Characterization of the Th cytokines profile in ovine spleen during early pregnancy

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ABSTRACT
The spleen combines the innate and adaptive immune systems, and the early conceptus must regulate the maternal intrauterine immune and systemic immune response during early pregnancy in sheep. However, it is unclear whether early pregnancy exerts its effects on the characterization of the T helper (Th) cytokines profile in the spleen during early pregnancy in ewes. In this study, spleens were obtained at day 16 of the oestrous cycle and at days 13, 16 and 25 of pregnancy (n = 6 for each group) from ewes, and qRT-PCR, western blot and immunohistochemistry analysis were used to analyze the Th cytokines profile of the spleens. Our results showed that there was down-regulation of IFN-γ at days 13 and 16 of pregnancy, but there was upregulation of IL-2, IL-5 and IL-6 at day 25 of pregnancy. TNF-β at days 16 and 25 of pregnancy, and IL-4 and IL-10 in all pregnant groups. Immunohistochemistry results showed that the IL-6 protein was limited to the capsule, trabeculae and splenic cords. This paper reported, for the first time, that characterization of the Th cytokines profile varied in the maternal spleen during early pregnancy, which may be essential for successful pregnancy in sheep.

Introduction
During pregnancy, there is an absence of any maternal immune response against the foetus and placenta, which is also known as maternal immune tolerance (Williams 2012). The chemokines and cytokines produced by the conceptus result in a variety of maternal immunological adjustments against the conceptus in sheep and cattle (Hansen 2011; Yang et al. 2014), and there is a shift of T helper 1 (Th1) cytokines to Th2 cytokines in the maternal endometrium induced by the conceptus and progesterone (P4) in ruminants (Hansen 2011; Zhang et al. 2015). Th1 cytokines include interleukin (IL)-2, interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-β), which are involved in cell-mediated cytotoxicity and inflammatory responses, whereas Th2 cytokines are anti-inflammatory cytokines, including IL-4, IL-5, IL-6 and IL-10 (Ott and Gifford 2010). Th2 cytokines promote a humoral response and are generally beneficial to a normal pregnancy, whereas Th1 cytokines normally suppressed in pregnancy, and significantly higher concentrations of Th1 cytokines are produced in spontaneous abortion groups (Raghupathy et al. 2000; Sykes et al. 2012a). There are trends towards changes in the Th2 cytokine profile and suppression of the Th1 cytokine profile in the peripheral blood and the maternal-fetal interface (Sykes et al. 2012b). We also revealed that IFN-γ is down-regulated, and IL-4, IL-5, IL-6, IL-10 and IL-13 are upregulated in peripheral blood mononuclear cells (PBMCs) during early pregnancy in cattle (Yang et al. 2016). There were a downregulation of TNF-β and IL-2 and an upregulation of IL-5 and IL-10 in the maternal lymph nodes during early pregnancy in sheep (Yang et al. 2019).

Lymphatic organs are the major parts of the immune system in the mammalian body, and the spleen is the largest lymphatic organ (de Porto et al. 2010). The spleen is one of the secondary lymphatic organs and has been described as a central organ of the immune system that combines the innate and adaptive immune system in a uniquely organized way (Mebius and Kraal 2005). The spleen is compartmentalized into red and white pulp. The red pulp participates in filtration of red blood cells and reserve of monocytes, and white pulp is implicated in active immune response (Swirski et al. 2009). The circulating immune cells actively contribute to the establishment of embryo implantation, and the first signal of pregnancy recognition works on the maternal immune system before implantation (Fujiwara et al. 2009). We recently reported that there are changes in the expression of the P4 receptor and P4-induced blocking factor in the ovine spleen (Yang et al. 2018a), which indicates that the early conceptus exerts its effects on the maternal spleen. There is a strong down-regulation of the expression of coillin-1, F-actin capping protein subunit alpha and malate dehydrogenase proteins in splenic CD4+ lymphocytes, which indicates that preimplantation pregnancy can alter the activation state of peripheral CD4+ lymphocytes in mice (Chelmonska-Soyta et al. 2014). The Th cytokines play an important role in the immune system. However, it has been unclear whether early pregnancy exerts its effects on the characterization of the Th cytokines profile in the ovine spleen. In this study, the spleens from nonpregnant and early pregnant ewes were sampled to study the expression of IL-2, IFN-γ, TNF-β, IL-4, IL-5, IL-6 and IL-10, which may be beneficial...
in understanding the effects of early pregnancy on maternal splenic immunomodulation in sheep.

Materials and methods

Animals and experimental design

All procedures were approved by the Hebei University of Engineering Animal Care and Use Committee, and all experiments were conducted following the guidelines of the National Standards for Laboratory Animals of China (GB 14925-2010). Small-tail Han ewes approximately 18 months of age were housed at the farm of Handan Boyuan Animal Husbandry Co., Ltd. in China, and ovine oestrus was detected daily by a vasectomized ram. Twenty-four ewes were randomly divided into a nonpregnancy group (day 16 of the oestrous cycle) and three pregnant groups (days 13, 16 and 25 of pregnancy; n = 6 for each group), and the first day of coition was counted as day 0 of pregnancy or nonpregnancy. The ewes in the pregnant groups were mated twice with fertile rams in a 12-h interval after the detection of sexual receptivity, but the ewes assigned to the nonpregnant group were not mated with an intact ram. The effects of early pregnancy on the expression of Th cytokines in the ovine spleens are mainly due to P4 and interferon-tau (IFNT). Therefore, splenic samples were collected at days 13, 16, 25 of pregnancy, and day 16 of the oestrous cycle at the time of slaughter. The spleens were obtained from the ewes on days 13, 16 and 25 of pregnancy, as well as day 16 of the oestrous cycle at the time of slaughter. The spleens were crushed into fine powders in liquid nitrogen, and the powders were dissolved in TRIzol (Invitrogen, California, USA), and then the total RNA was extracted according to the manufacturer’s instructions. Genomic DNA removal and cDNA synthesis were performed using a FastQuant RT kit (With gDNase, Tiangen Biotech Co., Ltd., Beijing). The primer sequences of IFN-γ, TNF-β, IL-2, IL-4, IL-5, IL-6, IL-10 and GAPDH were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Table 1), and assessed by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) at NCBI. PCR amplification efficiency of each pair of primers was assessed before quantification, and was found to be in an acceptable range (between 0.9 and 1.1). The primer product was sequenced to check for specificity, and the expression of the targeted genes was determined by a Bio-rad CFX96 real-time PCR system with 20 μl. The qPCR was performed using a SuperReal PreMix Plus kit (Tiangen Biotech), and the melting curve was analyzed to guarantee the specificity of the amplification after PCR reactions. The PCR amplifications were carried out at 95°C for 10 sec, 55–58°C (55°C for IL-5 and IL-6, 58°C for IFN-γ, TNF-β, IL-4, IL-2 and IL-10) for 20 sec, and 72°C for 25 sec, and the number of PCR cycles was 40. The GAPDH PCR reaction was the same as IFN-γ, TNF-β, IL-2, IL-4, IL-5, IL-6, and IL-10, respectively (Yang et al. 2019). The relative expression values for the qRT-PCR assay were calculated by the 2−ΔΔCt analysis method, with GAPDH used for normalization of the PCR data (Wong and Medrano 2005). The relative expression value was set as 1 for the group on day 16 of the oestrous cycle.

Western blot

The total proteins of the spleenic samples were extracted by RIPA Lysis Buffer (Biosharp, BL504A), and a BCA Protein Assay kit (Tiangen Biotech) was used to measure the protein concentration. Equal amounts of total proteins (10 μg/lane) were separated using 12% SDS-PAGE and the proteins were transferred to 0.22 μm polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). In addition, the control proteins of TNF-β (Sigma-Aldrich Co. LLC., T7799), IL-5 (Sigma-Aldrich Co. LLC., IS273) and IL-10 (Sigma-Aldrich Co. LLC., I9276) were used for validity of antibodies. IFN-γ, TNF-β, IL-2, IL-4, IL-5, IL-6 and IL-10 were detected by western blot using a mouse anti-IFN-γ monoclonal antibody (Abcam, ab27919, 1:1000), mouse anti-TNF-β monoclonal antibody (Santa Cruz Biotechnology, Inc., SC-28345, 1:1000), rabbit anti-IL-2 polyclonal antibody (Abcam, ab193807, 1:1000), mouse anti-IL-4 monoclonal antibody (Bio-Techne, MAB82469, 1:1000), mouse anti-IL-5 monoclonal antibody (Abcam, ab193853, 1:1000) and mouse anti-IL-10 monoclonal antibody (Santa Cruz Biotechnology, Inc., SC-32815, 1:1000), respectively. Secondary goat anti-mouse IgG-HRP (Biosharp, BL001A) and goat anti-rabbit IgG-HRP (Biosharp, BL003A) were diluted to 1:10000. A Pro-light HRP chemiluminescence detection reagent (Tiangen Biotech) was used to detect the immunoreactive bands. These procedures were validated using positive tissue (endometrium) and negative control tissue (fat), and the target bands were present in the endometrium without a band

| Gene | Primer | Sequence | Size (bp) |
|------|--------|----------|----------|
| IL-2 | Forward | AAACCTGAACACCAGAGAGAT | 117 |
|      | Reverse | GCCTTTACTGTCGCATCA  |
| IFN-γ| Forward | TTGAACGGCGAGCTCTGAGAA | 124 |
|      | Reverse | TGGGACACGAGCATTCACTC |
| TNF-β| Forward | CCACCTAGGCGGCTTTATCTT | 141 |
|      | Reverse | TGATGCGGCAAGAGGTGTTG |
| IL-4 | Forward | GCTGCCTGAGATTCTGCTCAA | 120 |
|      | Reverse | CATCCTGCCCTGACTGCTTG |
| IL-5 | Forward | AGTCTCCATCACCACACGCA | 139 |
|      | Reverse | CGATGGTGTTGTGCTTTGAGG |
| IL-6 | Forward | GTACGATGTTGTGCTTTGAGG | 118 |
|      | Reverse | CTGTTGCGCTGCTTCTCCT |
| IL-10| Forward | TGTCAGTTGGCTCTCATTITG | 169 |
|      | Reverse | GGTTCACTACATCTCCTGACCT | 176 |
| GAPDH | Forward | GCCATCAAGCTCCTGACCT | 176 |
|      | Reverse | GTCAATAGCTCCCTCAGCA  |
in the fat. Sample loading was monitored with the anti-GAPDH antibody (Santa Cruz Biotechnology, Inc., sc-20357) at a dilution of 1:1000. Quantity One V452 software (Bio-Rad Laboratories) was used to semi-quantify the intensity of the blots, and the relative levels were calculated using GAPDH. The expression of GAPDH protein was measured by western blot, and there was no difference among the four groups.

**Immunohistochemistry analysis**

The fixed splenic samples were embedded in paraffin, and the paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. The sections were stained by hematoxylin and eosin (HE). The endogenous peroxidase activity of the rehydrated sections was quenched using 3% H2O2, and 5% normal goat serum in PBS was used to reduce nonspecific binding. Immunohistochemical localization of IL-6 in the splenic tissue was performed using the rabbit anti-IL-6 polyclonal antibody (Abcam, ab193853, 1:100). For a negative control, nonimmune goat serum was used in place of the primary antibody. A DAB kit (Tiangen Biotech) was used to visualize the antibody binding sites in the tissue sections. Finally, the images were captured using a light microscope (Nikon Eclipse E800, Japan) with a digital camera (AxioCam ERc 5s), and the intensity of staining and density of the stained cells were analyzed through the images. The immunostaining intensity of the different splenic tissue samples from different ewes (n = 6 for each group) was rated by 2 different investigators in a blinded fashion, and histological subtypes were analyzed by assigning an immunoreactive intensity of a scale of 0–3, as described previously (Kandil et al. 2007). An intensity of 3+ was given to the cells with the highest staining intensity, and an intensity of 0 was assigned to the cells with no immunoreactivity.

**Statistical analyses**

The data for the relative expression levels of IFN-γ, TNF-β, IL-2, IL-4, IL-5, IL-6 and IL-10 mRNA and proteins were analyzed using a completely randomized design with six animals per group via the Proc Mixed model of SAS (Version 9.1; SAS Institute, Cary, NC). For spleens from different stages of gestation or pregnancy status, the model contained the random effect of the ewe and the fixed effects of the stage of gestation, pregnancy status and the interaction between the stage of gestation and pregnancy status. The comparisons among the relative expression levels of the different groups were performed using the Duncan method and controlling the experimentwise type ± error equal to 0.05. Data are presented as least squares means. Groups were considered significantly different at P < 0.05.

**Results**

**Relative expression levels of Th1 cytokines mRNA and proteins in the spleens**

The qRT-PCR assay and western blot revealed (Figures 1 and 2) that the relative expression levels of IFN-γ mRNA and protein were higher in the spleens at day 16 of the oestrous cycle and day 16 and 25 of pregnancy than that days 13 and 16 of pregnancy (P < 0.05), and the relative expression levels of TNF-β mRNA and protein were higher at days 16 and 25 of pregnancy than that at day 16 of the oestrous cycle and day 16 of pregnancy (P < 0.05). Furthermore, the relative expression levels of IL-2 mRNA and protein were the highest in the spleens at day 25 of pregnancy among nonpregnant and early pregnancy ewes (P < 0.05), but there was no significant difference among nonpregnant or days 13 and 16 the pregnant ewes (P > 0.05) (Figures 1 and 2).

**Relative expression levels of Th2 cytokines mRNA and proteins in the spleens**

There was an upregulation of the relative expression levels of IL-4 mRNA and protein at day 25 of pregnancy (P < 0.05), and the
Figure 2. Expression of Th1 cytokines (IL-2, IFN-γ and TNF-β) and Th2 cytokines (IL-4, IL-5, IL-6 and IL-10) proteins in spleens of nonpregnant and pregnant ewes analyzed by western blot.

Note: hTNF-β = Recombinant human TNF-β protein; hIL-5 = Recombinant human IL-5 protein; hIL-10 = Recombinant human IL-10 protein; DN16 = Day 16 of the oestrous cycle; DP13 = Day 13 of pregnancy; DP16 = Day 16 of pregnancy; DP25 = Day 25 of pregnancy. Significant differences (P < 0.05) are indicated by different superscript letters within the same colour column.
relative expression levels were the lowest in the nonpregnant group ($P < 0.05$), but there was no significant difference between days 13 and 16 of pregnancy ($P > 0.05$) (Figures 1 and 2). The relative expression levels of IL-5 and IL-6 mRNA and proteins were the highest in the spleens at day 25 of pregnancy among nonpregnant and early pregnancy ewes ($P < 0.05$), but there was no significant difference among nonpregnant or days 13 and 16 of the pregnant groups ($P > 0.05$) (Figures 1 and 2). Furthermore, the relative expression levels of IL-10 mRNA and protein were the lowest in the spleens at day 16 of the oestrous cycle among nonpregnant and early pregnancy ewes ($P < 0.05$), but there was no significant difference among the pregnant ewes ($P > 0.05$) (Figures 1 and 2).

**The immunohistochemistry for IFN-γ and IL-6 proteins in the spleens**

The immunohistochemistry for the IL-6 protein was limited to the capsule, trabeculae and splenic cords (Figure 3). Furthermore, the staining intensity for IL-6 was 1+, 1+, 1+, and 3+ for the spleens from day 16 of the oestrous cycle, and the spleens from days 13, 16 and 25 of pregnancy, respectively (Figure 3). The staining intensity was as follows: 0 = negative; 1+ = weak; 2+ = strong; 3+ = stronger.

**Discussion**

The spleen is part of the circulatory system and has an unusual structure of lymphoid compartments to combine the innate and adaptive immune system (Mebius and Kraal 2005). The size of the maternal spleen increases an average of 50% in late pregnancy in mice (Davis et al. 1961), and the relative content of splenic macrophages increases two-fold during mid-pregnancy in mice (Mattsson et al. 1984). In this study, there was a decrease in the levels of IFN-γ mRNA and protein at days 13 and 16 of pregnancy (Figures 1 and 2). IFN-γ is the only member of the type II class of interferon and it has antiviral, immunoregulatory, and antitumor properties (Schroder et al. 2004). IFN-γ plays an essential role in Th0 cells differentiating into Th1 cells, which induces a Th1-adapted immune response (Maley et al. 2006). IFN-γ is also an inducer of the Class II major histocompatibility complex (MHC) molecule that activates macrophages (Schoenborn and Wilson 2007), which is harmful to a normal pregnancy. It has been reported that there is a down-regulation of IFN-γ at the feto-maternal interface (Saito 2000), in the endometria (Beltman et al. 2013) and PBMCs (Yang et al. 2016). Therefore, a decrease in the level of IFN-γ in the maternal spleen at days 13 and 16 of pregnancy may be needed to maintain early pregnancy in sheep.

TNF-β, also known as lymphotixin-alpha (LT-α), has a significant impact on the maintenance of the immune system, including the development of secondary lymphoid organs (Ruddle 2014). As a signalling molecule, TNF-β can regulate cell survival, proliferation, differentiation and apoptosis (Bauer et al. 2012), prevent tumour growth and destroy cancerous cell lines, and it plays an essential role in innate immune regulation (Fernandes et al. 2016). It has been reported that TNF-α, another member of the tumour necrosis factor superfamily, is implicated...
in decidualization, placentation, and prevention of abortion (Ozgilin et al. 2015). Therefore, upregulation of TNF-β at days 16 and 25 in pregnant spleens may be involved in the regulation of maternal splenic immunity during pregnancy in sheep.

IL-2 is a pleiotropic cytokine and has direct effects on T-cell differentiation, which plays key roles in regulating functions of the immune system (Liao et al. 2011). IL-2 regulates the immune response by promoting naive CD4+ T-cell differentiation into Th1 and Th2 cells and is involved in mediating tolerance and limiting inappropriate immune reactions by regulating the development and maintenance of T regulatory cells and activation-induced cell death (Liao et al. 2013). The blood serum level of IL-2 increases during the second week of gestation compared with that in anoestrus and dioestrus, which is essential for the development and maintenance of pregnancy in bitches (Maciel et al. 2014). It has also been reported that recombinant bovine IL-2 can enhance immunity and protect against abortion induced by Brucella abortus vaccines in cattle (Wyckoff et al. 2005), and there is upregulated expression of IL-2 mRNA in pregnant PBMCs compared with that in nonpregnant PBMCs in cattle (Yang et al. 2016). Our results indicated that there was upregulated expression of IL-2 mRNA and protein in the spleens at day 25 of pregnancy (Figures 1 and 2), which may be helpful for establishing a successful pregnancy in sheep.

IL-4 induces the differentiation of naive Th cells (Th0 cells) into Th2 cells that subsequently produce additional IL-4 in a positive feedback loop, and IL-4 also decreases the production of Th1 cells and IFN-γ (Sokol et al. 2008). The serum concentration of IL-4 is elevated during early gestation in bitches, which depends on P4, and P4 can act as a potent inhibitor of Th1 cytokines (Pantaleo et al. 2013). Trophoblast IFN-γ enhances the expression of IL-4 mRNA by effector T cells in cattle (Tuo et al. 1999), and P4 also significantly upregulates the expression of IL-4 in PBMCs in pregnant cows (Maeda et al. 2013). IFN-γ induces upregulation of the conjugated proteins of interferon-stimulated gene 15 kDa protein in maternal spleen during early pregnancy in sheep (Yang et al. 2018b). We found that the relative levels of IL-4 mRNA and protein were higher in the pregnant groups (Figures 1 and 2), which may be due to the high serum concentrations of P4 and IFN-γ during early pregnancy.

Th2 cells and mast cells produce IL-5 that stimulates B cell growth and increases immunoglobulin secretion through binding to its receptor. The dendritic cells treated with placental pregnancy in bitches (Maciel et al. 2014). It has also been reported that in nonpregnant PBMCs in cattle (Yang et al. 2016). Our immunohistochemistry results showed that the immunostaining of the IL-6 protein was limited to the capsule, trabeculae and splenic cords (Figure 3). The staining intensity for IL-6 at day 25 of pregnancy was stronger than that at day 16 of the oestrous cycle, and at days 13 and 16 of pregnancy (Figure 3). The blood flowing in the splenic artery drains directly into the splenic sinuses of the red pulp (Mebius and Kraal 2005), and the monocytes in red pulp differentiate irreversibly into DCs or macrophages upon tissue entry to regulate systemic immunity through blood circulation (Swirski et al. 2009). The splenic monocytes increase their motility, in response to tissue injury, exit the spleen en masse to participate in wound healing (Swirski et al. 2009). Therefore, we suggest that the change in the expression of Th cytokines in maternal spleen may affect the function of the splenic monocytes. The monocytes exit the spleen, in response to the conceptus in maternal uterus, to regulate the uterine immune function, which is beneficial for embryonic development during early pregnancy in sheep.

In conclusion, there was upregulation of TNF-β, IL-2, IL-4, IL-5, IL-6 and IL-10 in the spleens during early pregnancy, but IFN-γ was down-regulated at days 13 and 16 of pregnancy. Furthermore, the IL-6 protein was limited to the capsule, trabeculae and splenic cords. Therefore, we suggest that early pregnancy
exerts its effects on the spleen to regulate the Th cytokines profile, which may be important for maintaining a normal pregnancy during early pregnancy in sheep.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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