Veterinary Immunology and Immunopathology 202 (2018) 1–10

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Expression of pattern recognition receptors in porcine uterine epithelial cells *in vivo* and in culture

Glenn Hamonic[a,b], J. Alex Pasternak[c], Nikki M. Forsberg[a], Tobias Käser[d], Heather L. Wilson[a,b,*]

[a] Vaccine and Infectious Disease Organization (VIDO) – International Vaccine Centre (InterVac), University of Saskatchewan, Saskatoon, SK, S7N 5E3, Canada
[b] Veterinary Microbiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5B4, Canada
[c] Large Animal Clinical Sciences (LACS), Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, S7N 5B4, Canada
[d] Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC, 27607, USA

ARTICLE INFO

Keywords:
Epithelial
Toll-like receptor
Pattern recognition receptor
Innate immunity
Pig
Uterus

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 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 subcellular localization of TLR3, TLR4 and TLR9 in both uterine tissue and in 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.

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. As well as forming a physical barrier, these cells express several pattern recognition receptors (PRRs) (Mair et al., 2014) which, upon stimulation, can direct the immune response through secretions of cytokines and chemokines. While the porcine uterine epithelia has 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., 2005).

PRRs are expressed by numerous cell types in the body and they bind pathogen-associated molecular patterns (PAMPs) enabling detection of a wide array of pathogens. PRRs are categorized as Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-Type lectin receptors (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 poly(I:C) (TLR3 ligand), lipopolysaccharide (TLR4 ligand), flagella (TLR5 ligand) and unmethylated CpG DNA (TLR9 ligand)(Kawai and Akira, 2010). Broad spectrum PAMP recognition

* Corresponding author at: Vaccine and Infectious Disease Organization (VIDO) – International Vaccine Centre (InterVac), University of Saskatchewan, Saskatoon, SK, S7N 5E3, Canada.
E-mail addresses: glenn.hamonic@usask.ca (G. Hamonic), alex.pasternak@usask.ca (J.A. Pasternak), nmf854@mail.usask.ca (N.M. Forsberg), tekaeser@ncsu.edu (T. Käser), heather.wilson@usask.ca (H.L. Wilson).

https://doi.org/10.1016/j.vetimm.2018.06.006
Received 2 February 2018; Received in revised form 25 May 2018; Accepted 6 June 2018

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 non-canonical subcellular localization of TLRs indicating that there is cell and tissue dependant localization of these receptors (Hamonic et al., 2018; 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 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). RIG-I-like receptors 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., 2005). 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 we 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.

2. Methods

2.1. 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 h 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 analysing the follicles on the ovaries. Tissue was collected from the middle of the left uterine horn and flash frozen in dry ice within 20 min 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 min 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 tissue 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 × g for 30 min. Buffy coats were resuspended in PBSA, layered on Ficol-Paque plus (GE Life Sciences) and separated at 400 × g for 40 min. PBMCs were collected and washed 3× in PBSA with centrifugation at 250 × g for 10 min. Finally, pellets were collected in Trizol (Invitrogen) for RNA isolation and cDNA preparation (described below).

2.2. 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 cryo-sectioned 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 s and 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 Fig. 1). The laser-captured uterine epithelial cells (LC-UE) were collected within 45 min 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).

2.3. 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 PBSA 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 PBSA 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 PBSA 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 Fig. 1). The sloughed cells were centrifuged at 400 × g for 10 min 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 min at room temperature (RT) to lyse red blood cells and then washed twice in PBSA. 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