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The Expression of IL-18Rα in Inferior Mesenteric Ganglion of Female Goat

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Authors' Contribution
YG, WW and YX conceived and designed the research. XG analyzed the data. YG, WW and HW performed the experiments. YG wrote the manuscript and YX revised it.

Key words
IL-18 receptor, IMG, Female goat, Reproductive, Immunity.

ABSTRACT

Immunity is the critical process by which organisms recognize and eliminate foreign antigens, and the process of local immunity in the female reproductive system is regulated by the coordinated integration of the nervous, immune and endocrine systems. The nerves that branch from the inferior mesenteric ganglion (IMG) are a major source of sympathetic nerves, which dominate the immune response of the female reproductive system. Interleukin-18 (IL-18) is an important Th1 cytokine and plays an important role in pregnancy. To explore whether or not Th1 cytokines influence the physiological state of the female reproductive system by sympathetic nerve pathways, we used immunohistochemistry, RT-PCR, Western blot and in situ hybridization to detect the expression and distribution of IL-18Rα in the inferior mesenteric ganglion of the female goat. Our results showed that IL-18Rα mRNA and proteins are expressed in the IMG, which provides molecular evidence for the study of immune and neuro-modulation in the female reproductive system.

INTRODUCTION

The uterus changes regularly in structure and physiology during pregnancy. These regular changes are influenced and regulated by the neuroendocrine-immune network. Local immunosuppression is necessary during the process of labor, and an inflammatory environment induces and improves labor performance (Bollapragada *et al*., 2009). Pregnancy, in fact, is one of the immune responses in which T or Th cells are confirmed to have immunosuppressive effects (Sakaguchi, 2000; de Lafaille *et al*., 2005; Zenclussen *et al*., 2005). In addition, IL-18 was found to be a key immunologic factor in the inflammatory reaction (Gracie *et al*., 2003; Lee *et al*., 2015), and its receptor IL-18R is characterized as one of the markers of Th1 immune type cells (Nakamura *et al*., 2000).

IL-18 is expressed in many cell types, including osteoplasts, microgliocytes, astrocytes, T cells, B cells, dendritic cells, macrophages and keratinocytes (Stoll *et al*., 1997; Prinz and Hanisch, 1999; Akira, 2000). IL-18 and IL-18 mRNA are also detected in the endometrium (Tsuji *et al*., 2001; Yoshino *et al*., 2001), and their expression levels are regulated by estrogen and progesterone during the gestation period (Murakami *et al*., 2005; Otsuki *et al*., 2007).

Recently, more and more attentions are paid to the effect of IL-18 on nervous system. Studies were found that IL-18 and it's receptor exist widely in central nervous system (CNS) (Andre *et al*., 2003), playing an important role in central nervous system inflammation, nervous system autoimmune disease, brain injury and so on (Hedtjarn *et al*., 2002; Yatsiv *et al*., 2002; Zwijnenburg *et al*., 2003; Kadhim *et al*., 2016). There were evidences demonstrated that IL-18R expressed in hypothalamus (Wheeler *et al*., 2000), hippocampus, striatum and cortex and in cultured astrocytes, microglia and neurons (Andre *et al*., 2003) by *in vitro* methods. Recently *in vivo* analysis also showed that IL-18R mRNA and protein are constitutively expressed in neurons throughout the brain (Otsuki *et al*., 2007; Andoh *et al*., 2008; Jeon *et al*., 2008; Alboni *et al*., 2009). However, there is no evidence that IL-18R expressed in peripheral nervous system.

Inferior mesenteric ganglion (IMG) is prevertebral ganglia, hypogastric nerve which is gave off by IMG is major source of sympathetic nerve that dominate uterus (Schofield, 1952; Owman, 1981). In addition, IMG is a critical integration neuro-center (Shi *et al*., 1995), the "viscera-ganglion-viscera" reflex center besides CNS, which regulate the immune response of uterus. The relationship between IMG and viscera is based on chemical substances (neurotransmitters, cytokines and so on) and the complex network they formed. IL-18 may be one of the cytokines connected the neuroendocrine-immune network

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between IMG, uterus and immune cells. However, until now there is no evidence that IL-18R expressed in peripheral nervous system. In order to investigate the putative effect of IL-18 on peripheral nervous system, we detected the expression and distribution of IL-18R in IMG in female goat by immunohistochemistry, western blot, PCR and in situ hybrization technology. Our results give molecular evidence for neuroendocrine-immune regulation in the microenvironment of uterus.

MATERIALS AND METHODS

RNA isolation, cDNA synthesis and PCR

The IGM of female goats (n=15) was dissected out and stored at -80 °C until use. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was treated with 10 U DNAse I (TaKaRa) for 15 min at 37°C to remove residual contaminating genomic DNA. cDNA templates for PCR amplification were synthesized from 2 μg of total RNA using a reverse transcriptase kit (Fermentas).

As the IL-18Rα gene sequences have not been deposited in the GenBank database, so we designed the primers according to the conserved region of human (GenBank Accession: BC143403), pig (GenBank Accession: AB079258) and Bos tarurs (GenBank Accession: XM_590497.4 and Accession: AB159085.1). The primers used for IL-18R amplification were: forward, 5'-AGACATGGTTGACATCCCAGGCCAC -3', and reverse, 5'- TCCACCAGTGCTTCATGGAGTCCAC -3'. Each PCR reaction contained 100 ng of cDNA template, 1μL Taq DNA polymerase buffer, 200 μM of each dNTP, 1 U of Taq DNA polymerase (ATGC), and 0.2 μM of specific primers, in a total volume of 50 μL. The conditions of amplification were 2 min at 94°C, followed by 35 cycles at 94°C for 30s, 58°C for 30s and 72°C for 30s. The PCR reactions would be expected to yield a product at 340 bp. Aliquots of the amplified products and 2000 bp DNA ladder were separated on a 1.2% agarose gel and visualized by Ethidium Bromide staining.

Sequencing of PCR products

To confirm the specificity of the RT-PCR amplified IL-18Rα, IL-18Rα products were purified from electrophoresis agarose gel using the DNA Gel Extraction Kit (BioTech). The purified IL-18Rα fragment was subcloned to the pGEM-T easy vector (Promega) and then sequenced.

Immunohistochemical analysis

IMG from female goats (n=15) was fixed by 4% paraformaldehyde and embedded by paraffin. Sections approximately 6μm thick of IMG tissue were then made by ultra-thin semiautomatic microtome (1900, Leika).

The sections were divided into two groups, one group was used for hematoxylin and eosin (HE) staining to confirming cell types; another group was used for immunohistochemistry staining using SP kit (MAIXIN). IL-18Rα immunodetection was performed using an indirect method (avidin-biotin-peroxidase complex method) following a standard protocol.

Heating induced epitope retrieval was conducted in citrate buffer (pH6.0) for 20 min. After cooling, endogenous peroxidase activity was blocked by incubating the slides in solution A for 10 min at room temperature. Slides were then incubated in solution B for 15 min at room temperature to reduce non-specific staining and then incubated with the anti-rabbit-IL-18Rα (SantaCruz) at 4°C overnight. After washing to remove excess primary antibodies, the slides were incubated with solution C (biotinylated goat-anti-rabbit IgG) for 20 min at room temperature. Washed in PBS, and then incubated in solution D (Streptomycin avidin–Peroxidase solution) for 20 min at room temperature. Diamino-benzidine (DAB)-peroxidase (ZHONGSHAN) was used as the colour developing reagent by 5-10 min room temperature incubation. Finally, the slides were dehydrated, cleared, and mounted. PBS instead of IL-18Rα was used as blank control.

Image analysis

Slides were examined by an OLYMPUS (Tokyo, Japan) CX41–32C02 microscope, and pictures were taken by an OLYMPUS DSE330 camera. Five visual fields were picked up randomly from every five slides. Relative expression (Relative expression = Microscope magnification × Positive area × Average optical density / Pixel) parameter was assessed by the high-definition image analysis system (Jie Da, Jiangsu).

Statistical analysis

All date were expressed as means ± standard error (SE). Comparisons of the result were performed using one-way ANOVA. All statistical analyses were done using the software SPSS18.0.

Western blot analysis

Samples of IGM were homogenized in an ice cold lysis buffer (10 mm Tris-Cl, pH 8.0; 150 mm NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 40 μM phenylmethylsulfonyl fluoride, and 1 μM leupeptin). This was followed by incubation for 30 min on ice and centrifugation at 10,000 g for 10 min at 4°C. The supernatant was transferred to new tubes, and stored at -80°C until the subsequent electrophoresis. Samples were denatured

by adding the denaturing buffer (62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.01% bromophe- nol blue), and then boiled for 5 min. Proteins were separated on 10% SDS-PAGE. Blots were incubated for 1 h at room temperature in 5% nonfat dry milk to block nonspecific binding, and then washed in TBST for 4-5 times (4-5 min every time). Blots were then incubated overnight at 4°C with the specific antibodies of IL-18Rα. After washing for five times (4-5 min every time) in TBST, membranes were incubated for 1 h with secondary antibodies, and finally washed three times for 15 min with TBST. Blots were then treated with enhanced chemiluminescence (Amersham Pharmacia) according to the manufacturer's instructions and exposed to x-ray film.

In situ *hybridization*

Samples of IGM was frozen on dry ice and stored at -80°C until use. IGM was fixed in 30% paraform for 1 h and precipitated with a solution of 15% sucrose. Serial cryosections (14 μm) were cut for the experiment of situ hybridization. The oligonucleotide complemented to the IL-18α which was sequenced was synthesized as the probe for situ hybridization (BOSTER, Wuhan). The probe sequence is 5'-ATTTTATAGA CATTTCATGGGAAGAGACGAAACCT-3'. The oligonucleotides were labeled with digoxin. Adjacent IGM sections were incubated in 30% H_2O_2 for 30 min at room temperature, and washed with distilled water for 3 times. Slides were then incubated in pepsin for 5-120 seconds at 37°C to exposure mRNA nucleic acid fragment and fixed in 1% paraform by immersion for 10 min. Remember that in every step the slides should be washed with ddH_2O for 3 times. Then, the slides were incubated for 2-4 h with the prehybridization solution and incubated with hybridization solution at 38-40°C overnight. The following day, sections were rinsed several times using SSC buffer (2×, 0.5× and 0.2×) at 37°C, and incubated in the confining liquid for 30min at 37°C. Then the slides were dropped in avidin–biotin-digoxin for 30 min at 37°C and SABC and biotin-peroxidase for 20 min at 37°C. In every step slides should be washed for several times with PBS. The slides were developed using a diamino- benzidine (DAB)-peroxidase substrate for 20-30 min at room temperature. Finally, the slides were, dehydrated, cleared, and mounted.

RESULTS

IL-18Rα immunoreactivity in the female goat IMG

HE staining in the IMG of female goats (Fig.1A) shows that there are a variety of cell types, including, but not limited to, neurons, endothelial cells and sertoli cells, with clearly distinguishable nuclei and nucleoli. The neurons are separated by connective tissue and surrounded by sertoli cells, with visible nuclei. The slices stained by the immunohistochemistry SP method show immunoreactive products as brown (Fig. 1B). The results were divided by degree of staining into strongly positive, positive and weakly positive. The background of the control staining groups was colorless or very light colored (Fig. 1C). The results of IL-18Rα stained by the SP method show brown immunoreactive products distributed in neurons, sertoli cells, endothelial cells, schwann cells and other cells (Fig. 1B), suggesting the very widespread distribution of IL-18Rα in the goat IMG.

Statistical analysis confirmed that there is a significant difference in expression levels between neurons and non-neurons in the female goat IMG (Table I).

Table I.- The expression of IL-18Rα in the female goat IMG.

Cell types	Dyeing strength	Relative expression
nerve cell	Brown	22.109±1.317[a]
non-neuronal structure	Light yellow	2.607±0.826[b]

a, means P-value ≤ 0.01; b, means P-value ≤ 0.05.

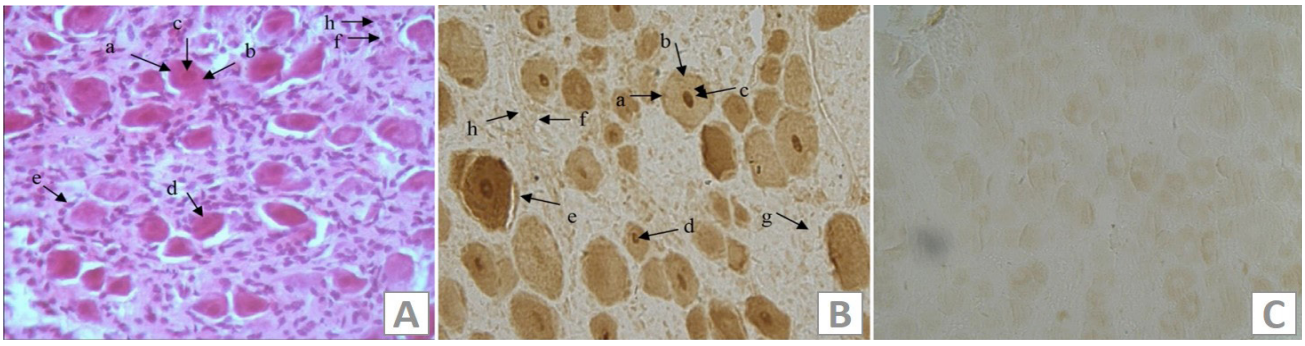

Fig. 1. Immunohistochemical analysis of IL-18 Rα in IMG. A, the HE stains of the IMG of female goat; B, the immunohistochemical stains of IL-18Rα in the IMG; C, the blank control. a, membrane of IMG neuron; b, cytoplasm of the IMG neuron; c, nucleus of the IMC neuron; d, nucleoli of the IMC neuron; e, sertoli cells; f, blood vessels; g, schwann cell; h, endothelial cell.

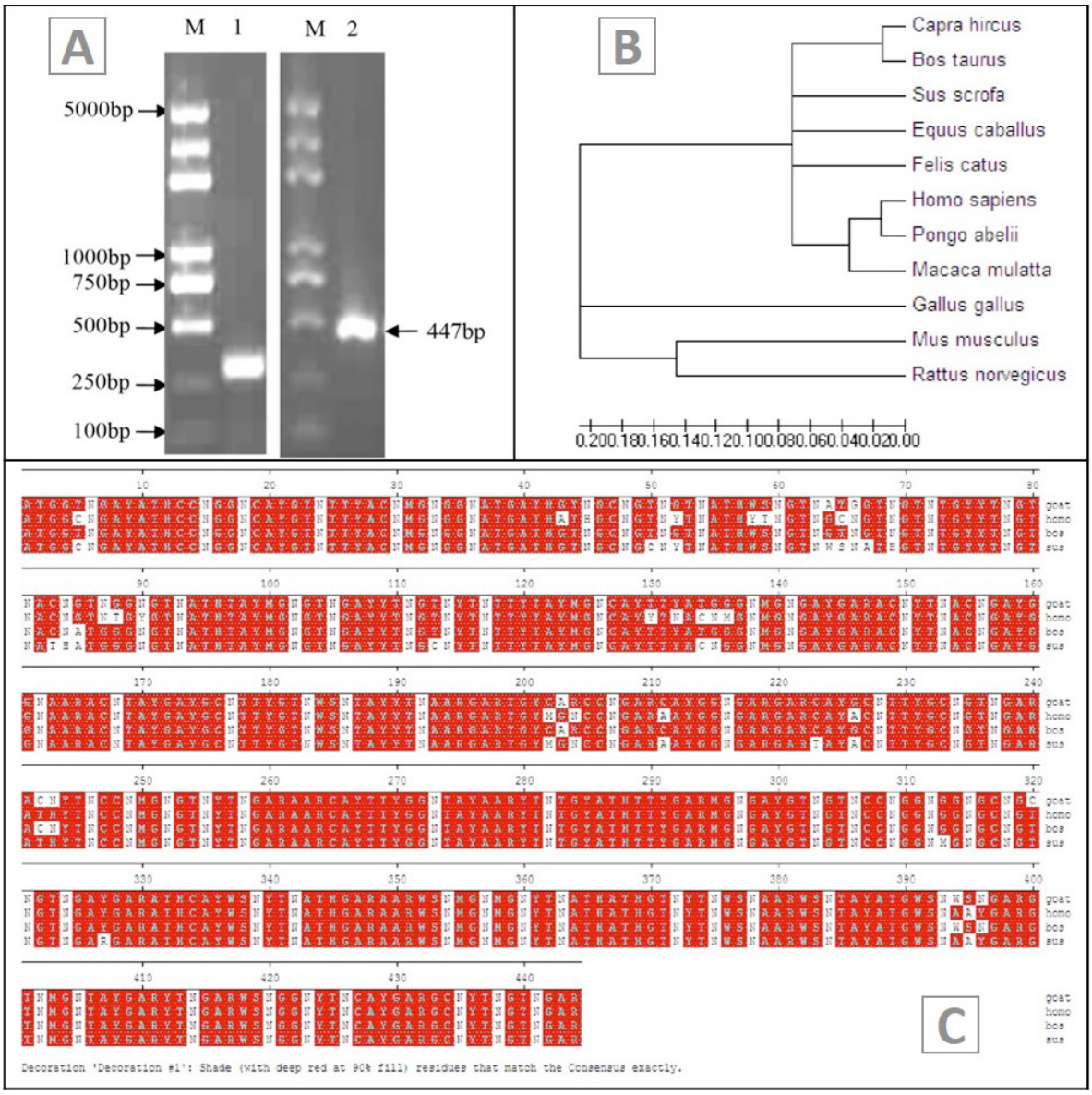

Fig. 2. PCR analysis of IL-18Rα in IMG. A, agarose gel electrophoresis analysis of the PCR product of female goat IL-18Rα; M, DNA marker DL5000; Line 1, β-actin; Line 2, PCR product of IL18Rα; B, phylogenetic analysis of IL-18Rα; C, the alignment of goat IL-18 Rα between *Homo sapiens*, *Sus scrofa* and *Bos taurus*. Goat IL-18 Rα is 91%, 88% and 87% homologous to *Bos taurus, Sus scrofa* and *Homo sapiens*, respectively.

IL-18Rα mRNA expression in the female goat IMG

The presence of IL-18Rα mRNA transcripts in the female goat IMG were demonstrated by RT–PCR (Fig. 2A). The PCR reactions yield a product near 500 bp, which is close to the target band (447 bp). The PCR product was confirmed by DNA sequencing (Fig. 2C). This study is the first to clone the gIL-18Rα gene, as the gIL-18 Rα mRNA has not been previously reported. Analysis by the NCBI Blast Program shows that the nucleotide sequences of the amplified fragments were 91%, 88%, and 87% homologous to *Bos taurus*, *Sus scrofa*, and *Homo sapiens*, respectively (Fig. 2C).

Phylogenetic analysis of IL-18Rα

The unrooted phylogenetic tree was made by using MEGA4 software (Fig. 2B). We discovered that the genetic relationship between *Capra hircus* and *Bos taurus* is closer than other relationships, and that the region of IL-18Rα is conserved in these species.

In situ *hybridization*

In situ hybridization analysis was performed using probes specific for IL-18Rα. The results show that strong specific hybridization signals are collected in the neurons and satellite cells, and that there are also weak hybridization signals between the positive cells. However, there are no positive products of IL-18Rα mRNA in the nucleolus (Fig. 3).

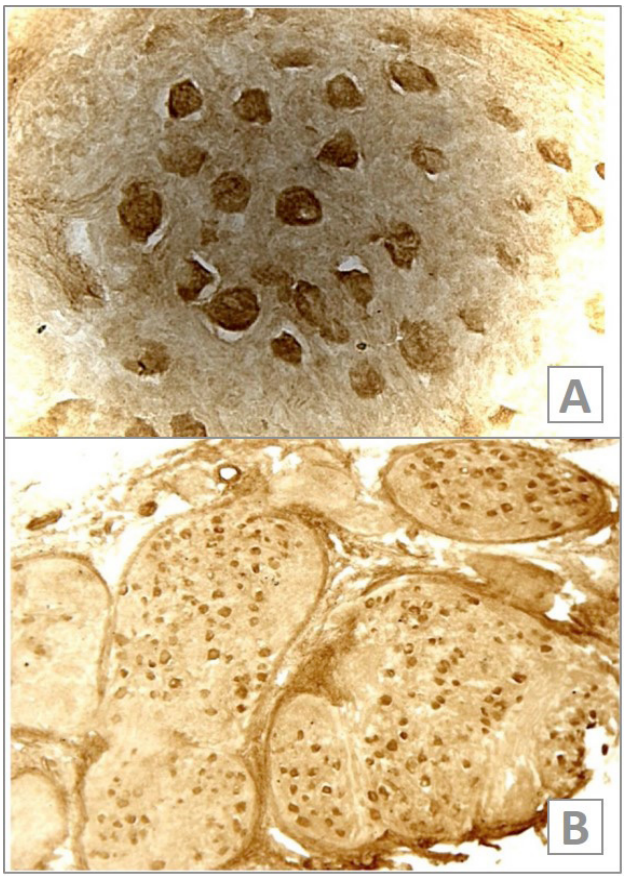

Fig. 3. Analysis of IL-18Rα mRNA expression in IMG by in situ hybridization. Representative hybridization signals in IMG are brown (A, ×400; B, ×100).

IL-18Rα protein expression in the female goat IMG

Western blot analysis demonstrates the presence of IL-18Rα protein in the female goat IMG (Fig. 4). The immunoreactive band expected for IL-18Rα (about 62 kDa) was detected in the female goat IMG, although the expression level is low.

DISCUSSION

In this study, we investigated the expression and distribution of IL-18Rα in the IMG by immunochemistry, RT-RCR, Western blot, and in situ hybridization. The results show that IL-18 Rα mRNA and protein are expressed in the IMG of female goat.

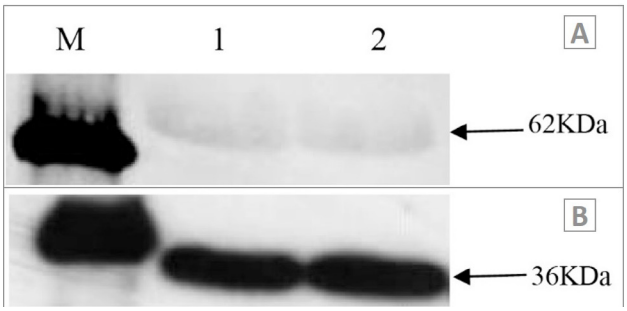

Fig. 4. Analysis of IL-18Rα expression in IMG of female goat by Western blot. A, there is one band expected for gPR (about 62 kDa) was detected in the female goat IMG; B, the expression of β-actin; M, protein marker; 1 and 2, two repeats.

An increasing number of experiments have demonstrated that the nervous system regulates the immune system through its widespread synapses, neurotransmitters, various endocrine hormones (Besedovsky *et al*., 1975; Martinez-Jaimes *et al*., 2016; Yu *et al*., 2016) and cytokines secreted by nerve cells. In conjunction, the immune system feeds back to the neuroendocrine system through various cytokines and hormone-like substances secreted by immune cells (Dunn and Berridge, 1990; Malagoli and Ottaviani, 2016). The cell surface of the two systems was confirmed to have corresponding receptors accepting information from the other side (Blalock and Costa, 1989; Ashley and Demas, 2017). This complex bidirectional effect between the two systems comprises the immune-neuroendocrine network and maintains the homeostasis of the body (Besedovsky *et al*., 1991; Ashley and Demas, 2017). There is evidence to show that neural activity influences the local immune functions of the uterus; and mast cells in uterine tissue were one of the critical working sites in the immune-neuroendocrine network. As pregnancy progresses, nerves in the uterus change correspondingly in structure and function. Is this change proactive by the nerve system itself, or is it a passive adaptation to the immune response of the uterus and reproductive hormones? What is the intrinsic relationship between typical cytokines and the nerve system in the immune response of the uterus?

During early pregnancy, the conceptus induces the expression of IL-18 in the endometrium to assist with development and connection to the placenta. The activity of IL-18 in the uterine cavity of estrogen treated piglets was shown to be reduced, which caused arrested

development of the conceptus and embryo implantation failure (Ashworth *et al*., 2010). Thus, the IL-18 level in uterine mucus may have a critical influence on embryo implantation and pregnancy. Nerves branching from the IMG are a major source of the sympathetic nerves that dominate the female reproductive system. In addition, IMG is a critical integration neurocenter, and the "viscera-ganglion-viscera" reflex center besides CNS. During the periodic changes of the uterus, the nerves in the uterus change correspondingly in structure and function (Klukovits *et al*., 2002). An injection of a tracer into the perioviductal area of the right uterine horn revealed tracer-positive neurons bilaterally in the IMG and the paracervical ganglia and single cells in the ipsilateral paravertebral ganglia (Wasowicz *et al*., 2002). In this study, we found IL-18Rα mRNA and proteins expressed in neuronal cytoplasm, SCGs and Schwann cells. This discovery is first demonstration that IL-18Rα exists in the peripheral nervous system, suggesting that IL-18 affects sympathetic postganglionic neurons directly, while affecting the neural-immune condition of the reproductive system in different physiological time.

In conclusion, IL-18 may be the critical cytokine that acts as a bridge between the immune-neuroendocrine networks of the reproductive system.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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