The early stage of pregnancy modulates toll-like receptor signaling in the ovine liver

Meihong Gao*, Chunjiang Cai*, Xu Han*, Luyu Wang, Weifeng Zhang, Leying Zhang and Ling Yang

Department of Animal Science, School of Life Sciences and Food Engineering, Hebei University of Engineering, Handan, People's Republic of China

ABSTRACT
Toll-like receptors (TLRs) participate in maternal immune regulation at multiple maternal-fetal interfaces. The liver is crucial for innate and adaptive immunity during pregnancy. The objective of this study was to measure the expression of genes and proteins related to TLR signaling. In this study, livers were obtained from ewes on day 16 of the estrous cycle, and on days 13, 16 and 25 of gestation, and RT-qPCR, western blot and immunohistochemistry were employed to measure the expression of genes and proteins related to TLR signaling, including TLR2, TLR3, TLR4, TLR5, TLR7, TLR9, myeloid differentiation primary-response protein 88 (MyD88), tumor necrosis factor receptor-associated factor 6 (TRAF6) and interleukin-1-receptor-associated kinase 1 (IRAK1). Our data showed that the expression levels of TLR2, TLR3, TLR5, TLR9 and TRAF6 were increased, but those of TLR4 and TLR7 were decreased in the liver during early pregnancy. In addition, the expression levels of MyD88 and IRAK1 were the highest on days 13 and 16 of pregnancy, respectively, and the MyD88 protein was located in endothelial cells of proper hepatic arteries and portal veins and in hepatocytes. In conclusion, early pregnancy modulates TLR signaling in the maternal liver in sheep, which may participate in maternal immune regulation.

INTRODUCTION
The maternal immune system tolerates fetal antigens by modulating maternal adaptive immune responses during pregnancy. It is known that disturbances in maternal immune responses result in reproductive failure (Díaz-Peña et al. 2019). Toll-like receptors (TLRs) play key roles in host defense, autoimmune diseases and effective immune responses (Kawai and Akira 2010). There is an increase in the innate immune response during normal pregnancy, but excessive upregulation of TLRs at multiple maternal-fetal interfaces is related to the pathology of pregnancy complications (Riley and Nelson 2010; Afkham et al. 2019). The expression patterns of TLRs at the maternal-fetal interface are dependent on the stages of pregnancy (Koga and Mor 2010), and TLRs participate in regulating innate immunity at the maternal-fetal interface (Abrahams and Mor 2003). The expression of TLRs in trophoblast cells is implicated in innate immunity during embryo implantation (Koga et al. 2009). Exosomes act on trophectoderm cells to regulate the secretion of interferon-tau (IFN-tau, IFNT) via TLR-mediated cell signaling in pregnant ewes (Ruiz-González et al. 2015). Embryo- and/or pregnancy-related factors change the expression of some components of TLRs in trophoblasts, which are involved in immune regulation at the maternal-fetal interface during early pregnancy in ewes (Koga et al. 2017). Early pregnancy regulates the expression of TLRs in the corpus luteum (CL) and thymus, which participate in the establishment of pregnancy in ewes (Atli et al. 2018; Li et al. 2020). However, it is unclear whether early pregnancy induces expression of TLRs in the maternal liver in sheep.

Interferon-stimulated gene (ISG) 15-kDa protein (ISG15) mRNA is upregulated in the CL and liver on day 15 of pregnancy in ewes (Bott et al. 2010), and pregnancy upregulates ISG15 and myxovirus-resistance protein-1 gene expression in the liver on day 18 of pregnancy in cows (Meyerholz et al. 2016). Early pregnancy also induces the upregulation of ISGs in bone marrow (Yang et al. 2017), the thymus (Zhang et al. 2018; 2020b), the spleen (Yang et al. 2018; Wang et al. 2019), lymph nodes (Yang et al. 2019; Zhang et al. 2020a), and the liver (Yang et al. 2020b) on day 16 of pregnancy in sheep.

The liver is a crucial immune tissue that is involved in maintaining the balance between immunity and tolerance (Kubes and Jenne 2018). There are changes in the liver biochemical profile during normal pregnancy (Ma et al. 2019). Hepatic drug metabolism is changed in a cytochrome (P450)-specific manner during pregnancy (Koh et al. 2011), and asialoglycoprotein receptor and mannose receptor mRNA are upregulated in the liver during pregnancy in mice (Mi et al. 2014). Maternal metabolic demands induce a conspicuous change in maternal liver growth and the gene expression profile during pregnancy in rats (Bustamante et al. 2010). We recently reported that progesterone receptor, progesterone-induced blocking factor (Zhang et al. 2019), cyclooxygenase-2, prostaglandin (PG) E synthase, PGF synthase (Yang et al. 2020a), ISGs (Yang et al. 2020b), interleukin (IL)-5 (Yang et al. 2019a) and gonadotropin

CONTACT Ling Yang yangling@hebeu.edu.cn Department of Animal Science, School of Life Sciences and Food Engineering, Hebei University of Engineering, Handan 056038, People’s Republic of China

*These authors contributed equally to this work as co-first author.

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releasing hormone and receptor (Cao et al. 2021) are upregulated in the ovine maternal liver during early pregnancy. Furthermore, the protein levels of melatonin receptor 1 and cluster of differentiation 4 are increased in the liver during early pregnancy in sheep (Bai et al. 2020). However, it was unclear whether early pregnancy affects on the expression of TLRs and adaptor molecules in the maternal liver. Therefore, we hypothesize that early pregnancy induces changes in TLR signaling in the maternal liver. The objective of this study was to measure and compare the expression of genes and proteins related to TLR signaling, including TLR2, TLR3, TLR4, TLR5, TLR7, TLR9, myeloid differentiation primary-response protein 88 (MyD88), tumor necrosis factor receptor associated factor 6 (TRAF6) and interleukin-1-receptor-associated kinase 1 (IRAK1), in the maternal liver during the estrous cycle and in early pregnancy in ewes. In addition, immunohistochemical analysis was used to visualize the protein expression of MyD88 in the liver.

Materials and methods
Animals and experimental design
All procedures were approved by the Hebei University of Engineering Animal Care and Use Committee (AEEI-16015), and humane animal care and handling procedures were followed throughout the experiments. Ewes (Small-tail Han sheep) of similar age (18 ± 2 months) were housed at a local farm in the Hebei Province (China), and were selected and assigned randomly to four groups (n = 6 for each group). All ewes received the same diet after the detection of estrus (day 0) through the experiments. Ewes (Small-tail Han sheep) of similar age (18 ± 2 months) were housed at a local farm in the Hebei Province (China), and were selected and assigned randomly to four groups (n = 6 for each group). All ewes received the same diet after the detection of estrus (day 0) through the experiments. All animals were sacrificed at day 16 of the estrous cycle (DN16). Ewes in the other three groups were mated to fertile rams and were sacrificed on days 13, 16 and 25 after coition (DP13, DP16 and DP25, respectively). All animals were sacrificed at an abattoir for human consumption, and liver samples were collected from the ewes. Pregnancy was confirmed by dissecting the uterus and finding a conceptus for all pregnant ewes. Hepatic samples were immediately snap-frozen in liquid nitrogen for RNA extraction and RT-qPCR assay.

RNA extraction and RT-qPCR assay
Total RNA was extracted using a TRizol reagent (Tiangen Biotech Co., Ltd., Beijing) according to the manufacturer’s instructions. A NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was used to determine the content and purity of the total RNA by measuring the absorbance ratio of all samples at 230 and 260 nm, and 260/230 values ranged from 2.0–2.2. Approximately 1 μg of the total RNA was reverse transcribed into cDNA using a FastQuant RT kit with DNase (Tiangen Biotech) according to the kit instructions. Primers (Table 1) for the target genes (TLR2, TLR3, TLR4, TLR5, TLR7, TLR9, MYD88, TIRAP6 and IRAK1) and reference gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) were designed and synthesized by Sangon Biotech Biotech Co., Ltd. (Shanghai, China). Amplification efficiencies of the primer sequences were evaluated before quantification. The PCR conditions consisted of 40 cycles of 95°C for 10 s, 60–62°C (60°C for TLR2, TLR3, TLR4, TLR7 and TRAF6, or 62°C for TLR5, TLR9, IRAK1 and MYD88) for 20 s, and 72°C for 25 s on a Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, Inc., CA, USA). Data were calculated using the 2^–ΔΔCt analysis method (Livak and Schmittgen 2001), with GAPDH as the housekeeping gene to normalize the data. The group (DN16) was used as a calibrator to compare the effects of early pregnancy on gene expression.

Western blot analysis
Hepatic tissues were lysed by a RIPA Lysis Buffer containing protease inhibitor (Biosharp, BLS04A), and the supernatant was collected. Protein concentration in the supernatant was detected using a BCA Protein Assay kit (Tiangen Biotech). Protein samples (10 μg/lane) were subjected to SDS-PAGE using 12% gel, and electroblotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk at 4°C overnight, and then incubated with primary antibodies at 37°C for 1 h. The primary antibodies included a rabbit anti-TLR2 polyclonal antibody (Abcam, Cambridge, UK, ab191458, 1:1000), a mouse anti-TLR3 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-32232, 1:1000), a mouse anti-TLR4 monoclonal antibody (Santa Cruz Biotechnology, sc-293072, 1:1000), a mouse anti-TLR5 monoclonal antibody (Santa Cruz Biotechnology, sc-517439, 1:1000), a rabbit anti-TLR7 polyclonal antibody (Abcam, ab113524, 1:1000), a mouse anti-TLR9 monoclonal antibody (Santa Cruz Biotechnology, sc-52966, 1:1000), a mouse anti-MyD88 monoclonal antibody (Santa Cruz Biotechnology, sc-136970, 1:1000), a rabbit anti-TRAF6 monoclonal antibody (Santa Cruz Biotechnology, sc-8409, 1:1000) and a rabbit anti-IRAK1 polyclonal antibody (Abcam, ab137327, 1:1000), respectively. The membranes were incubated with a horseradish peroxidase conjugated goat anti-mouse (Biosharp, BL001A) or anti-rabbit IgG (Biosharp, BL003) at a 1:10,000 dilution for 1 h at room temperature. Immunoreactive protein bands were visualized using a pro-light HRP chemiluminescence kit (Tiangen Biotech). The relative expression levels of the target proteins were normalized using an anti-GAPDH antibody (Santa Cruz Biotechnology, sc-47724, 1:1000). The relative expression values of the target proteins were quantified by Quantity One software (Bio-Rad Laboratories).

Immunohistochemical analysis
Pieces of livers were dehydrated using methanol, and then paraffin-embedded. Paraffin-embedded sections (5 μm in thickness) were deparaffinized in xylene, and rehydrated in ethanol. Some sections were stained by hematoxylin and eosin (HE). Tissues were boiled in citrate solution for antigen retrieval, and blocked endogenous peroxidase activity using 3% hydrogen peroxide. Normal goat serum (5% in PBS) was used for
blocking nonspecific binding sites. The sections were incubated in a humidified chamber using the mouse anti-MyD88 monoclonal antibody (Santa Cruz Biotechnology, sc-136970, 1:200) at 4 °C overnight. Negative controls were the sections those were treated with non-immune goat serum instead of the anti-MyD88 antibody at the same concentration in the same procedure. A DAB kit (Tiangen Biotech) was used to detect specific binding sites. The sections were incubated in a humidified chamber using the mouse anti-MyD88 monoclonal antibody (Santa Cruz Biotechnology, sc-136970, 1:200) at 4 °C overnight. Negative controls were the sections those were treated with non-immune goat serum instead of the anti-MyD88 antibody at the same concentration in the same procedure. A DAB kit (Tiangen Biotech) was used to detect specific staining, and nuclei were stained by hematoxylin. Images were taken using a light microscope (Nikon Eclipse E800, Japan) with a digital camera (AxioCam ERC s5). Finally, the images were analyzed by two investigators in a blinded fashion, according to the following scale: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining (Kandil et al. 2007).

**Statistical analysis**

Each group consisted of six replicates. Data of relative expression levels of TLR2, TLR3, TLR4, TLR5, TLR7, TLR9, IRAK1, TRAF6 and MYD88 mRNA and proteins were analyzed in a completely randomized design using the Proc Mixed models of SAS (Version 9.1; SAS Institute, Cary, NC, USA). Tukey’s multiple comparison test was used to compare the relative expression levels of the different groups. Data were considered statistically significant when P values were lower than 0.05.

**Results**

Relative mRNA expression levels of TLR2, TLR3, TLR4, TLR5, TLR7, TLR9, IRAK1, TRAF6 and MYD88 in the liver

The RT-qPCR data revealed the upregulation of TLR2 and TRAF6 mRNA during early pregnancy (P < 0.05; Figure 1), but there was no significant difference between DN16 and DP13 or between DP16 and DP25 (P > 0.05). The mRNA levels of TLR3, TLR5 and TLR9 on DP25 were higher than those on DN16, DP13 and DP16 (P > 0.05), and there was no significant difference on DN16, DP13 or DP16 (P > 0.05). However, the mRNA expression levels of TLR4 and TLR7 were decreased during early pregnancy (P < 0.05), but there was no significant difference in TLR4 on DP13, DP16 and DP25, or in TLR7 on DN16, DP13 and DP16 (P > 0.05). Furthermore, there were peaks of MYD88 and IRAK1 mRNA on days 13 and 16 of pregnancy, respectively (P < 0.05; Figure 1), but there was no significant difference in MYD88 on DN16, DP16 and DP25, or in IRAK1 on DN16, DP13 and DP25 (P > 0.05).

**Immunohistochemical analysis of the MyD88 protein in the liver**

Immunohistochemical analysis revealed that the protein levels of TLR2, TLR3, TLR5, TLR9, IRAK1, TRAF6 and MyD88 were increased in the maternal liver during early pregnancy (P < 0.05; Figure 2), but there was no significant difference on DN16 or DP13, and or on DP16 or DP25 in TLR2 and TRAF6 expression (P > 0.05). In addition, there was no significant difference in TLR3 on DN16, DP13 and DP16 (P > 0.05), and TLR5 and TLR9 were undetectable on DN16, DP13 and DP16. However, TLR4 protein expression was only detected on DN16, and was undetectable during early pregnancy. Furthermore, IRAK1 protein peaked on DP16, but there was no significant difference on DN16, DP13 and DP25. In addition, the MyD88 protein was expressed on DP13 (P < 0.05), but it was undetectable on DN16, DP16 and DP25.

**Discussion**

TLRs are implicated in immune regulation at the maternal-fetal interface during early pregnancy in ewes (Kaya et al. 2017), and
early pregnancy modulates the expression of TLRs in the CL and thymus in ewes (Atli et al. 2018; Li et al. 2020). It has been reported that there are changes in the expression of progesterone receptor, T helper cytokines, PG synthases, ISGs, melatonin receptor 1, cluster of differentiation 4, and gonadotropin releasing hormone and its receptor in the liver during early pregnancy in sheep (Zhang et al. 2019; Yang et al. 2019a; 2020a; 2020b; Bai et al. 2020; Cao et al. 2021). In this study, our results showed for the first time that early pregnancy modulates TLR signaling in the maternal liver.

The TLR system (including TLR2) regulates the function of nonparenchymal liver cells, which are a major component of the innate and adaptive immune systems of the liver and are involved in the tolerogenic functions in the liver (Wu et al. 2010). Our results revealed that there was an upregulation in TLR2 in the maternal liver at DP16 and DP25 compared to DP13 and DN16. TLR2 is involved in initiating the innate immune response and maintaining the intact placental barrier in placental antiparasitic defenses (Castillo et al. 2017). The mRNA expression of TLR2 in the endometrium is increased in a pregnancy stage-dependent manner in pigs (Yoo et al. 2019).

It has been reported that TLR3 is expressed in the liver, and plays a key role in the innate immune response against viral infection in humans (Yin and Gao 2010). It was revealed in the present study that TLR3 mRNA and protein were upregulated on DP25 compared to DN16, DP13, and DP16. TLR3 can regulate T cell responses to exogenous antigens by enhancing immune responses and maintaining tolerance (Salio and Cerundolo 2005). TLR3 can induce the expression of indoleamine 2,3-dioxygenase, which plays a role in inhibiting the proliferation of T cells in human first-trimester trophoblasts, suggesting that TLR3 is helpful in inhibiting fetal rejection and establishing pregnancy in mice (Wang et al. 2011).

TLR4 is involved in leflunomide-induced hepatotoxicity and is increased in a dose-dependent manner in the liver (Elshaer et al. 2019). Our data showed that TLR4 mRNA and protein levels were decreased in the maternal liver during early pregnancy compared to DN16. The TLR4 gene is upregulated in neutrophils from women with preterm birth compared to those in the normal control group (Prearo Moço et al. 2018), suggesting that the downregulation of TLR4 in maternal neutrophils is beneficial for normal pregnancy in humans.

TLR5 is expressed in the liver and is involved in promoting bacterial clearance and protecting the liver against diet-induced hepatic steatosis (Etienne-Mesmin et al. 2016). This study showed that there was an increase in the expression of TLR5 in the maternal liver on DP25 compared to DP13, DP16 and DN16. It has been reported that the concentration of progesterone is increased during the first three weeks of pregnancy in ewes (Roman-Ponce et al. 1983). There is an increase...
in the mRNA expression of TLR5 in the porcine endometrium with increasing doses of progesterone during mid-to-late pregnancy (Yoo et al. 2019).

TLR7 mRNA is expressed in the liver, and TLR7 participates in the immunopathogenesis of chronic hepatitis C in humans (Tarantino et al. 2013). Our data demonstrated that TLR7 was
downregulated on DP25 compared to DP13, DP16 and DN16. Treatment with the TLR7-specific agonist imiquimod leads to pregnancy-dependent hypertension, endothelial dysfunction, and placental inflammation in mice, and the expression of TLR7 is increased in women with pre-eclampsia (Chatterjee et al. 2012). The downregulation of TLR7 in the maternal liver on day 25 of pregnancy may be beneficial for successful pregnancy in sheep.

TLR9 is increased in livers with nonalcoholic steatohepatitis and is involved in inflammatory recruitment and cell survival in humans and mice (Mridha et al. 2017). Our results showed that TLR9 was increased in the maternal liver on DP25 compared to DP13, DP16 and DN16. TLR9 can recognize foreign DNA and self-DNA to activate innate and adaptive immune responses (Lamphier et al. 2006). TLR9 protein expression is downregulated in splenic B cells from abortion-prone mice compared with that in healthy women, suggesting that a high level of TLR9 is the cause of immune intolerance in the fetal-placental unit (Zhao et al. 2018). TLR9 is increased in livers with nonalcoholic steatohepatitis and related to the increased progesterone levels during early pregnancy in sheep.

In a review, Kawai and Akira (2010) showed that TLR2, TLR4 and TLR5 participate in immune responses via MyD88, IRAK1 and TRAF6, that TLR4 and TLR3 are involved in immune regulation via Toll/IL-1R domain-containing adaptor-inducing IFN-β and TRAF6, that TLR7 and TLR9 are implicated in immune responses via MyD88 and TRAF6. In this study, two adaptors (MyD88 and IRAK1) were upregulated on days 13 and 16 of pregnancy (the period of pregnancy recognition), respectively, which may be related to the complicated regulation induced by altering the expression of TLR2, TLR4, TLR5, TLR7 and TLR9 in a MyD88-dependent manner. In addition, the upregulation of TRAF3 and/or downregulation of TLR4 on day 25 of pregnancy may be related to pregnancy maintenance in a MyD88-independent manner in the maternal liver.

In conclusion, early pregnancy modulated Toll-like receptor signaling in ovine livers and changed the protein expression of MyD88 in the endothelial cells of the proper hepatic arteries and portal veins and in hepatocytes. These findings suggest that the modulation of TLR signaling induced by early pregnancy may be due to the high levels of progesterone and PAG in the plasma of ewes, which is related to pregnancy recognition, maintaining immune tolerance and pregnancy maintenance in sheep.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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ORCID
Ling Yang http://orcid.org/0000-0003-4385-0024

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