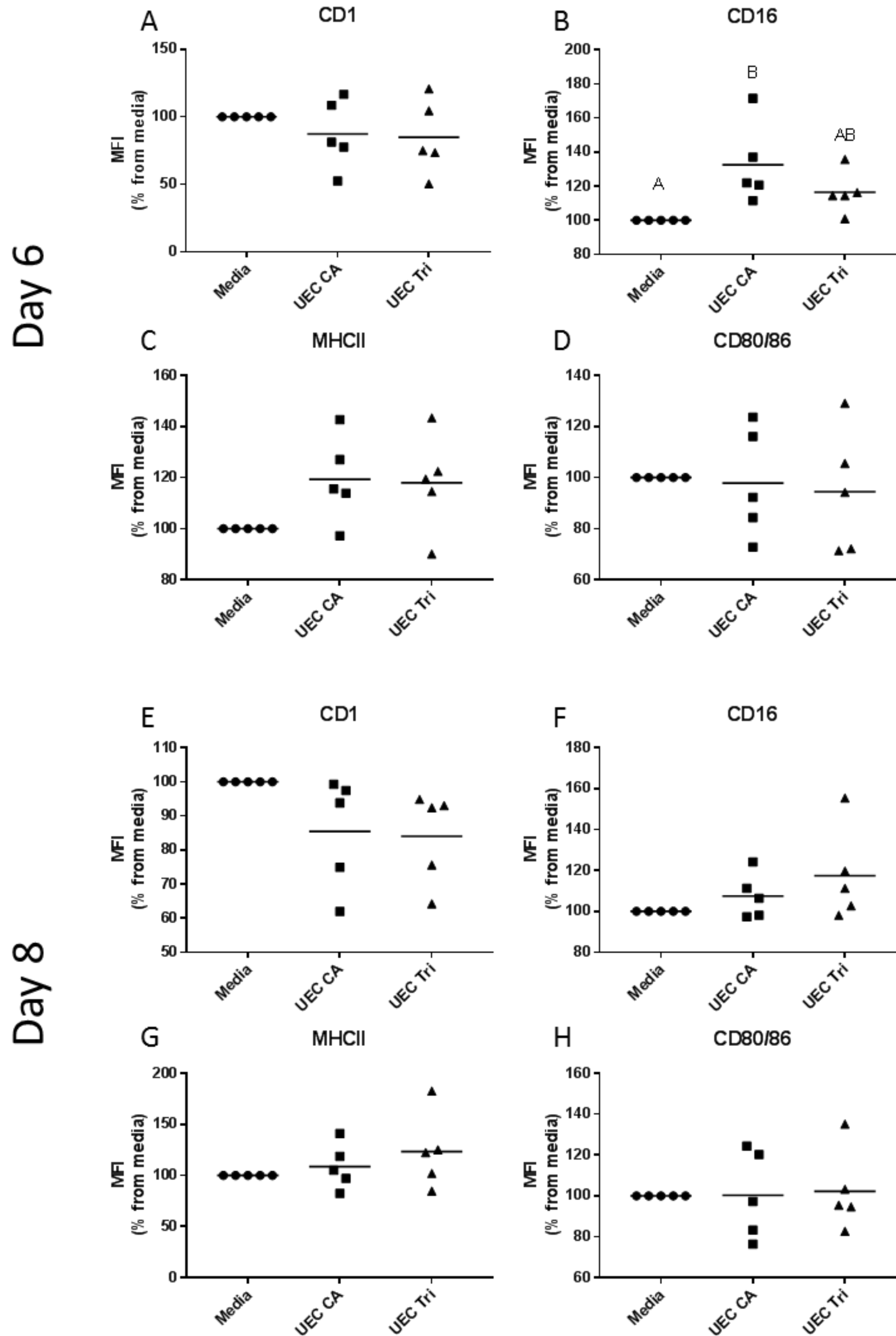


Figure 5-3. Expression levels of CD1, CD16, MHCII, and CD80/86 of monocyte-derived dendritic cells after 6 days (A, B, C, D) and 8 days (E, F, G, H) differentiation in media alone, unstimulated uterine epithelial cell (UEC CA) supernatant and triple adjuvant combination stimulated UECs (UEC Tri). Cells were collected, stained, and analyzed by flow cytometry, and protein levels were expressed as the median fluorescence intensity (MFI) collected across 100,000 events. Statistical analysis was carried out by ordinary one way ANOVA and differences between groups determined

by Dunnett's multiple comparison tests. Significantly different groups ($p<0.05$) represented by a differing letter. Each circle, square, or triangle indicates a unique biological replicate.

Next, we measured whether moDC differentiation in response to secretions from UEC CA or UEC Tri impacted maturation. moDCs were differentiated for 6 days in the conditions described above and were then stimulated for 48 hours in the presence of three distinct TLR ligands: 100 ng/ml LPS (Figure 5-4A), 25 µg/ml polyI:C (Figure 5-4B) or 500 ng/ml Pam3CSK4 (Figure 5-4C) or media to promote maturation (Figure 5-4A-C). Regardless of which maturation factor was used or how the moDCs were differentiated, there was no significant change in surface expression of CD1, CD16, or MHCII. All TLR ligands triggered increased expression of CD80/86 relative to differentiated moDCs treated with media alone however, there were no significant differences in the level of expression between the media, UEC CA and UEC Tri treatments (Figure 5-4A, B and C). Thus moDCs responded to maturation with all 3 TLR ligands in the same manner.

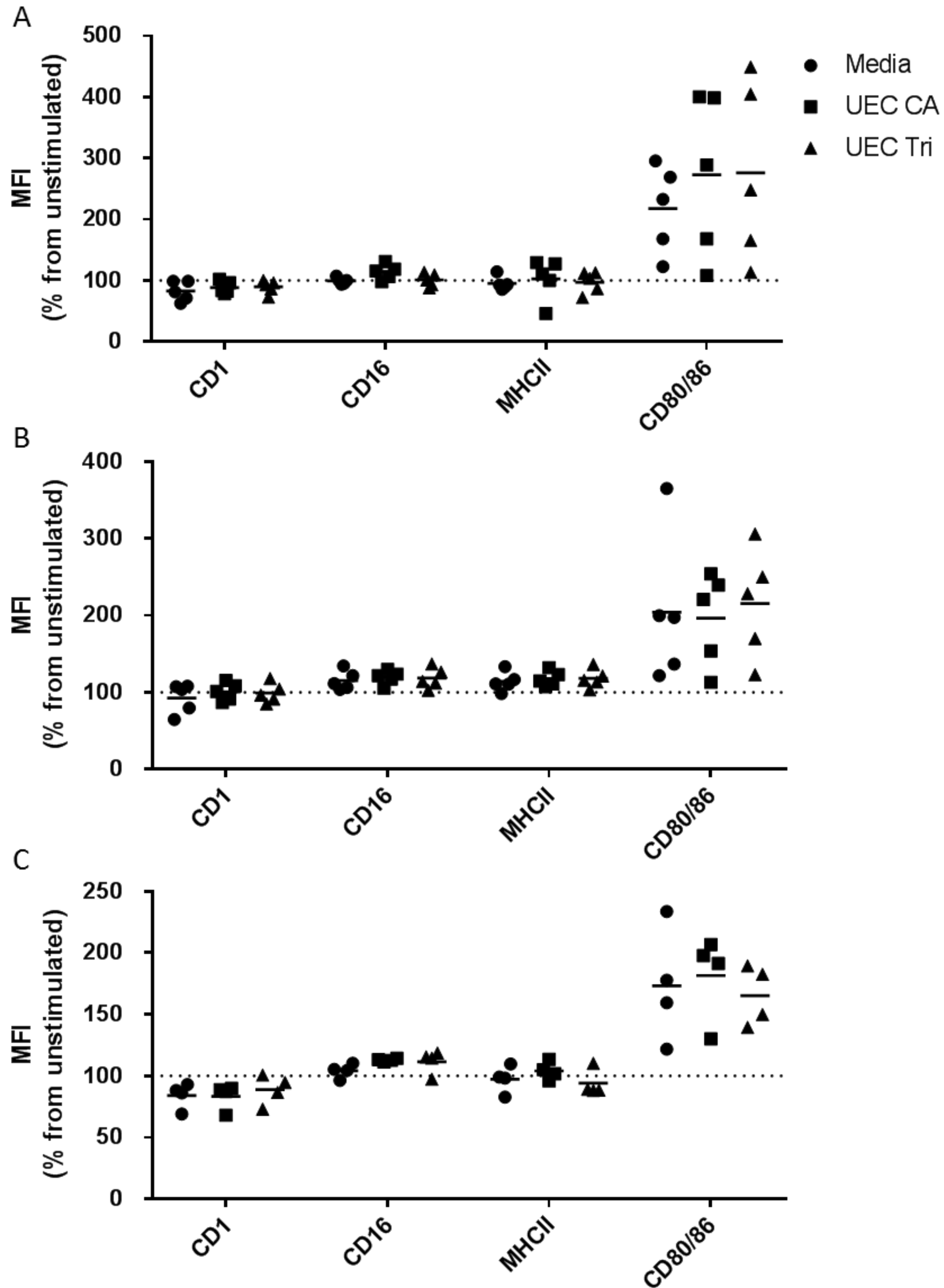


Figure 5-4. Effect of maturation on the expression levels of CD1, CD16, MHCII and CD80/86 by monocyte-derived dendritic cells (moDCs) that were differentiated in media, unstimulated uterine epithelial supernatant (UEC Media) and triple adjuvant combination stimulated UEC supernatant (UEC Tri). Maturation was induced in moDCs by LPS (A), polyI:C (B) or Pam3CSK4 (C) for 48 hours before staining and analysis by flow cytometry. Data are presented as the percent change in

median fluorescent intensity (MFI) when compared to each samples unstimulated control. Statistical analysis was carried out by ordinary one way ANOVA and differences between groups determined by Dunnett's multiple comparison tests. Each circle, square, or triangle indicates a unique biological replicate.

The endocytic activity of moDCs.

moDCs were differentiated as described above under the influence of media, UEC CA supernatants or UEC TriAdj supernatants for six days to promote differentiation. The endocytic activity of the moDCs was then evaluated to determine if the UEC supernatants had an impact on the moDCs ability to take up dextran. The percentage of moDCs which endocytosed FITC dextran showed the mean percentage of endocytic cells between 68-70% at 0.1 mg/ml FITC dextran and 88-94% at 1 mg/ml FITC dextran for moDC differentiated with media, UEC CA or UEC Tri (Figure 5-5A). We quantified the MFI to determine whether the amount of endocytosed FITC dextran was impacted by moDC differentiation. No significant difference in the amount of FITC dextran endocytosed was observed between any of the treatments with mean MFI at 0.1 mg/ml FITC dextran between 66-75 MFI and at 1 mg/ml FITC dextran between 173-196 MFI (Figure 5-5B). Differentiation of moDCs in the presence of unstimulated or stimulated UEC secretions did not appear to impact the cells ability to endocytose FITC dextran.

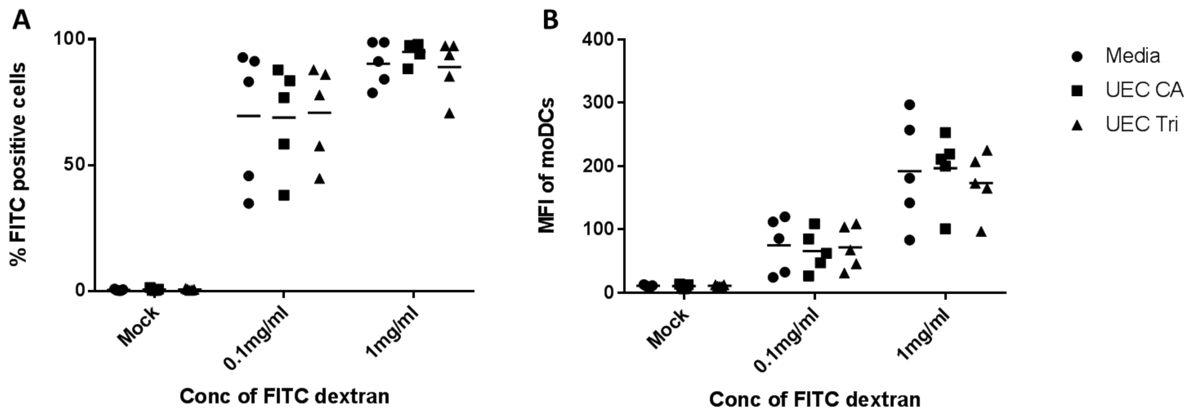


Figure 5-5. Endocytosis of FITC dextran by monocyte-derived dendritic cells (moDCs) when differentiated in differing conditions. moDCs were differentiated in media alone (Media), unstimulated uterine epithelial cells supernatant (UEC CA), and triple adjuvant combination stimulated uterine epithelial cells (UEC Tri). Once differentiated, moDCs were incubated in different concentrations of FITC dextran for 60 min at 37°C before being analyzed for the number of cells that took up FITC dextran (A) and the median fluorescence intensity (MFI) following FITC dextran endocytosis. Statistical analysis determined between differentiation conditions at each FITC dextran concentration by ordinary one way ANOVA and differences between groups determined by Dunnett's multiple comparison tests. Each circle, square, or triangle indicates a unique biological replicate.

The proliferation of T cells co-cultured with moDCs.

Continuing to determine if porcine UEC supernatants modulate moDC functionality, moDCs were subjected to the mentioned differing conditions, and they were then matured in the presence of Pam3CSK4, and co-cultured with T cells to determine their ability to induce proliferation in T cell populations. Mature moDCs differentiated without UEC secretions were able to induce T cell proliferation in a ratio dependent manner (1:5, 1:10 and 1:100 moDCs to T (data not shown)) and a 1:5 ratio of mature moDCs: T cells (Figure 5-6). Mature moDCs induced 1.35% T cell proliferation when differentiated with IL-4 and GM-CSF alone (media), 1.92% T cell proliferation when differentiated with IL-4 and GM-CSF plus secretions from UEC CA and 2.01% T cell proliferation when differentiated with IL-4 and GM-CSF plus secretions from UEC Tri, which were all statistically similar (Figure 5-6A). When the T cell subsets were investigated further, 63-66% of proliferating T cells in response to cells from all three moDC differentiating conditions were γδ T cells (Figure 5-6B). moDC differentiation in UEC supernatants did not appear to impact their ability to induce proliferation in T cells.

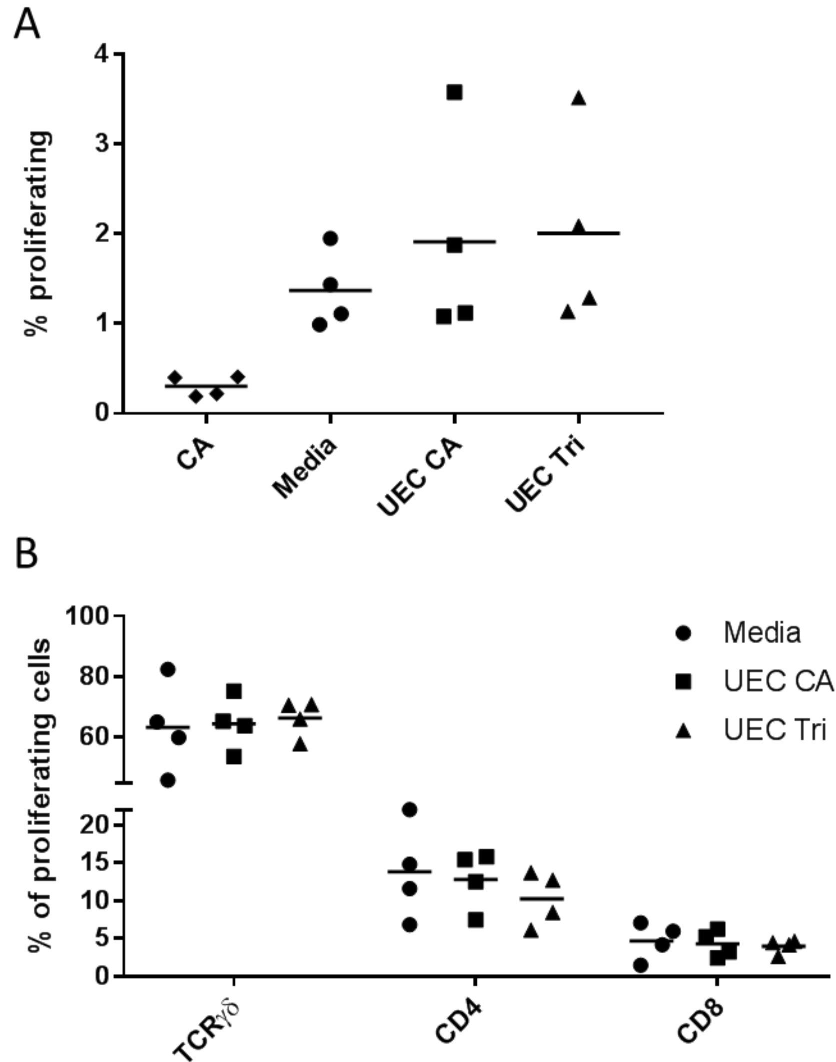


Figure 5-6. The proliferation of T cells in response to co-culture with Pam3CSK4 stimulated monocyte-derived dendritic cells (moDCs) differentiated in differing conditions. moDCs were differentiated in media alone (Media), unstimulated uterine epithelial cells supernatant (UEC CA), and triple adjuvant combination stimulated uterine epithelial cells (UEC Tri). Once differentiated, moDCs were matured with Pam3CSK4 for 24 hours before being plated on T cells at differing conditions and proliferation was measured by flow cytometry following four days of co-culture. Data presented show the results of 1:5 moDC to T cells, with statistical analysis between differentiation conditions determined by ordinary one way ANOVA and differences between groups determined by Dunnett's multiple comparisons test. Each circle, square, or triangle indicates a unique biological replicate.

5.4 Discussion

The upper reproductive tract requires a highly regulated immune system to be effective in generating a response to pathogens as they invade the uterus while avoiding generating an immune response to the semi-allogenic fetuses present during pregnancy. As the development of intrauterine vaccinations continues in pigs and other species, it is important to understand what role the uterine epithelia play in initiating the immune response. In humans, unstimulated UECs secretions significantly suppressed the maturation of moDCs through reduced induction of CD80, CD83, and CD86 expression (Ochiel et al., 2010). We previously showed that polarized pig UECs stimulated with TriAdj showed induced the expression of several cytokines and chemokines genes (Chapter 4) including CCL2, CCL3, and CCL4. Chemokines CCL2, CCL3, and CCL4 all induce monocyte recruitment through the chemokine receptors CCR2 (CCL2), CCR1, CCR4 and CCR5 (CCL3), CCR5 and CCR8 (CCL4) (Ondrackova et al., 2013). Interestingly, although it was previously shown that TriAdj induced a 4.3 fold increase in CCL2 gene expression, 3.33 fold increase in CCL3, and a 2.77 fold increase in CCL4 (Chapter 4), TriAdj stimulation of UECs showed no significant impact on the secretion of CCL2.

Neither secretions from unstimulated or TriAdj-stimulated UECs induced monocyte recruitment in our *in vitro* system, which may indicate a lack of functional response or that UEC production of these chemokines was too low to induce recruitment in our treatment. This result was partially confirmed by quantification of CCL2, which showed levels of the chemokine to be 100X below the level necessary to induce significant monocyte recruitment in our system. One component of the TriAdj platform, polyI:C, significantly increased gene expression of CCL2 in porcine UECs (Chapter 4), which was also observed in primary human UECs when protein secretion was quantified (Schaefer et al., 2005). A human UEC cell line (ECC-1 cells) stimulated

with polyI:C showed no significant increase in CCL2 protein secretion (Schaefer et al., 2004), as was observed in our study. Although we considered the possibility that the lack of secretion from UECs is an artifact of cell culture as is seen in the cell line, this is unlikely as the lack of monocyte recruitment corresponds with our previous study in which TriAdj did not significantly increase luminal CCL2 or increase the numbers of CD163^{+} cells in the endometrium (Chapter 4). The limitations of gene expression analysis are that although they typically correlate with protein production, they do not always correlate with protein expression levels (Liu et al., 2016b). There are several mechanisms which can impact the translation and secretion of CCL2, including stabilization of the mRNA through binding of the RNA-binding protein HuR (also referred to as ELAVL1). In human airway epithelial cells, HuR was found to be an important stabilizer of CCL2 mRNA promoting its translation (Fan et al., 2011) and although the gene is present in pigs, no studies have investigated its functionality in pigs. Additionally, once CCL2 is translated, it requires secretion, and that is further complicated as several proteins have been found to be important in the secretion of CCL2 (Deshmane et al., 2009). Due to the lack of study of these pathways in pigs, it remains to be determined if the blocking of CCL2 secretion occurs at the level of chemokine secretion or in the protein expression of the chemokine, however it does appear that there is some degree of post-transcriptional control of CCL2 in UECs. As the porcine immunological toolbox continues to expand, secretion of CCL3 and CCL4 should be quantified in addition to evaluating chemokine post-transcriptional control.

Due to their relatively low frequencies in blood and difficulty in isolating them from other tissues, it can be difficult to study DCs, *in vitro.* Thus, it is common to generate DCs from blood monocytes to acquire enough DCs to characterize their response to stimuli in an attempt to better understand DC response to stimuli *in vivo (Carrasco et al., 2001).* moDCs closely resemble blood

DCs in regards to DC cell surface markers (CD172, MHC class II, CD16, and CD80/86) (Facci et al., 2010). Although there are minor differences in the intensity of their responses, both cell types effectively induce proliferation of naïve or primed T cells (Facci et al., 2010; Summerfield and McCullough, 2009). Differentiation of monocytes to moDCs in the presence of unstimulated human UEC cell secretions resulted in moDCs with significantly reduced costimulatory molecule expression (CD83 and CD86) (Ochiel et al., 2010). Interestingly we show that pig UEC cell secretions had no impact on pig moDCs expression of CD80/86. moDC differentiation in the presence of unstimulated UEC cell secretions resulted in increased transient induction of CD16 expression at day six which was not evident in the moDCs differentiated in the presence of TriAdj stimulated UEC secretions.

Despite observing no significant impact on the differentiation of moDCs in response to UEC secretions from pigs, we wanted to validate that the functional responses of the moDCs to supernatants from UEC-stimulated cells would not be suppressive as observed in humans. Porcine moDCs express several TLRs including TLR2, TLR3 (receptor for polyI:C), and TLR4 (receptor for LPS). Stimulation of pig moDCs with ligands for these receptors promote their maturation and increased expression of the costimulatory molecules CD80/86 (Auray et al., 2010; Hartmann et al., 2016). We showed that moDCs differentiated in the presence of secretions from TriAdj-stimulated and unstimulated UECs matured in response to TLR ligands in a similar fashion, which suggest that porcine UEC secretions do not suppress maturation of moDCs. These results are in contrast to what was observed when human primary UEC secretions caused moDCs to express lower levels of the co-stimulatory molecules indicating suppression of DC maturation (Ochiel et al., 2010). Our results showed no difference in the maturation response between moDC differentiated in the presence of secretions from unstimulated or stimulated UEC. We previously

showed that stimulation of UECs with TriAdj induced gene expression of IFNβ (chapter 4) which is known to modulate moDC maturation and leads to increased CD86 expression (Remoli et al., 2007). Because we did not observe a change in the maturation of moDCs, it is likely that the cytokines produced and secreted by UECs are below the levels necessary to impact moDC function.

Formulation of a vaccine with TriAdj and administered to the rabbit uterus induced a strong systemic and mucosal immune response (Pasternak et al., 2017). In order for intrauterine immunization to be successful, the vaccine would need to either traverse the luminal UEC barrier or recruit local APCs into the uterine lumen (or promote the extension of DC dendrites through UEC wall) to endocytose the antigen. The APCs would then need to undergo maturation such as increased expression of MHC class II and costimulatory molecules CD80 and CD86) to begin their migration to the draining lymph node. Here, the mature DCs would induce proliferation of cognate T cells. Our study evaluated the impact of UEC secretions had on each of these stages and determined that unlike in humans where UECs were shown to suppress DC function, our study indicates porcine UECs do not appear to suppress moDC function. moDCs differentiated in either UEC secretions and stimulated with Pam3CSK4 were successful in inducing proliferation of naïve T cells to similar levels as has previously been observed (Liu et al., 2016a), confirming our observed normal moDC functionality. Although human UEC secretions have been shown to suppress DC function, stimulation of the human UECs with polyIC restored IL-12 secretion by DC and T cell co-cultures (Sathe and Reddy, 2014). Because there is no modulation of pig moDC function in response to secretions from stimulated or unstimulated UECs, we anticipate that in response to intrauterine immunization of pigs, local APCs in the endometrium will be primed to endocytose and present the delivered antigens.

As characterization of the upper reproductive tract in both swine and humans expands, notable differences in the functionality become clearer, which is not unexpected as there are notable differences in the reproductive physiology of the two species. One of the most significant differences in physiology is placentation in the two species as humans have a significantly more invasive hemochorial placenta as opposed to the epitheliochorial placenta of pigs (Moffett and Loke, 2006). Due to the more intimate interaction with maternal blood and thus immune cells in the human placenta, it is possible that the modulation of the immune response during pregnancy may be more significant in humans compared to the immune response observed in pigs. It should also be noted that our *in vitro* system is hormonally neutral and that these experiments do not take into consideration the potential impact of both high estrogen and progesterone conditions, which impacts the upper reproductive tract. Although the impact of these hormones has been studied to a lesser extent in pigs than in humans, it is clear that during estrous there is increased numbers of neutrophils and macrophages within the endometrium during proestrus and estrous (higher estrogen) and lymphocytes peak during early metestrus (higher P4) (Kaeoket et al., 2002a; Kaeoket et al., 2002b; Lorenzen et al., 2015). Although our data show that porcine UECs do not modulate moDCs functionality under hormonally neutral conditions, it would be valuable to evaluate whether exogenous hormones and/or primary UECs harvested under distinct stages of the estrus cycle impact moDC differentiation and functionality.

Despite several chemokines genes being induced in UECs stimulated with TriAdj, secretions from TriAdj-stimulated UECs do not promote significantly higher secretion of CCL2 or monocyte recruitment relative to unstimulated UECs. Porcine UEC secretions from TriAdj-stimulated or unstimulated UECs did not impact moDC differentiation, maturation, or functionality, including endocytosis or induction of T cell proliferation.

6 GENERAL DISCUSSION AND FUTURE DIRECTIONS

The innate immune responses of the porcine uterus have had limited study, with most research evaluating the immune response generated to breeding. Reproductive health in gilts and sows is incredibly important to the success of the commercial swine industry, and continued research into the responses in the uterus needs to be carried out to understand better how the uterus responds to infection and how it may respond to vaccination. Although UECs have been well characterized in other species, this thesis is the first extensive characterization of UECs and their role in the local immune system of the uterus.

It is widely recognized that *in vitro* experiments can reduce the number of animals and the associated ethical concerns and cost associated with animal experimentation. The epithelial cell *in vitro* models are particularly beneficial in studying infection, or drug delivery as they are the first contact point for anything attempting to cross or interacting with mucosal surfaces (Duell et al., 2011). Although there are limitations with *in vitro* models, they can be effective tools to evaluate epithelial responses to stimulation/infection or transport of materials across the epithelial barrier before moving forward into *in vivo* models. The primary limitation of *in vitro* models is that cells may not respond as they do *in vivo* due to the cells be in isolation, altered pH or hormone levels from the *in vivo* environment (Gamboa and Leong, 2013). Transwell systems have been thoroughly evaluated in gut epithelial studies and have demonstrated to better represent the phenotypes and characteristics of *in vitro* cultures (Hilgers et al., 1990). Results using our cultures system wherein primary UECs were polarized on transwell membranes, showed that they very closely resembled UECs *in vivo* both in the expression of tight junction proteins, localization of both tight junction proteins and TLRs, and maintenance of PRR gene expression. Also, responses of UECs observed both *in vivo* and *in vitro* were comparable, with the TriAdj stimulating chemokine expression.

Although we did observe differences in the number of genes observed in response to TriAdj stimulation *in vivo*, this can be partially accounted for by the lack of proper no-semen control to compare to in the *in vivo* samples. It is anticipated that the presence of spermatozoa will impact the pro-inflammatory response as was observed in a study that found spermatozoa suppressed the pro-inflammatory response generated to seminal plasma and the semen extender Androhep (Taylor et al., 2009). Unfortunately, a significant limitation of the *in vitro* model was the inability to include extended semen in UEC stimulations due to cytotoxicity issues and the inability to effectively remove spermatozoa prior to UEC cell collection for gene expression analysis. Regardless, primary polarized UECs maintained their epithelial phenotype when cultured on transwell membranes and are an effective *in vitro* model for studying the responses of UECs.

Epithelial cells are considered sentinels of the immune response, a notion that is supported by the expression of several PRRs and expression of TLRs in uterine epithelial cells is highly conserved across species. Both bovine, porcine, human, and murine primary UECs lack TLR8, and TLR10 expression, additionally primary human and murine UECs lack TLR7 expression (Davies et al., 2008b; Soboll et al., 2006b; Young et al., 2004b). Although TLR expression is relatively conserved, the responses to particular TLR ligands between species or between primary cells and cell lines can vary. This is notably observed comparing a mouse, and human primary UECs response to LPS where mouse cells induced CCL2 expression and human UECs suppressed CCL2 expression (Schaefer et al., 2004; Soboll et al., 2006b). When comparing the human UEC cell line ECC-1 and the primary UECs in response to stimulation with polyI:C, this TLR3 agonist did not affect the cell line, but it induced secretion of CCL2 in the primary UECs (Schaefer et al., 2004; Schaefer et al., 2005). Beyond these inherent inconsistencies between species and cell lines vs. primary cells, a limitation of these studies is that lack of biological response of these secretions

is difficult to attribute to the biological function or simply that the levels are below those necessary for biological impact. Our initial data in porcine UECs indicated they might play a role as sentinels of the immune response, however, following evaluation of their impact on cellular recruitment or modulation of APCs, the response of UECs to vaccine adjuvants may be too insignificant to modulate the immune response. Further *in vivo* studies should be carried out to determine if responses generated by UECs in response to TLR ligands or adjuvants have the capacity to modulate the immune response or induce cellular recruitment into the endometrium.

Based on our data and comparisons between studies in other species, it is likely that regulation of UEC responses to stimulation is mediated by both signalling cascades of the PRRs and post-transcriptional control on certain genes. TLR3 stimulation was the only examined PRR which demonstrated the classical activation with the induction of pro-inflammatory, type I IFNs and chemokines in response to its ligand. TLR3 is unique in that it is the only TLR in which the signalling cascade is always independent of the MyD88 adapter (Kawai and Akira, 2007), and this mechanism may indicate that the TLR3 signalling pathway through the TRIF adapter protein is not impacted in UECs. However, the other TLRs stimulated which often respond signalling through the MyD88 adapter displayed no pro-inflammatory immune response and instead displayed increased IL-10 in response to CpG (TLR9 signalling) or IL-13 in response to LPS (TLR4 signalling). Dysregulation in MyD88 dependent signalling may account for the lack of pro-inflammatory cytokine response from TLR4 and TLR9, as was observed in MyD88 deficient mice that lacked inflammatory cytokine expression in response to those ligands, however, these studies did not investigate any non-pro-inflammatory cytokines (Akira et al., 2000; Kawai and Akira, 2007). Additionally, TLR4 stimulation was shown to induce non-pro-inflammatory cytokines such as IL-13 through MyD88 signalling in mouse bone marrow-derived mast cells, however, this

response was dependent on leukotriene B4 receptor-2 (Lee et al., 2017). As we continue to study UECs and their impact in uterine immunity, it would be valuable to evaluate the signalling cascade of each response to understand how these cells may respond both to pathogens as well as stimulation from vaccine adjuvants. Further, control of the uterine epithelial cells could be mediated by post-transcriptional regulation as is observed in the regulation of CCL2 secretion in response to polyI:C. Lastly, as the porcine UECs displayed both relatively high levels of TLR3, RIG1, MDA5, and LGP2 in addition to the induction of both pro-inflammatory cytokines and IFNβ, they may be pre-disposed to play a role in the local anti-viral immune response.

The TriAdj formulation has been evaluated in several species, and several delivery methods with success, and in particular we have shown it to be successful in developing strong mucosal IgG responses when delivered via the intrauterine route in rabbits (Pasternak et al., 2017; Pasternak et al., 2018). Although the innate immune response generated towards the intrauterine vaccine was not, these studies confirm that intrauterine delivery of an antigen with TriAdj has the potential to generate a strong immune response. When we compare the gene expression data from *in vitro* stimulation of the UECs compared to murine macrophages, similar induction of chemokines and the pro-inflammatory cytokines TNF-α and IL-6 were observed, although they were induced to a greater magnitude (Sarkar et al., 2018). These studies indicate that the UECs may respond to the intrauterine vaccine formulated with TriAdj and that they may play a role in initiating the response. Greater scrutiny must be undertaken to determine a biologically significant response. Additionally, the large numbers of APCs present within the endometrium following breeding may negate the need for UECs to induce cellular recruitment and their role in the initial immune response to an intrauterine vaccine may be limited to transporting the vaccine across the epithelial barrier. UECs in other species such as humans may respond to a greater extent to the intrauterine vaccine as

polyI:C has been shown to induce increased secretion of CCL2 (Schaefer et al., 2005) and human UECs have demonstrated the ability to endocytose and present tetanus toxin to T cells (Wallace et al., 2001). As intrauterine vaccination is explored as a delivery route for mucosal vaccines in both pigs and other species, the impact of UECs to respond to stimulation and interact with other cells of the immune system may be species-dependent.

UECs have the capacity to respond to several vaccine adjuvants through the expression of their receptors. However, limited adjuvants will induce a significant pro-inflammatory cytokine and chemokine response desired by an intrauterine vaccine. Inclusion of TriAdj in an extended semen dose does lead to a significant drop in γδ T cells and monocytes following breeding. However, this did not result in a significant increase in either cell population with the uterine lumen. It remains to be determined whether adjuvants are necessary to include in an intrauterine vaccine when delivered with an extended semen dose, and adjuvants may be more important in vaccines delivered in the absence of extended semen. However, the use of primary UECs as an *in vitro* model to evaluate UECs responses to stimulants or pathogens is a valuable tool that thus far closely resembles responses generated *in vivo*.

Future Directions

Although it does not appear that the response generated in UECs is of a high enough magnitude to impact APC recruitment or activity, UECs are still required to transport an intrauterine vaccine across the epithelial barrier. It would be valuable to study the transport mechanisms utilized by the vaccine (pinocytosis, FcRN mediated transport, etc.) which could be evaluated in the *in vitro* polarized UEC model. In addition, as we observed a decrease in TEER following stimulation with the vaccine, it remains to be determined if the corresponding temporary loosening of the tight

junctions is sufficient to enable pericellular transport of the vaccine molecules between the UECs. Once the mechanism of transport has been evaluated, it would be valuable to determine the rate *in vivo* that vaccine molecules both cross the epithelial barrier and are transported by APCs to the draining lymph nodes. A concern with mucosal vaccines is the unknown dose of vaccine that required to be transported across the epithelial barrier and enter the body, this could be investigated in part by flushing the reproductive tract and measuring unabsorbed vaccine during the *in vivo* trials evaluating vaccine transport rates.

More thorough experiments evaluating the cell's response to both damage and infection may aid in determining if UECs (through their PRRSs) act as sentinels of the uterine immune response in the pig. In addition to evaluating the magnitude and direction (pro-inflammatory or anti-inflammatory) of UECs following active infection or damage, the response of UECs to different types of infection (viral, bacterial or fungal) could indicate potential adjuvant components that may be more effective for inclusion in an intrauterine vaccine. Studies focusing on the signalling cascades of activated TLRs in UECS and the post-transcriptional control on their expression may determine whether other components included in an intrauterine vaccine will amplify the response of UECs.

Our data indicate that only one component of the TriAdj, polyI:C, has any impact in the response of UECs to an intrauterine vaccine, while PCEP and HDP do not aid or hinder this response. As our data show high levels of immune cells present in the endometrium following breeding, *in vivo* vaccine trials should be carried out evaluating an intrauterine vaccine using extended semen as the sole adjuvant, as well as polyI:C compared to the TriAdj formulation. This will determine if the inclusion of polyI:C or the whole combination impact the ability of APCs to endocytose the vaccine and initiate a strong protective immune response to the vaccine. If extended

semen alone is a sufficient vaccine adjuvant in swine, the cost per dose of vaccine and any complications associated with formulating combinatorial vaccines could be avoided, resulting in a more cost-effective and simple to produce a vaccine.

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APPENDIX

A.1 Additional publications during my studies

Porcine retinal cell line VIDO R1 and Chlamydia suis to modelize ocular chlamydiosis.

T. Käser, T. Cnudde, G. Hamonic, M. Rieder, J.A. Pasternak, K. Lai, S.K. Tikoo, H.L. Wilson, F. Meurens

Published in Veterinary Immunology and Immunotherapeutic, 2015. Vol 166.

Human ocular Chlamydia trachomatis infections can lead to trachoma, the major cause of infectious blindness worldwide. Trachoma control strategies are very helpful but logistically challenging, and a trachoma vaccine is needed but not available. Pigs are a valuable large animal model for various immunological questions and could facilitate the study of human ocular chlamydial infections. In addition, a recent study identified the zoonotic potential of Chlamydia suis, the natural pathogen of pigs. In terms of the One Health Initiative, understanding the host–pathogen-interactions and finding a vaccine for porcine chlamydia infections would also benefit human health. Thus, we infected the porcine retinal cell line VIDO R1 with C. suis and analyzed the chlamydial life cycle and the innate immune response of the infected cells. Our results indicate that C. suis completes its life cycle in VIDO R1 cells within 48 h, comparable to C. trachomatis in humans. C. suis infection of VIDO R1 cells led to increased levels of various innate immune mediators like pathogen recognition receptors, cytokines and chemokines including IL6, TNFα, and MMP9, also most relevant in human C. trachomatis infections. These results illustrate the first steps in the host–pathogen-interactions of ocular C. suis infections in pigs and show their similarity to C. trachomatis infections in humans, justifying further testing of pigs as an animal model for human trachoma.

Extended semen for artificial insemination in swine as a potential transmission mechanism for infectious Chlamydia suis.

G. Hamonic, J.A. Pasternak, T. Käser, F. Meurens, H.L. Wilson

Published in Theriogenology, 2016. Vol 86.

Although typically unnoticed, Chlamydia infections in swine have been shown to be both widespread and may impact production characteristics and reproductive performance in swine. Serum titers suggest Chlamydia infection within boar studs is common, and infected boars are known to shed chlamydia in their ejaculates. Although the transmission of viruses in chilled extended semen (ES) is well established, the inclusion of antibiotics in commercially available extender is generally believed to limit or preclude the transmission of infectious bacteria. The objective of this study was to evaluate the potential of ES used in artificial insemination to support transmission of the obligate intracellular bacteria Chlamydia suis (C suis) under standard industry conditions. First, the effect of C suis on sperm quality during storage was assessed by flow cytometry. Only concentrations above 5 x 10^5 viable C suis/mL caused significant spermicidal effects which only became evident after 7 days of storage at 17 °C. No significant effect on acrosome reaction was observed using any chlamydial concentration. Next, an in vitro infection model of swine testicular fibroblast cells was established and used to evaluate the effect of chilled storage on C suis viability under variable conditions. Storage in Androhep ES reduced viability by 34.4% at a multiplicity of infection of 1.25, an effect which increased to 53.3% when the multiplicity of infection decreased to 0.1. Interestingly, storage in semen extender alone (SE) or ES with additional antibiotics had no effect on bacterial viability. To rule out a secondary effect on extender resulting from metabolically active sperm, C suis was stored in fresh and expended SE and again no significant effect on bacterial viability was observed. Fluorescent microscopy of C suis in ES shows an association between bacteria and the remaining gel fraction after storage suggesting that the apparent reduction of bacterial viability in the presence of semen is due to adherence to gel fraction. Taken together, the results of this study suggest that C suis remains viable and infectious during chilled storage and is globally unaffected by antibiotics in extender. Thus, ES used in artificial insemination may act as a viable transmission mechanism for C suis in swine.

Flow Cytometry as an Improved Method for the Titration of Chlamydiaceae and Other Intracellular Bacteria

T. Kaeser, J.A. Pasternak, G. Hamonic, M. Rieder, K. Lai, M. Delgado-Ortega, V. Gerdts, F. Meurens.

Published in Cytometry Part A, 2016. Vol 89

Chlamydiaceae is a family of intracellular bacteria causing a range of diverse pathological outcomes. The most devastating human diseases are ocular infections with C. trachomatis leading to blindness and genital infections causing pelvic inflammatory disease with long-term sequelae including infertility and chronic pelvic pain. In order to enable the comparison of experiments between laboratories investigating host chlamydia interactions, the infectious titer has to be determined. Titer determination of chlamydia is most commonly performed via microscopy of host cells infected with a serial dilution of chlamydia. However, other methods including fluorescent ELISpot (Fluorospot) and DNA Chip Scanning Technology have also been proposed to enumerate chlamydia-infected cells. For viruses, flow cytometry has been suggested as a superior alternative to standard titration methods. In this study we compared the use of flow cytometry with microscopy and Fluorospot for the titration of C. suis as a representative of other intracellular bacteria. Titer determination via Fluorospot was unreliable, while titration via microscopy led to a linear read-out range of 16264 dilutions and moderate reproducibility with acceptable standard deviations within and between investigators. In contrast, flow cytometry had a vast linear read-out range of 1,024 dilutions and the lowest standard deviations given a basic training in these methods. In addition, flow cytometry was faster and material costs were lower compared to microscopy. Flow cytometry offers a fast, cheap, precise, and reproducible alternative for the titration of intracellular bacteria like C. suis.

Postnatal regulation of MAMDC4 in the porcine intestinal epithelium is influenced by bacterial colonization

J.A. Pasternak, G. Hamonic, A. Van Kessel, H.L. Wilson

Published in Physiological Reports, 2016. Vol 4.

The MAM domain-containing 4 (MAMDC4) protein is associated with the unique endocytotic mechanism observed in the intestine of mammals during the immediate postnatal period. Transcriptional expression of MAMDC4 was substantially upregulated at birth in both the piglet jejunum and ileum and its expression decreases after birth. The protein was found localized specifically to the apical region of the luminal epithelium, however, MAMDC4 protein expression was lost at day 10 and 15 in the jejunum and ileum, respectively, and was not associated with "fetal" enterocyte replacement. Although spatial variation in the subcellular localization of Claudin 1 (CLDN1) was noted at day 3, the loss of MAMDC4 at later stages of development did not appear to have any effect on the tight junction structure. Germ-free (GF) piglets and piglets whose gastrointestinal flora consists exclusively of Escherichia coli (EC) or Lactobacillus fermentum (LF) maintained MAMDC4 protein expression to 14 days of age in distal regions of the small intestine whereas those with conventionalized intestinal flora (CV) showed no MAMDC4 protein at this age. MAMDC4 protein expression was most pronounced in the LF and GF colonized piglets which showed staining in the epithelial cells at 75% and 95% of the length of the small intestine, respectively, which matched that of the newborn. In contrast, EC animals showed only a low abundance at these regions as well as a discontinuous staining pattern. Collectively these results suggest that maturation of MAMDC4 expression in the porcine epithelium occurs more rapidly than what is reported in previously studied rodent species. Furthermore, intestinal bacterial colonization is a major regulator of MAMDC4 in a manner specific to bacterial species and independent of enterocyte turnover.

Dual infections of CD163 expressing NPTr epithelial cells with influenza A virus and PRRSV

C. Provost, G. Hamonic, C.A. Gagnon, F. Meurens

Published in Verteinary Microbiology, 2017. Vol 207.

In the pig, respiratory co-infections involving various pathogens are far more frequent than single infections. Amongst respiratory viruses, swine influenza type A virus (swIAV) and porcine reproductive and respiratory syndrome virus (PRRSV) are frequently associated. Previously, we performed co-infections with swIAV and PRRSV in porcine alveolar macrophages (PAM) and precision cut lung slices (PCLS). With these two approaches it was practically impossible to have co-infections of the same cells as the main target cell of swIAV is the epithelial cell while the main target of PRRSV is the PAM. This constraint makes the study of interference between the two viruses difficult at the cellular level. In the current report, an epithelial cell line expressing, CD163, the main receptor of PRRSV was generated. This cell line receptive for both viruses was used to assess the interference between the two viruses. Results showed that swIAV as well as PRRSV, even if they interacted differently with the modified epithelial cells, were clearly interfering with each other regarding their replication when they infected a same cell with consequences within the cellular antiviral response. Our modified cell line, receptive to both viruses, can be used as a tool to assess interference between swIAV and PRRSV in a same cell as it probably happens in the porcine host.

Intrauterine delivery of subunit vaccines induces a systemic and mucosal immune response in rabbits

J.A. Pasternak, G. Hamonic, N.M. Forsberg, C.L. Wheler, M.K. Dyck, H.L. Wilson

Published in the American Journal of Reroductive Immunology, 2017. Vol 78.

Problem: Mucosal vaccines have long been sought after to improve protection though the production of both a mucosal and systemic immune response, and are thought to be particularly effective at the site of induction. Development of such vaccines has, however, been delayed by the general propensity to develop immune tolerance to antigens encountered at mucosal sites. This study aimed to determine whether an appropriately formulated subunit vaccine delivered to the uterine lumen would effectively trigger induction of immunity over tolerance.

Methods: Ovalbumin (OVA), truncated glycoprotein D (tGD) from bovine herpesvirus, and a fusion protein of porcine parvovirus VP2 and bacterial thioredoxin (rVP2-TrX) were each formulated with a tri-adjuvant combination of Poly(I : C) (PIC), a host defense peptide (HDP), and a polyphosphazene (PCEP). A single dose of vaccine was delivered either intramuscularly (IM) or into the uterine lumen of intact female rabbits, and the humoral response subsequently evaluated both systemically and at local and distal mucosal sites.

Results: Vaccination through either route-induced antigen-specific humoral responses systemically and within the local (uterus) and distal mucosa (lungs and vagina). The observed mucosal response was not compartmentalized to, or within, the upper genital tract and the degree of response appeared to be at least in part antigen dependant.

Conclusion: The results of this study provide proof of principle that the uterus can be used as an induction site for subunit vaccination and that vaccine formulation with appropriate adjuvants can trigger both systemic and mucosal immunity when administered IM or into the uterine lumen.

Chlamydia suis and Chlamydia trachomatis induce multifunctional CD4 T cells in pigs

T. Käser, J.A. Pasternak, M. Delgado-Ortega, G. Hamonic, K. Lai, J. Erickson, S. Walker, J.R. Dillon, V. Gerdts, F. Meurens.

Published in Vaccine, 2017. Vol. 35.

Chlamydia trachomatis infections are the most prominent bacterial sexually-transmitted disease worldwide and a lot of effort is put into the development of an effective vaccine. Pigs have been shown to be a valuable animal model for C. trachomatis vaccine development. The aim of this study was to decipher the T-cell-mediated immune response to chlamydial infections including C. trachomatis and C. suis, the chlamydia species naturally infecting pigs with a demonstrated zoonotic potential. Vaginal infection of pigs with C. suis and C. trachomatis lasted from 3 to 21 days and intra-uterine infection was still present after 21 days in 3 out of 5 C. suis- and 4 out of 5 C. trachomatis-inoculated animals and caused severe pathological changes. Humoral immune responses including neutralizing antibodies were found predominantly in response to C. suis starting at 14 days post inoculation. The T-cell-mediated immune responses to C. trachomatis and C. suis-infections started at 7 days post inoculation and consisted mainly of CD4+ T cells which were either IFN-c single cytokine-producing or IFN-c/TNF-a double cytokine-producing Thelper 1 cells. IL-17-producing CD4+ T cells were rare or completely absent. The T-cell-mediated immune responses were triggered by both homologous or heterologous re-stimulation indicating that crossprotection between the two chlamydia species is possible. Thus, having access to a working genital C. suis and C. trachomatis infection model, efficient monitoring of the host-pathogen interactions, and being able to accurately assess the responses to infection makes the pig an excellent animal model for vaccine development which also could bridge the gap to the clinical phase for C. trachomatis vaccine research.

Recognizing conserved non-canonical localization patterns of toll-like receptors in tissues and across species

G. Hamonic, J.A. Pasternak, H.L. Wilson

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Toll-like receptors (TLR) 1, 2, 4, 5 and 6 were originally characterized as exclusively expressed on the cell surface and TLR 3, 7, 8 and 9 were said to be localized to the endosomes. However, continued research in this area shows that TLR localization may be altered across cell-types, and in response to stimulation, age or disease. Mucosal surfaces must remain tolerant to the commensal flora and thus intracellular or basal lateral localization of TLRs at mucosal surfaces may be necessary to prevent induction of an inflammatory response to commensal flora while still allowing the possibility for the receptors to prime an immune response when a pathogen has crossed the epithelial barrier. Here, we highlight the research specifying 'non-canonical' localization of TLRs in human and animal mucosal tissues and blood-derived cells,while excluding cultured polarized immortalized cells. Reports that only indicate TLR gene/protein expression and/or responsiveness to agonists have been excluded unless the report also indicates surface/ intracellular distribution in the cell. Understanding the tissue- and species-specific localization of these specific pattern recognition receptors will lead to a greater appreciation of the way in which TLR ligands promote innate immunity and influence the adaptive immune response. A more comprehensive understanding of this information will potentially aid in the exploitation of the therapeutic or adjuvant potential of selectively localized TLRs and in opening new perspectives in understanding the basis of immunity.

Intrauterine vaccination induces a dose-sensitive primary humoral response with limited evidence of recall potential

J.A. Pasternak, G. Hamonic, J. Van Kessel, C.L. Wheler, M.K. Dyck, H.L. Wilson

Published in the American journal of Reproductive Immunology, 2018. Vol. 80.

Problem: Induction of the local mucosal immune system within the reproductive tract is widely considered to be a key component in the development of effective prophylactic vaccines to control the spread of sexually transmitted infections. Here, we examine the capacity of the upper reproductive tract to act as a site of immune induction following.

Method of study: Two vaccines formulated with a triple adjuvant combination and either recombinant bovine herpesvirus (tgD) protein or ovalbumin (OVA) were delivered at varying doses to the uterine lumen of rabbits and the resulting immune response evaluated after 32 days.

Results: Intrauterine vaccination produced a dose-dependent induction of both antigen-specific IgG and IgA in serum. Both uterine and broncheoalveolar lavage of the high and medium-dose vaccine group contained a significant increase in both anti-OVA and anti-tgD IgG, but no significant quantities of antigen-specific IgA were observed. The restimulation of splenocytes from the high-dose vaccine group with ovalbumin (OVA) only resulted in a small but significant increase in gene expression of the Th1 cytokines (IL2/IFNγ) in the absence of an observable increase in proliferation.

Conclusion: Collectively, the results confirm the capacity of the uterine immune system to generate a primary response following stimulation.

Molecular and Physiological Effects on the Small Intestine ofWeaner Pigs Following Feeding with Deoxynivalenol-Contaminated Feed

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We intended to assess how exposure of piglets to deoxynivalenol (DON)-contaminated feed impacted their growth, immune response and gut development. Piglets were fed traditional Phase I, Phase II and Phase III diets with the control group receiving 0.20–0.40 ppm DON (referred to as the Control group) and treatment group receiving much higher level of DON-contaminated wheat (3.30–3.80 ppm; referred to as DON-contaminated group). Feeding a DON-contaminated diet had no impact on average daily feed intake (ADFI) ($p < 0.08$) or average daily gain (ADG) ($p > 0.10$) but it did significantly reduce body weight over time relative to the control piglets ($p < 0.05$). Cytokine analysis after initial exposure to the DON-contaminated feed did not result in significant differences in serum interleukin (IL) IL-1β, IL-8, IL-13, tumor necrosis factor (TNF)-α or interferon (IFN)-γ. After day 24, no obvious changes in jejunum or ileum gut morphology, histology or changes in gene expression for IL-1β, IL-6, IL-10, TNF-α, or Toll-like receptor (TLR)-4 genes. IL-8 showed a trend towards increased expression in the ileum in DON-fed piglets. A significant increase in gene expression for claudin (CLDN) 7 gene expression and a trend towards increased CLDN 2-expression was observed in the ileum in piglets fed the highly DON-contaminated wheat. Because CLDN localization was not negatively affected, we believe that it is unlikely that gut permeability was affected. Exposure to DON-contaminated feed did not significantly impact weaner piglet performance or gut physiology.

A.2 Oral presentations given during my studies

Hamonic G., Pasternak, JA., Wilson HL. (2018) Immune cell recruitment in response to vaccine adjuvants delivered by intrauterine vaccination in sows. 2019 meeting of the Canadian society for Immunology, Apr 12-15th. Banff, AB.

Hamonic G., Pasternak, JA., Wilson HL. (2018) Evaluating Immune cell recruitment in response to vaccine adjuvants delivered by intrauterine vaccination in sows. Conference of Research Workers in Animal Diseases, Dec 1-4th. Chicago, Illinois.

Hamonic, G., Pasternak, JA., Forsberg, N., Wilson, HL. (2017) Characterizing the innate immune response to vaccine adjuvants delivered by intrauterine vaccination in sows. Conference of Research Workers in Animal Diseases, Dec 2-5th. Chicago, Illinois.

Hamonic, G., Pasternak, JA., Forsberg, N., Wilson, HL. (2017) The initial immune response to vaccine adjuvants in the porcine uterus. Sask. Pork Symposium, Nov 14-15, Saskatoon, SK.

A.3 Scholarships and Fellowships received during my studies

NSERC Postgraduate Scholarship (2018-2020), Doctoral program. ($42,000)

Saskatchewan Innovation and Opportunity Scholarship (2017-2018), University of Saskatchewan ($20,000).

Devolved Graduate Scholarship (2016-2019), Department of Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan ($30,000).

Hope Haanen Memorial Scholarship (2016), Western College of Veterinary Medicine, University of Saskatchewan ($1,000).

Graduate Research Fellowship (2015-2016), College of Graduate Studies and Research, University of Saskatchewan, University of Saskatchewan ($8,000).