J Anim Reprod Biotechnol 2023;38:89-98
pISSN: 2671-4639 • eISSN: 2671-4663
https://doi.org/10.12750/JARB.38.3.89

JARB
Journal of Animal Reproduction and Biotechnology
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Original Article

Innate lymphoid cell markers: expression, localization, and regulation at the maternal-conceptus interface in pigs

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Received May 31, 2023
Revised July 3, 2023
Accepted July 4, 2023

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ABSTRACT
Background: The regulation of maternal immunity is critical for the establishment and maintenance of successful pregnancy. Among many cell types regulating the immune system, innate lymphoid cells (ILCs) are known to play an important role in innate immunity. Although some reports show that ILCs are present at the maternal-conceptus interface in humans and mice, the expression and function of ILCs in the endometrium have not been studied in pigs.
Methods: Thus, we determined the expression, localization, and regulation of ILC markers, *CD127* (a common marker for ILCs), *BCL11B* (a ILC2 marker), and *RORC* (a ILC3 marker) at the maternal-conceptus interface in pigs.
Results: The expression of *BCL11B* and *RORC*, but not *CD127*, in the endometrium changed during pregnancy in a stage-specific manner and the expression of *CD127*, *BCL11B*, and *RORC* was greatest on Day 15 during pregnancy. *CD127*, *BCL11B*, and *RORC* were also expressed in conceptus tissues during early pregnancy and in chorioallantoic tissues during the later stage of pregnancy. BCL11B and RORC proteins were localized to specific cells in endometrial stroma. The expression of *CD127* and *BCL11B*, but not *RORC*, was increased by the increasing doses of interferon-γ (IFNG) in endometrial explants.
Conclusions: These results suggest that ILCs present at the maternal-conceptus interface may play a role in the establishment and maintenance of pregnancy by regulating the innate immunity in pigs.

Keywords: endometrium, innate lymphoid cell, pig, pregnancy

INTRODUCTION

The appropriate immune reaction at the maternal-conceptus interface is essential to establish and maintain a successful pregnancy in mammals (Bazer and Johnson, 2014). The immune system is divided into the innate immune system and the adaptive immune system and the innate immune system first acts as a barrier to prevent pathogenic microorganisms or external immunogen (Mei et al., 2019). The innate immune system consists of epithelial cell barriers, various immune cells such as macrophages, natural killer (NK) cells, dendritic cells, innate lymphoid cells (ILCs), and soluble effectors, including cytokines, chemokines, complements, and antimicrobial peptides (AMPs) (Hoffmann and Akira, 2013). Epithelial cells function in either eradicating the pathogen

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independently or communicating with immune cells to orchestrate pathogen clearance (Sharma et al., 2020). Antigen presenting cells play a role in phagocytosis, processing, and delivering pathogens to cells involved in the adaptive immune system (Gaudino and Kumar, 2019). Cytokines, chemokines, and AMPs secreted by epithelial cells and various immune cells also act on removal of invading pathogens and communication with other immune cells (Gaudino and Kumar, 2019; Sharma et al., 2020). Likewise, these cells and effector molecules of the innate immune system present in the female reproductive tract, including the endometrium, play a critical role during the estrous cycle and pregnancy to maintain fertility by protecting the mother from infections (Mor and Cardenas, 2010).

Among many cell types in the innate immune system, ILCs are morphologically similar to B cells and T cells, but lack rearranged antigen receptors (Vivier et al., 2016; 2018). ILCs are present in lymphoid and non-lymphoid organs and particularly abundant at the mucosal tissue barriers, where they are exposed to allergens, commensal microbes, and pathogens (Vivier et al., 2016; 2018). ILCs have a similar functional diversity to T cells and are divided into five types: groups 1, 2, and 3 ILCs, NK cells, and lymphoid tissue inducer (LTi) cells (Vivier et al., 2016; 2018). Among these ILCs, group 1 ILCs (ILC1s) are stimulated by tumors and infection of viruses or intracellular bacteria and induce type 1 immunity by producing interferon (IFN)-γ (IFNG) and tumor necrosis factor-α but have no or weak cytotoxic effects (Vivier et al., 2016; 2018). Group 2 ILCs (ILC2s) are activated by large extracellular parasites, helminths, and allergens and induce type 2 immunity as well as tissue repair and homeostasis by secreting interleukin (IL)-4, IL-5, and IL-13 (Artis and Spits, 2015; Vivier et al., 2016; 2018). Group 3 ILCs (ILC3s) found mainly in mucosal tissues are stimulated by extracellular microbes such as bacteria and fungi and induce type 3 immunity by producing IL-17, IL-22, and granulocyte-macrophage colony stimulating factor (Vivier et al., 2016; 2018). ILCs in each group express specific cell markers compared to other immune cells in humans and mice (Guia and Narni-Mancinelli, 2020). CD127 (IL-7 receptor-α) is a common marker for ILC1s, ILC2s, and ILC3s, B-cell lymphoma/leukemia 11B (BCL11B) for ILC2s, and retinoic acid receptor-related orphan receptor (ROR)-γ (RORC), a transcription factor, for ILC3s (Guia and Narni-Mancinelli, 2020).

A variety of immune cells, including ILCs, are distributed in the uterus during the reproductive cycle and pregnancy to protect the uterine environment from microbial infections and tumor development (Monin et al., 2020). Uterine NK cells, a type of ILCs, play an important role in spiral artery remodeling during pregnancy in mice (Fraser et al., 2015). ILC1s and ILC2s in the uterus are barely detectable during the estrous cycle, and ILC1s increase during pregnancy compared to non-pregnancy state in mice (Doisne et al., 2015; Miller et al., 2018). In humans, ILC1s, ILC2s, and ILC3s are present in the endometrium during the menstrual cycle and pregnancy and function in neutrophil migration and cytokine secretion (Fraser et al., 2015; Croxatto et al., 2016; Miller et al., 2018). However, the exact localization and function of ILCs in the endometrium during the estrous cycle and pregnancy are not fully understood in any species. In pigs, there are some studies reported on localization of uterine NK cells (Dimova et al., 2008), but localization and function of other ILCs in the endometrium during the estrous cycle and pregnancy has not been studied so far. Therefore, to initiate our study of the role of ILCs in the endometrium during the estrous cycle and at the maternal-conceptus interface during pregnancy in pigs, we determined: 1) the expression of ILC markers in the endometrium during the estrous cycle and pregnancy and in early-stage conceptus and chorioallantoic tissues during pregnancy; 2) localization of ILC markers in the endometrium during the estrous cycle and pregnancy; and 3) the effect of IFNG on the expression of ILC markers in endometrial tissues.

MATERIALS AND METHODS

Animals and tissue preparation

All experimental procedures involving animals were conducted by the Guide for Care and Use of Research Animals in Teaching and Research and approved by the Institutional Animal Care and Use Committee of Yonsei University (No. YWC-P120). Sexually mature crossbred female gilts of similar age (6-8 months) and weight (100-120 kg) were assigned randomly to either cyclic or pregnant status. Gilts assigned to the pregnant uterus status group were artificially inseminated with fresh boar semen (Darby AI Center, Ansung, Korea) at the onset of estrus (Day 0) and 12 h later. The reproductive tracts of gilts were ob-

tained immediately after slaughter on either Days 12 or 15 of the estrous cycle and either Days 10, 12, 15, 30, 60, 90, or 114 of pregnancy (n = 3-6/day/status). Pregnancy was confirmed by the presence of apparently normal spherical to filamentous conceptuses in uterine flushings on Days 10, 12, and 15 and presence of embryos and placenta on the later days of pregnancy. Uterine flushings were obtained by introducing and recovering 25 mL phosphate buffered saline (PBS; pH 7.4) into each uterine horn. Chorioallantoic tissues were obtained from Days 30, 60, 90, and 114 of pregnancy (n = 3-4/day). Endometrial tissue, dissected free of myometrium, was collected from the middle portion of each uterine horn, snap-frozen in liquid nitrogen, and stored at -80℃ prior to RNA extraction. For immunohistochemistry, cross-sections of endometrium were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 h and then embedded in paraffin as previously described (Seo et al., 2008).

Endometrial explant cultures

To determine the effects of IFNG on the expression of *CD127*, *BCL11B*, and *RORC* mRNAs in the endometrium, endometrial explant tissues obtained from gilts on Day 12 of the estrous cycle were cultured as previously described (Jang et al., 2017). Endometrium was dissected from the myometrium and placed into warm phenol red-free Dulbecco's modified Eagle's medium/F-12 culture medium (DMEM/F-12; Sigma) containing penicillin G (100 IU/mL) and streptomycin (0.1 mg/mL). The endometrium was minced into small pieces using scalpel blades (2-3 mm^3), and aliquots of 500 mg were placed into T25 flasks with serum-free modified DMEM/F-12 containing 10 μg/mL insulin (Sigma), 10 ng/mL transferrin (Sigma), and 10 ng/mL hydrocortisone (Sigma). To assess the effect of IFNG, endometrial tissues obtained from gilts on Day 12 of the estrous cycle were cultured in the presence of progesterone (P4; 30 ng/mL), estradiol-17β (E2; 10 ng/mL), and IL-1β (IL1B; 10 ng/mL; Sigma) for 24 h with rocking in an atmosphere of 5% CO_2 in air at 37℃ and additional 24 h with 0, 1, 10, or 100 ng/mL of IFNG (R&D Systems) in the presence of 30 ng/mL P4, 10 ng/mL E2, and 10 ng/mL IL1B, as previously described (Cross and Roberts, 1989; Mirando et al., 1990; La Bonnardière et al., 1991). Explant tissues were then harvested, and total RNA was extracted for real-time RT-PCR to determine the expression of *CD127*, *BCL11B* and *RORC* mRNAs.

Total RNA extraction, reverse transcription–polymerase chain reaction (RT–PCR), and cloning of ILC marker cDNAs

Total RNA was extracted from endometrial, conceptus, and chorioallantoic tissues using TRIzol reagent (Invitrogen Life Technology, Carlsbad, CA, USA) according to the manufacturer's recommendations. The quantity of RNA was assessed spectrophotometrically, and integrity of RNA was validated following electrophoresis in 1% agarose gel. Four micrograms of total RNA from endometrial, conceptus, and chorioallantoic tissues were treated with DNase I (Promega, Madison, WI, USA) and reverse transcribed using SuperScript II Reverse Transcriptase (Invitrogen) to obtain cDNAs. The cDNA templates were then diluted 1:4 with sterile water and amplified by PCR using Taq polymerase (Takara Bio, Shiga, Japan). The final PCR reaction volume of 50 μL included 3 μL of cDNA, 5 μL of 10X PCR buffer, 4 μL of dNTP mix (2.5 mM), 1 μL of each primer (20 μM), and 0.3 μL Taq polymerase (Takara Bio), and 36.7 μL of ddH_2O. PCR conditions, sequences of primer pairs for ILC markers, and expected product sizes are listed in Table 1. PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining. The identity of each amplified PCR product was verified by sequence analysis after cloning into the pCRII vector (Invitrogen).

Quantitative real–time RT–PCR

Levels of expression of *CD127*, *BCL11B*, and *RORC* mRNAs in endometrial and chorioallantoic tissues were analyzed by real-time RT-PCR using the StepOnePlus System (Applied Biosystems, Foster City, CA, USA). Power SYBR Green PCR Master Mix (Applied Biosystems) was used for PCR reactions. The final reaction volume of 20 μL included 2 μL of cDNA, 10 μL of 2X Master mix, 2 μL of each primer (2 μM), and 4 μL of ddH_2O. PCR was performed with an initial incubation at 95℃ for 10 min, followed by 40 cycles of 15 sec at 95℃ and 30 sec at 60℃. The sequences of primer pairs are listed in Table 1. The results are reported as expression relative to that detected on Day 12 of the estrous cycle in endometrial tissues, on Day 30 of pregnancy in chorioallantoic tissues, or detected in control explant tissues after normalization of the transcript amount to the geometric mean of endogenous porcine ribosomal protein L7 (*RPL7*), ubiquitin B (*UBB*), and TATA box binding protein (*TBP*) controls by the $2^{-\Delta\Delta Ct}$ method as previously described (Livak and Schmittgen, 2001).

Table 1. Summary of primer sequences for RT-PCR and real-time RT-PCR and expected product sizes

Primer	Sequence of forward (F) and reverse (R) primers (5' → 3')	Annealing temperature (℃)	Product size (bp)	GenBank accession no.
CD127	F: TCCGCTGTCCCGATTTCTAC R: TTACTTTCCCTGCTGCCTCC	62	289	NM_001146128.2
BCL11B	F: TCAAGAACTGCAGCAACCTG R: GCTCGATTTTGACGTCGTTAG	60	255	NM_001297633.1
RORC	F: GAAGTGGTGCTGGTCAGGAT R: CGGGAGAAGTCAAAGATGGA	60	140	XM_013997125.2
RPL7	F: AAGCCAAGCACTATCACAAGGAATACA R: TGC ACACCTTTCTGACCTTTG	60	172	NM_001113217
UBB	F: GCATTGTTGGCGGTTTCG R: AGACGCTGTGAAGCCAATCA	60	81	NM_001105309.1
TBP	F: AACAGTTCAGTAGTTATGAGCCAGA R: AGATGTTCTCAAACGCTTCG	60	262	DQ845178.1

Immunohistochemistry

Immunohistochemistry was performed to determine the type of cells expressing BCL11B and RORC protein in the porcine endometrium. Uterine tissue sections (5 μm thick) were deparaffinized and rehydrated in an alcohol gradient. For BCL11B and RORC antigen retrieval, tissue sections were boiled in citrate buffer (pH 6.0) for 10 min. Tissue sections were washed with PBS with 0.1% (v/v) Tween-20 (PBST) and endogenous peroxidase activity was blocked with 0.5% (v/v) H_2O_2 in methanol for 30 min. Tissue sections were then blocked with 10% normal goat serum for 30 min at room temperature. Rabbit polyclonal anti-BCL11B antibody (5 μg/mL; OriGene Technologies, Rockville, MD, USA) and Rabbit polyclonal anti-RORC antibody (3 μg/mL; Aviva Systems Biology, San Diego, CA, USA) was added, and sections were incubated overnight at 4℃ in a humidified chamber. For each tissue tested, purified normal rabbit IgG was substituted for the primary antibody as a negative control. Tissue sections were washed intensively with PBST. Biotinylated goat anti-rabbit secondary antibody (1 μg/mL; Vector Laboratories, Burlingame, CA, USA) was added, and sections were incubated for 1 h at room temperature. Following washes with PBST, a streptavidin peroxidase conjugate (GBI Labs, Bothell, WA, USA) was added to the tissue sections, which were then incubated for 10 min at room temperature. The sections were washed with PBST, and aminoethyl carbazole substrate (Vector Laboratories) was added to the tissue sections, which were then incubated for 20 min at room temperature. The tissue sections were washed in water, counterstained with Mayer's hematoxylin, and coverslipped. Images were captured using an Eclipse TE2000-U microscope (Nikon, Seoul, Korea) and processed with Adobe Photoshop CS6 software (Adobe Systems, Seattle, WA, USA).

Statistical analysis

Data from real-time RT-PCR for *CD127*, *BCL11B*, and *RORC* expression were subjected to ANOVA using the General Linear Models procedures of SAS (Cary, NC, USA). As sources of variation, the model included day, pregnancy status (cyclic or pregnant, Days 12 and 15 post-estrus), and their interactions to evaluate steady-state levels of *CD127*, *BCL11B*, and *RORC* mRNAs. Data from real-time RT-PCR performed to assess the effects of day of pregnancy (Days 10, 12, 15, 30, 60, 90, and 114) in the endometrium, the effects of day of pregnancy in chorioallantoic tissues (Days 30, 60, 90, and 114), and the effect of IFNG doses in explant tissues on *CD127*, *BCL11B*, and *RORC* expression were analyzed by least squares regression analysis. Prior to the analysis, all data were tested for normality and homogeneity of variances, and log transformation was performed when necessary. Data are presented as mean with SEM. A p-value < 0.05 was considered significant, and p-values 0.05-0.10 were considered to indicate a trend toward significance.

RESULTS

Expression of ILC markers, *CD127*, *BCL11B*, and *RORC* mRNAs in the endometrium during the estrous cycle and pregnancy in pigs

To determine whether *CD127*, *BCL11B*, and *RORC* mRNAs were expressed in the endometrium during the estrous cycle and pregnancy in pigs, we measured their relative abundance in the endometrium during the estrous cycle and pregnancy using real-time RT-PCR analysis (Fig. 1). On Days 12 and 15 post-estrus, the expression of *CD127* and *RORC* was not affected by day, pregnancy status, or day x status interaction (Fig. 1A and 1C). The expression of *BCL11B* mRNA was affected by day ($p < 0.05$), pregnancy status ($p < 0.01$), and day x status interaction ($p < 0.05$), and the abundance of *BCL11B* expression was greater on Day 15 of pregnancy than on Day 15 of the estrous cycle (Fig. 1B). During pregnancy, the abundance of *BCL11B* and *RORC* mRNAs changed in the endometrium with the greatest abundance on Day 15 of pregnancy (linear effect of day; $p = 0.0719$ for *BCL11B*, $p < 0.05$ for *RORC*) (Fig. 1B and 1C).

Expression of ILC markers, *CD127*, *BCL11B*, and *RORC*, in conceptuses during early pregnancy and chorioallantoic tissues during later stage of pregnancy

We determined whether conceptuses during the peri-implantation period of pregnancy expressed *CD127*, *BCL11B*, and *RORC* mRNAs by RT-PCR using cDNAs from conceptuses from Days 12 and 15. The expression of *CD127*, *BCL11B*, and *RORC* mRNAs was detected in conceptus tissues on both days of pregnancy (Fig. 2A). We also performed real-time RT-PCR analysis to determine if the expression of *CD127*, *BCL11B*, and *RORC* changed in chorioallantoic tissues during Day 30 to term pregnancy. The expression of *CD127*, *BCL11B*, and *RORC* in chorioallantoic tissues during mid- to term pregnancy changed toward term pregnancy (linear effect of day, $p < 0.001$ for *CD127*; $p = 0.078$ for *BCL11B*; $p < 0.05$ for *RORC*) (Fig. 2B).

Localization of BLC11B and RORC protein in the endometrium during the estrous cycle and pregnancy in pigs

We determined whether cells expressing ILC markers, BCL11B and RORC, were present in the endometrium on Days 12 and 15 of the estrous cycle and pregnancy using immunohistochemistry (Fig. 3). BCL11B- and RORC-expressing cells were primarily localized to stroma in the endometrium during the estrous cycle and pregnancy, but cells expressing BCL11B and RORC were not abundant in the endometrium. BCL11B and RORC proteins were also detected in duodenum and small intestine, respectively, that was used as a positive control. Immunohistochemical analysis of CD127 could not be done due to the lack of available antibody targeting porcine CD127 protein.

Effects of IFNG on *CD127*, *BCL11B* and *RORC* mRNA expression in endometrial tissues

Because the expression of ILC marker mRNAs changed in the endometrium with the greatest abundance of *BCL11B* and *RORC* mRNAs on Day 15 of pregnancy, when

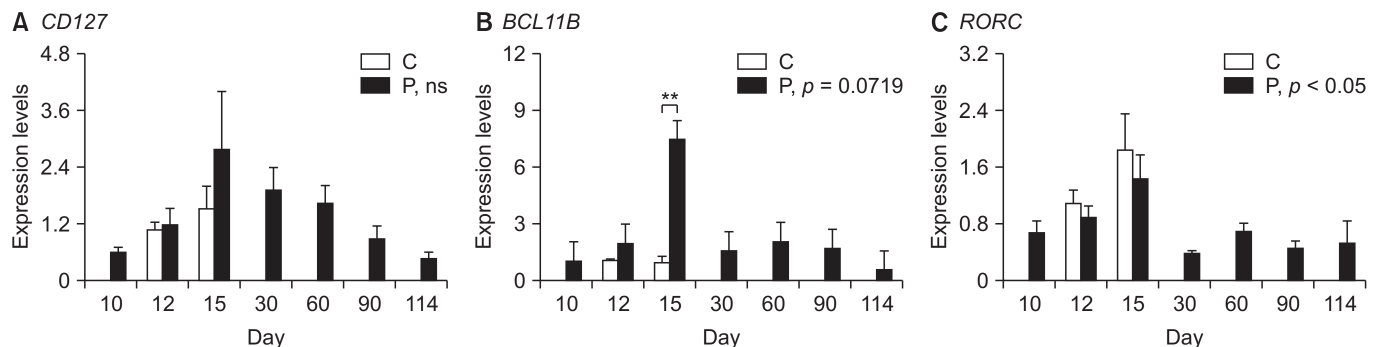

Fig. 1. Expression of *CD127* (A), *BCL11B* (B), and *RORC* (C) in the endometrium during the estrous cycle and pregnancy. Endometrial tissue samples from cyclic (Cy) and pregnant gilts (Px) were analyzed by real-time RT-PCR, and data are reported as expression relative to that detected on Day 12 of the estrous cycle after normalization of the transcript amount to the endogenous ribosomal protein L7 (*RPL7*), ubiquitin B (*UBB*), and TATA binding protein (*TBP*) controls. Data are presented as mean with standard error. ns, not significant. $^{**}p < 0.01$.

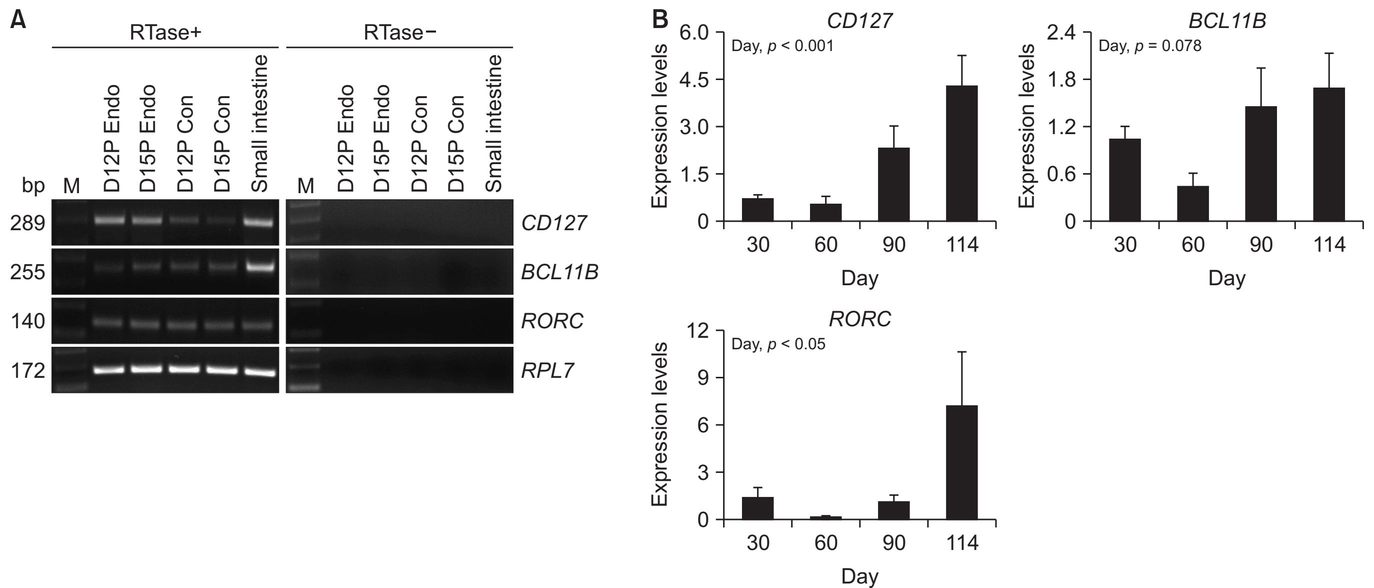

Fig. 2. Expression of *CD127*, *BCL11B* and *RORC* in conceptuses from Days 12 and 15 of pregnancy and in chorioallantoic tissues during mid- to late pregnancy. (A) RT-PCR analysis of ILC markers in conceptuses on Days 12 and 15 of pregnancy. *RPL7* was used as a positive control for PCR reaction. RTase +/-, with (+) or without (-) reverse transcriptase; M, molecular marker; D12 Endo, endometrium on Day 12 of pregnancy; D12 Con, Day 12 conceptus; D15 Con, Day 15 conceptus. (B) Real-time RT-PCR analysis of the expression of ILC markers in chorioallantoic tissues on Days 30, 60, 90, and 114 of pregnancy. Data are reported as expression relative to that detected on Day 30 of pregnancy after normalization of the transcript amount to the endogenous ribosomal protein L7 (*RPL7*), ubiquitin B (*UBB*), and TATA binding protein (*TBP*) controls, and data are presented as means with standard errors.

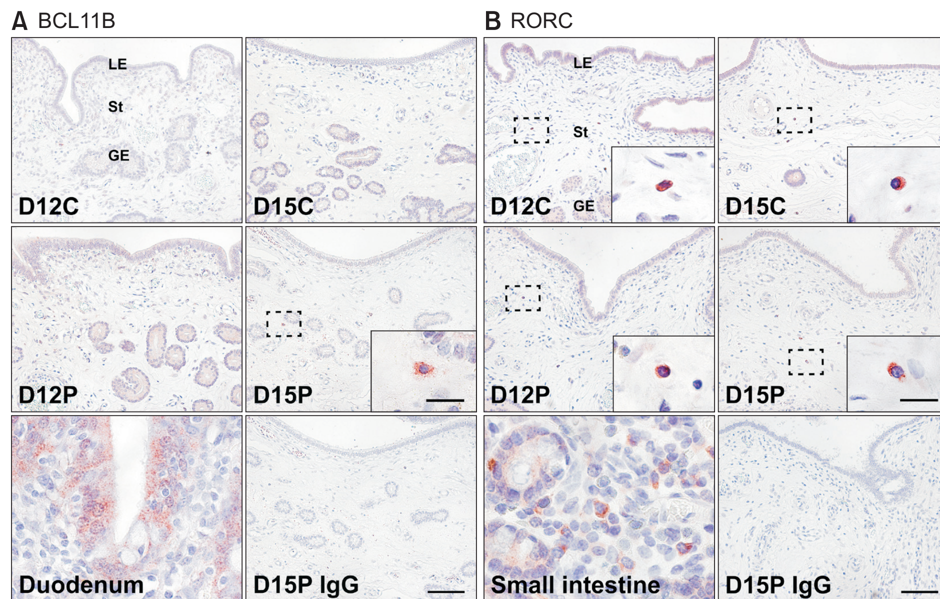

Fig. 3. Immunohistochemistry analysis of BCL11B (A) and RORC (B) proteins in the endometrium on Days 12 and 15 of the estrous cycle and pregnancy in pigs. Representative uterine sections from Day 15 of pregnancy immunostained with normal IgG (IgG) are shown as negative controls, and tissue sections from the duodenum for BCL11B and small intestine for RORC serve as a positive control, respectively. D, Day; C, estrous cycle; P, pregnancy; LE, luminal epithelium; GE, glandular epithelium. Bars = 100 μm, 20 μm in inset.

porcine conceptuses secrete IFNs, we hypothesized that IFNG may affect the expression of ILC markers in the endometrium during early pregnancy. Thus, we treated endometrial explant tissues from Day 12 of the estrous cycle with increasing doses of IFNG and determined the effect of IFNG on the expression of ILC markers. IFNG increased the expression of *CD127* and *BCL11B* mRNAs (linear effect of dose, $p < 0.05$ for *CD127* and *BCL11B*), but not *RORC* mRNA (Fig. 4).

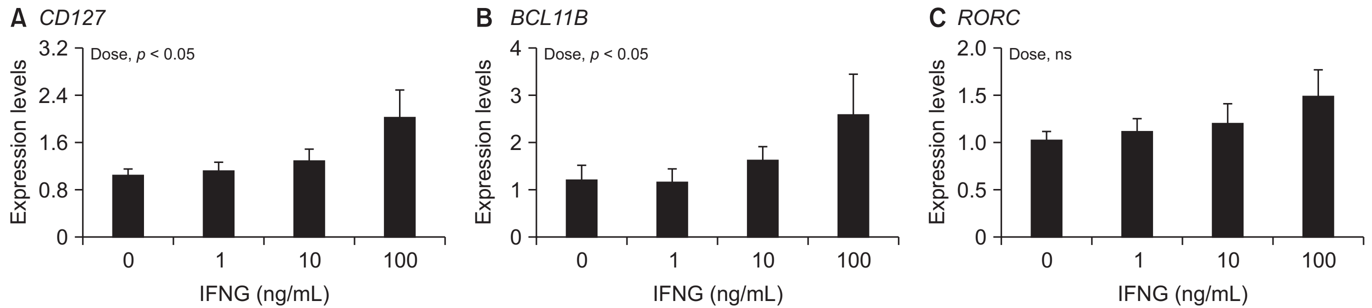

Fig. 4. Effects of IFNG on *CD127* (A), *BCL11B* (B), and *RORC* (C) in endometrial explant cultures. Endometrial explants from gilts on Day 12 of the estrous cycle were cultured with increasing doses of IFNG (0, 1, 10, and 100 ng/mL) in the presence of E2 (10 ng/mL), P4 (30 ng/mL), and IL1B (10 ng/mL). Abundance of mRNA expression based on real-time RT-PCR analyses is relative to that for ILC markers mRNA in the control group of endometrial explants after normalization of transcript amounts to ribosomal protein L7 (*RPL7*), ubiquitin B (*UBB*), and TATA binding protein (*TBP*) mRNAs. Data are presented as means with standard errors. These treatments were performed in triplicate using tissues obtained from each of three gilts. ns, not significant.

DISCUSSION

The novel findings of this study in pigs were that: 1) ILC markers, *CD127*, *BCL11B*, and *RORC*, were expressed in the endometrium during the estrous cycle and pregnancy; 2) the abundance of *BCL11B* and *RORC* expression in the endometrium during pregnancy was greatest on Day 15; 3) conceptuses on Days 12 and 15 of pregnancy and chorioallantoic tissues during mid- to late pregnancy expressed *CD127*, *BCL11B*, and *RORC*; 4) BCL11B and RORC proteins were mainly localized to specific stromal cells in the endometrium on Days 12 and 15 of the estrous cycle and pregnancy; and 5) IFNG increased the expression of *CD127* and *BCL11B* in endometrial explant tissues.

All groups of ILCs are present in the uterus and involved in the establishment and maintenance of pregnancy by regulating immune response against foreign antigens in mice and humans (Doisne et al., 2015; Fraser et al., 2015; Croxatto et al., 2016; Miller et al., 2018). ILC1s are found but rare in the decidua and in the endometrium and play a minor role in pregnancy and uterine immunity in humans (Mendes et al., 2020). ILC2s are the most common ILC subtype in the uterus with more population during pregnancy than non-pregnancy in humans and mice (Miller et al., 2018; Xu et al., 2018). ILC3s are also present at the maternal-conceptus interface during pregnancy in humans and mice (Miller et al., 2018). ILC3s increase in the decidua in women with preterm labor and the expression of IL-22, IL-17A, IL-13, and IFNG in the decidua is greater in ILC3s than ILC2s (Xu et al., 2018). In mice, the population of ILC3s in the uterus is greatest during early to mid-pregnancy and greater during pregnancy than the estrous cycle (Li et al., 2017). Results of this study in pigs showed that ILC markers were expressed in the endometrium during the estrous cycle and pregnancy. The expression of CD127, BCL11B, and RORC in the endometrium during the estrous cycle and pregnancy suggests that ILC1s, ILC2s, and ILC3s are present in the endometrium.

Although the characterization and function of other ILCs at the maternal-conceptus interface are still not fully understood, the localization and function of NK cells among several ILC subtypes has been relatively well studied in the endometrium during pregnancy in humans, mice, and pigs (Dimova et al., 2008; Santoni et al., 2008). The major function of uterine NK is to activate angiogenesis in the decidua and to regulate trophoblast invasion, but not to induce cytotoxicity in humans and mice (Wallace et al., 2014). In pigs, it has been shown that CD16+, CD56+, CD3-CD8+ NK cells are present in the endometrium during the estrous cycle and pregnancy, and the number of CD16+ NK cells decreases at the implantation sites during early pregnancy, suggesting the possibility of reduced cytolytic activity against the semi-allogenic fetus (Dimova et al., 2008). However, detailed function of uterine NK cells in pigs has not been studied.

Our results also show that BCL11B- and RORC-expressing cells were present in stroma of the endometrium on Days 12 and 15 of the estrous cycle and pregnancy, suggesting that ILC2s and ILC3s are present in stroma of the endometrium in pigs. However, the number of these cells in stroma was very low, although we did not measure the exact number of ILCs in the endometrium in this study. Indeed, there are a variety of immune cell types, including eosinophils, basophils, neutrophils, macrophages, NK

cells, B cells, and T cells, in the endometrium during the estrous cycle and pregnancy (Kaeoket et al., 2001; 2003; Croy et al., 2009; McLendon et al., 2020) and the number of T cells increases dramatically in the endometrium on Day 15 of pregnancy in pigs (Han et al., 2017). Results of this study showed that the ILCs are also present in the porcine endometrium. Nevertheless, further analysis is needed to analyze the distribution and changes in exact ILC numbers in the endometrium during the estrous cycle and at the maternal-conceptus interface during pregnancy. ILC markers were also expressed in early-stage conceptus and chorioallantoic tissues during mid- to late pregnancy in this study. Especially, the expression of ILC markers in chorioallantoic tissues increased toward term pregnancy with greatest levels on Day 114 of pregnancy. Although ILCs are found in fetal liver, lung, spleen, intestine, and amnion at the second and third trimester in humans (Miller et al., 2018), development of the innate immune system has not been fully determined in the implanting conceptus and fetal tissues during pregnancy in pigs and the ILC markers are also involved in many other cellular functions (Eberl and Littman, 2003; Holmes et al., 2021; Winer et al., 2022). Thus, one can speculate that the ILC markers expressed in the early-stage conceptus and chorioallantoic tissues may have different roles rather than mediating the innate immunity in porcine conceptus tissues.

Results of this study showed that the expression of *CD127*, *BCL11B*, and *RORC* in the endometrium during pregnancy was greatest on Day 15. In pigs, IFNG secreted by the conceptus at around Day 15 of pregnancy regulates the expression of endometrial genes necessary for the establishment and maintenance of pregnancy (Bazer and Johnson, 2014). Thus, we hypothesized that IFNG of conceptus origin might affect the endometrial expression of *CD127*, *BCL11B*, and *RORC*. Indeed, the results of this study showed that the expression of *CD127* and *BCL11B* were increased by IFNG in endometrial tissues, suggesting that the implanting conceptus affects the expression of ILC markers in the endometrium. The expression of *RORC* was not affected by IFNG in this study, although cytokines affect the expression of *RORC* through the nuclear factor of activated T-cell and nuclear factor-kB signaling pathways (Jetten et al., 2001; Yahia-Cherbal et al., 2019). Because the implanting porcine conceptus secretes IFND, and the maternal-conceptus interface during the implantation period is a rich source of cytokines and chemokines (Bazer and Johnson, 2014), it may be possible that other factors may affect the expression of *RORC* in the endometrium. A variety of effector molecules in the innate immune system, including cytokines, AMPs, β-defensins, and complements, are present in the endometrium during the estrous cycle and at the maternal-conceptus interface in pigs (Ka et al., 2018; Jang et al., 2022; Lee et al., 2023a; 2023b). The expression of these effector molecules is regulated by sex steroid hormones and/or conceptus-derived signals in the endometrium during the estrous cycle or pregnancy, indicating that sex steroid hormones or conceptus signals regulate the innate immunity in the endometrium during the estrous cycle and pregnancy. Together with the results in this study, conceptus signals may also affect the innate immunity by regulating the expression of effector molecules and ILC markers at the maternal-conceptus interface in pigs.

CONCLUSION

The innate immune system composed of various immune cells and effector molecules is the primary line of defense of immune response and a link to adaptive immunity (Palucka and Banchereau, 1999). Although several cell types of the innate immunity have been characterized in the endometrium during the estrous cycle and pregnancy in pigs (Kaeoket et al., 2001; 2003; Croy et al., 2009; McLendon et al., 2020), the presence of ILCs has not been studied so far. Results of this study showed that ILC markers, *CD127*, *BCL11B*, and *RORC*, were expressed in the endometrium during the estrous cycle and pregnancy, BCL11B and RORC proteins were localized to specific stromal cells in the endometrium, and IFNG of conceptus origin increased the expression of *CD127* and *BCL11B* in the endometrium. These results suggest that ILCs are present in the endometrium and play a role in regulating the innate immunity along with other effector molecules during the estrous cycle and pregnancy. Further studies are still needed to determine the detailed function of ILCs in the endometrium to establish and maintain pregnancy in pigs.

Author Contributions: Conceptualization, Y.C. and H.K.; methodology, Y.C. and H.K.; Investigation, Y.C., S.L., I.Y., and H.K.; data curation, Y.C. and H.K.; writing—original

draft preparation, Y.C.; writing—review and editing, Y.C., I.Y., S.L., and H.K.; supervision, H.K.; funding acquisition, H.K.

Funding: This work was supported by the National Research Foundation grant funded by the Korean Government (NRF-2019R1A2C1004670), Republic of Korea.

Ethical Approval: Institutional Animal Care and Use Committee of Yonsei University (No. YWC-P120).

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: Not applicable.

Acknowledgements: The authors thank all the members of the Animal Biotechnology Laboratory, Yonsei University, for their support and assistance throughout this project.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

REFERENCES

Artis D and Spits H. 2015. The biology of innate lymphoid cells. Nature 517:293-301.

Bazer FW and Johnson GA. 2014. Pig blastocyst-uterine interactions. Differentiation 87:52-65.

Cross JC and Roberts RM. 1989. Porcine conceptuses secrete an interferon during the preattachment period of early pregnancy. Biol. Reprod. 40:1109-1118.

Croxatto D, Micheletti A, Montaldo E, Orecchia P, Loiacono F, Canegallo F, Calzetti F, Fulcheri E, Munari E, Zamò A, Venturini PL, Moretta L, Cassatella MA, Mingari MC, Vacca P. 2016. Group 3 innate lymphoid cells regulate neutrophil migration and function in human decidua. Mucosal Immunol. 9:1372-1383.

Croy BA, Wessels J, Linton N, Tayade C. 2009. Comparison of immune cell recruitment and function in endometrium during development of epitheliochorial (pig) and hemochorial (mouse and human) placentas. Placenta 30 Suppl A:S26-S31.

Dimova T, Mihaylova A, Spassova P, Georgieva R. 2008. Superficial implantation in pigs is associated with decreased numbers and redistribution of endometrial NK-cell populations. Am. J. Reprod. Immunol. 59:359-369.

Doisne JM, Balmas E, Boulenouar S, Gaynor LM, Kieckbusch J, Gardner L, Hawkes DA, Barbara CF, Sharkey AM, Brady HJ, Brosens JJ, Moffett A, Colucci F. 2015. Composition, development, and function of uterine innate lymphoid cells. J. Immunol. 195:3937-3945.

Eberl G and Littman DR. 2003. The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. Immunol. Rev. 195:81-90.

Fraser R, Whitley GS, Thilaganathan B, Cartwright JE. 2015. Decidual natural killer cells regulate vessel stability: implications for impaired spiral artery remodelling. J. Reprod. Immunol. 110:54-60.

Gaudino SJ and Kumar P. 2019. Cross-talk between antigen presenting cells and T cells impacts intestinal homeostasis, bacterial infections, and tumorigenesis. Front. Immunol. 10: 360.

Guia S and Narni-Mancinelli E. 2020. Helper-like innate lymphoid cells in humans and mice. Trends Immunol. 41:436-452.

Han J, Gu MJ, Yoo I, Choi Y, Jang H, Kim M, Yun CH, Ka H. 2017. Analysis of cysteine-X-cysteine motif chemokine ligands 9, 10, and 11, their receptor CXCR3, and their possible role on the recruitment of immune cells at the maternal-conceptus interface in pigs. Biol. Reprod. 97:69-80.

Hoffmann J and Akira S. 2013. Innate immunity. Curr. Opin. Immunol. 25:1-3.

Holmes TD, Pandey RV, Helm EY, Schlums H, Han H, Campbell TM, Drashansky TT, Chiang S, Wu CY, Tao C, Shoukier M, Tolosa E, Von Hardenberg S, Sun M, Klemann C, Marsh RA, Lau CM, Lin Y, Sun JC, Månsson R, Cichocki F, Avram D, Bryceson YT. 2021. The transcription factor Bcl11b promotes both canonical and adaptive NK cell differentiation. Sci. Immunol. 6:eabc9801.

Jang H, Choi Y, Yoo I, Han J, Kim M, Ka H. 2017. Expression and regulation of prostaglandin transporters, ATP-binding cassette, subfamily C, member 1 and 9, and solute carrier organic anion transporter family, member 2A1 and 5A1 in the uterine endometrium during the estrous cycle and pregnancy in pigs. Asian-Australas. J. Anim. Sci. 30:643-652.

Jang H, Lee S, Yoo I, Choi Y, Han J, Cheon Y, Ka H. 2022. Calcium-binding proteins S100A8, S100A9, and S100A12: expression and regulation at the maternal-conceptus interface in pigs. Biol. Reprod. 106:1098-1111.

Jetten AM, Kurebayashi S, Ueda E. 2001. The ROR nuclear orphan receptor subfamily: critical regulators of multiple biological processes. Prog. Nucleic Acid Res. Mol. Biol. 69:205-247.

Kaeoket K, Persson E, Dalin AM. 2001. The sow endometrium at different stages of the oestrous cycle: studies on morphological changes and infiltration by cells of the immune system. Anim. Reprod. Sci. 65:95-114.

Kaeoket K, Persson E, Dalin AM. 2003. Influence of pre-ovulatory insemination and early pregnancy on the infiltration by cells of the immune system in the sow endometrium. Anim. Reprod. Sci. 75:55-71.

Ka H, Seo H, Choi Y, Yoo I, Han J. 2018. Endometrial response to conceptus-derived estrogen and interleukin-1β at the

time of implantation in pigs. J. Anim. Sci. Biotechnol. 9:44.

La Bonnardière C, Martinat-Botte F, Terqui M, Lefèvre F, Zouari K, Martal J, Bazer FW. 1991. Production of two species of interferon by Large White and Meishan pig conceptuses during the peri-attachment period. J. Reprod. Fertil. 91:469-478.

Lee S, Yoo I, Cheon Y, Hong M, Jeon BY, Ka H. 2023a. Antimicrobial peptides β-defensin family: expression and regulation in the endometrium during the estrous cycle and pregnancy in pigs. Dev. Comp. Immunol. 139:104596.

Lee S, Yoo I, Cheon Y, Ka H. 2023b. Conceptus-derived cytokines interleukin-1β and interferon-γ induce the expression of acute phase protein serum amyloid A3 in endometrial epithelia at the time of conceptus implantation in pigs. Anim. Biosci. 36:441-450.

Li M, Gao Y, Yong L, Huang D, Shen J, Liu M, Ren C, Hou X. 2017. Molecular signature and functional analysis of uterine ILCs in mouse pregnancy. J. Reprod. Immunol. 123:48-57.

Livak KJ and Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408.

McLendon BA, Seo H, Kramer AC, Burghardt RC, Bazer FW, Johnson GA. 2020. Pig conceptuses secrete interferon gamma to recruit T cells to the endometrium during the periimplantation period. Biol. Reprod. 103:1018-1029.

Mei C, Yang W, Wei X, Wu K, Huang D. 2019. The Unique microbiome and innate immunity during pregnancy. Front. Immunol. 10:2886.

Mendes J, Areia AL, Rodrigues-Santos P, Santos-Rosa M, Mota-Pinto A. 2020. Innate lymphoid cells in human pregnancy. Front. Immunol. 11:551707.

Miller D, Motomura K, Garcia-Flores V, Romero R, Gomez-Lopez N. 2018. Innate lymphoid cells in the maternal and fetal compartments. Front. Immunol. 9:2396.

Mirando MA, Harney JP, Beers S, Pontzer CH, Torres BA, Johnson HM, Bazer FW. 1990. Onset of secretion of proteins with antiviral activity by pig conceptuses. J. Reprod. Fertil. 88: 197-203.

Monin L, Whettlock EM, Male V. 2020. Immune responses in the human female reproductive tract. Immunology 160:106-115.

Mor G and Cardenas I. 2010. The immune system in pregnancy: a unique complexity. Am. J. Reprod. Immunol. 63:425-433.

Palucka K and Banchereau J. 1999. Linking innate and adaptive immunity. Nat. Med. 5:868-870.

Santoni A, Carlino C, Gismondi A. 2008. Uterine NK cell development, migration and function. Reprod. Biomed. Online 16:202-210.

Seo H, Kim M, Choi Y, Lee CK, Ka H. 2008. Analysis of lysophosphatidic acid (LPA) receptor and LPA-induced endometrial prostaglandin-endoperoxide synthase 2 expression in the porcine uterus. Endocrinology 149:6166-6175.

Sharma L, Feng J, Britto CJ, Dela Cruz CS. 2020. Mechanisms of epithelial immunity evasion by respiratory bacterial pathogens. Front. Immunol. 11:91.

Vivier E, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, Koyasu S, Locksley RM, McKenzie ANJ, Mebius RE, Powrie F, Spits H. 2018. Innate lymphoid cells: 10 years on. Cell 174:1054-1066.

Vivier E, van de Pavert SA, Cooper MD, Belz GT. 2016. The evolution of innate lymphoid cells. Nat. Immunol. 17:790-794.

Wallace AE, Fraser R, Gurung S, Goulwara SS, Whitley GS, Johnstone AP, Cartwright JE. 2014. Increased angiogenic factor secretion by decidual natural killer cells from pregnancies with high uterine artery resistance alters trophoblast function. Hum. Reprod. 29:652-660.

Winer H, Rodrigues GOL, Hixon JA, Aiello FB, Hsu TC, Wachter BT, Li W, Durum SK. 2022. IL-7: comprehensive review. Cytokine 160:156049.

Xu Y, Romero R, Miller D, Silva P, Panaitescu B, Theis KR, Arif A, Hassan SS, Gomez-Lopez N. 2018. Innate lymphoid cells at the human maternal-fetal interface in spontaneous preterm labor. Am. J. Reprod. Immunol. 79:e12820.

Yahia-Cherbal H, Rybczynska M, Lovecchio D, Stephen T, Lescale C, Placek K, Larghero J, Rogge L, Bianchi E. 2019. NFAT primes the human RORC locus for RORγt expression in CD4+ T cells. Nat. Commun. 10:4698. (Erratum published 2019, Nat. Commun. 10:5450)