Possible role of interferon tau on the bovine corpus luteum and neutrophils during the early pregnancy

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Abstract

When pregnancy is established, interferon tau (IFNT), a well-known pregnancy recognition signal in ruminants, is secreted by embryonic trophoblast cells and acts within the uterus to prepare for pregnancy. IFNT acts as an endocrine factor on the corpus luteum (CL) to induce refractory ability against the luteolytic action of PGF2 α. Hypothesising that IFNT may influence not only the uterine environment but also the CL in cows via local or peripheral circulation, we investigated qualitative changes in the CL of pregnant cows during the maternal recognition period (day 16) and the CL of non-pregnant cows. The CL of pregnant animals had a higher number of neutrophils, and the expression of interleukin 8 (IL8) mRNA and its protein was higher as well compared with the CL of non-pregnant animals. Although IFNT did not affect progesterone (P4) secretion and neutrophil migration directly, it stimulated IL8 mRNA expression on luteal cells (LCs), influencing the neutrophils, resulting in the increased migration of IFNT-activated neutrophils. Moreover, both IFNT-activated neutrophils and IL8 increased P4 secretion from LCs in vitro. Our novel finding was the increase in neutrophils and IL8 within the CL of pregnant cows, suggesting the involvement of IFNT function within the CL toward establishment of pregnancy in cows. The present results suggest that IFNT upregulates neutrophil numbers and function via IL8 on LCs in the CL of early pregnant cows and that both neutrophils and IL8, stimulated by IFNT, are associated with an increase in P4 concentrations during the maternal recognition period in cows.

Introduction

The corpus luteum (CL) is formed from the ovulated follicle and grows rapidly to secrete progesterone (P₄), thereby supporting the implantation and maintenance of pregnancy. When pregnancy is established, interferon tau (IFNT), a well-known pregnancy recognition signal in ruminants (Imakawa et al. 1987), is secreted by embryonic trophoblast cells and indirectly maintains the CL by attenuating (cow) (Meyer et al. 1995) or altering (ewe) (Zarco et al. 1988) luteolytic pulses of uterine PGF₂α, beginning at 16–18 days after insemination. Therefore, this period is defined as the maternal recognition period (Spencer et al. 2004).

Although it has been considered that IFNT only acts within the uterus to prepare for pregnancy, it appears to additionally act as an endocrine factor on the CL to induce refractory ability against the luteolytic action of PGF₂α in the ewe (Antoniazzi et al. 2013). Interestingly, the interferon-stimulated gene 15 (ISG15), a marker gene of IFN response, was upregulated in peripheral blood cells, the CL and the endometrium during the maternal recognition period in the pregnant cow and ewe (Han et al. 2006, Gifford et al. 2007, Hansen et al. 2010, Yang et al. 2010, Shirasuna et al. 2012). Furthermore, IFNT enhanced the resistance of the CL to the luteolytic effect of PGF₂α in an endocrine manner, and the infusion of IFNT into the uterine vein clearly inhibited spontaneous luteolysis in ewes (Bott et al. 2010). These findings suggest that IFNT may influence not only the uterine environment but also the CL in the cow via local or peripheral circulation.

Recently, attention has focused on the multiple roles of immune cells in luteal function, not only in luteolysis (CL disruption by immune responses involving T lymphocytes and macrophages) but also in CL development (CL remodelling by different immune responses involving neutrophils and macrophages). Indeed, Care et al. (2013) demonstrated the fundamental role of macrophages on the...
followed 7 days later by PGF2 hormone (GnRH; 0.01 mg Buserelin and 2.5 ml of Recepta), ovulation, all of the cows received gonadotrophin-releasing hormone confirmed by ultrasonography in all animals. Two days after GnRH, ovulation (day 1) was confirmed by ultrasonography in all cows. Therefore, we hypothesised that IFNT-mediated immune response is involved in the functional change to the CL in preparation for pregnancy in cows. To confirm this hypothesis, we investigated the qualitative changes in levels of ISG15, the neutrophilic chemokine IL8 and polymorphonuclear neutrophils (PMNs) in the CL of pregnant cows during the maternal recognition period (day 16) and in the CL of non-pregnant cows. To examine the role of IFNT, using in vitro experiments, the effects of IFNT on ISG15 and IL8 levels on luteal cells (LCs), migration capacity of PMNs and PMN function on luteal P4 secretion were investigated.

Materials and methods
The CL and plasma collections were conducted at the Clinic for Cattle, University of Veterinary Medicine, Hannover, Germany, and blood collection was conducted at the Field Center for Animal Science and Agriculture, Obihiro University, Obihiro, Japan. All of the experimental procedures complied with the Ethics Committee on Animal Rights Protection, Oldenburg, Germany; in accordance with German legislation on animal rights and welfare (file reference number 33.9-42502-04-07/1275) and the Guidelines for the Care and Use of Agricultural Animals of Obihiro University. All animal protocols were approved by the committees.

Collection of the bovine CL and plasma
The collection of the ovaries with CLs was conducted at the University of Veterinary Medicine, Hannover, Germany. To collect the CL samples, 11 normal cyclic non-lactating German Holstein cows were used, in a similar way to our previous in vivo study in Germany (Beindorff et al. 2010). Briefly, to synchronise and determine the exact day of ovulation, all of the cows received gonadotrophin-releasing hormone (GnRH; 0.01 mg Buserelin and 2.5 ml of Recepta), followed 7 days later by PGF2a and then GnRH at 48 h after PGF2a. Only the animals that possessed a pre-ovulatory follicle by the last GnRH application checked by ultrasonography were used in the study. The animals were randomly divided into two groups as non-pregnant and pregnant (inseminated). Artificial inseminations were carried out 12 and 24 h after GnRH application. Two days after GnRH, ovulation (day 1) was confirmed by ultrasonography in all animals.

We selected day 16 after artificial insemination as the transitional phase to pregnancy, in comparison with the late luteal phase (day 16) during the oestrous cycle. Ovariectomy was performed on day 16 (n=4) of the oestrous cycle (not inseminated) as well as on day 16 (n=7) of pregnancy. Collection of the ovaries was conducted by laparotomy (Beindorff et al. 2010). After administration of epidural anaesthesia (2% procaine hydrochloride, Procasel, Selectavet, Weyarn-Holzolling, Germany), the long incision line was infiltrated subcutaneously and intramuscularly with 2% procaine hydrochloride with epinephrine (Isocain, Selectavet). After removing the CL-bearing ovary with an effenimator (Reisinger, modified by Richter), the laparotomy was closed in layers. All procedures to correct the ovaries were conducted within 30 min. To check the pregnancy status for day 16 inseminated cows, the cows were slaughtered after ovariectomy, and the presence of an embryo within the uterus was confirmed. At a local abattoir, the reproductive tract was recovered within 20 min after slaughter, and the uterus was ligated cranial to the cervix to prevent embryo loss and transported to the laboratory within 30 min of slaughter. In two animals, no embryo was found by ultrasonography and the tracts were flushed to detect an embryo; therefore, these animals were excluded from the experiment.

The collection of blood samples was conducted at Obihiro University. For pregnancy blood samples, 31 normal cyclic cows were used. The animals were divided into two groups, and blood samples were taken on day 16 (n=10) of the oestrous cycle (not inseminated), as well as on day 16 (n=21) of pregnancy. Within the study of pregnant cows, artificial inseminations were carried out after an observation of oestrus (day 0) and ovulation (day 1) was confirmed by ultrasonography in all animals. Pregnancy was checked by ultrasonography at day 15 and again confirmed at day 40. In ten animals, no embryo was found by ultrasonography at day 40; therefore, these animals were excluded from the experiment.

Processing of the CL
The CL was enucleated from the ovary and dissected free of connective tissue as previously described (Shirasuna et al. 2008a). Subsequently, the CL cut into a cube shape (about 5 mm), was fixed with 10% formaldehyde for 24 h at room temperature and embedded in paraffin wax according to the standard histological technique. In addition, the remaining CL was prepared for molecular biological purposes. For analysis of mRNA expression, the luteal tissue samples were collected, minced and immediately placed into a 1.5 ml microcentrifuge tube with 400 µl TRIzol reagent (Invitrogen Corp.) and stored at −80 °C until analysis. For analysis of protein expression, the luteal tissue samples were placed into a 1.5 ml microcentrifuge tube with protein lysis buffer as the following describes.

RNA extraction, cDNA production and quantitative PCR
Total RNA was extracted from cells following the protocol of Chomczynsky & Sacchi (1987) using TRIzol reagent and was treated with DNase using a commercial kit (SV Total RNA
Isolation System, Promega Co.). Subsequently, RNA was frozen at –20 °C in THE RNA Storage Solution (Ambion, Inc., Austin, TX, USA). The cDNA was produced using the method of a previous study (Shirasuna et al. 2008b). The mRNA expression for ISG15, IL8, chemokine (C-X-C motif) ligand 6 (CXCL6), chemokine (C-C motif) ligand 2 (CCL2), tumour necrosis factor alpha (TNFα) and β-actin were examined by quantitative PCR with a LightCycler (Roche Diagnostics Co.), as per our previous study (Shirasuna et al. 2008b). RT-qPCR conditions were 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 20 s. The primers used for real-time PCR were as follows: forward, 5’-GGGATGATGAGCT-CAAGCATACT-3’ and reverse, 5’-ACCTCTTGCTGTAAGGT-3’ for ISG15 (accession no. NM_173925); forward, 5’-TGAGCCTGGAACTCAGGTC-3’ and reverse, 5’-TCTGGTTGTGGAGTGAGTC-3’ for IL8 (accession no. NM_001040606); forward, 5’-CTCCTTGTCAATATGACTTC-3’ and reverse, 5’-GGGCTACCTCTCAATAAATCTC-3’ for β-actin (accession no. NM_173925); forward, 5’-CTGGCCTTGAAGCACA-3’ and reverse, 5’-AAATACGGAGACAGAAATA-3’ for CXCL6 (accession no. AF_149924); forward, 5’-AAGCCTACCTGGCGCAAGGA-3’ and reverse, 5’-TGGGTGTTGGAGTGATGC-3’ for CCL2 (accession no. NM_281043); forward, 5’-TGACGGCTTACCTCTACTC-3’ and reverse, 5’-TGATGCCAGAGATGTT-3’ for TNFα (accession no. AF_348421) and forward, 5’-CCAGGGCAAGCTGAGAAAT-3’ and reverse, 5’-CCA-5CATTCCGTGAGGACTTTCC-3’ for β-actin (accession no. MN_173979.3). The PCR products were subjected to electrophoresis, and the target band was cut out and purified using a DNA purification kit (SUPRECTM-01, TaKaRa Bio, Inc., Otsu, Japan). The relative amount of each PCR product was calculated in comparison using β-actin as the internal standard. For each run, a standard curve was generated using tenfold serial dilution.

**Western blotting**

The luteal tissue samples were homogenised in a protein lysis buffer containing 25 mM Tris–HCl pH 7.4, 0.3 M sucrose, 2 mM Na2EDTA and protease inhibitor cocktail and then filtered through a 70 μm filter. The proteins were dissolved in a sample buffer (0.5 M Tris–HCl pH 6.8, glycerol, 10% SDS and 0.5% bromophenol blue) and steamed for 5 min. All of the samples were subjected to electrophoresis on 15% SDS–PAGE gels for 50 min at 200 V. The proteins were then transferred to PVDF membranes for 2 h at 60 V. The membranes were blocked with 4% Block ACE powder in TBS with 0.5% Tween-20 for 1 h at room temperature. The membranes were then incubated with a rabbit anti-bovine IL8 polyclonal antibody (1:1000 dilution; Kingfisher Biotech, Inc., St Paul, MN, USA) and a mouse anti-β-actin monoclonal clone AC-5 antibody (1:10 000 dilution). The membranes were then washed three times in TBS with 0.5% Tween-20, incubated with HRP-conjugated anti-rabbit (1:10 000 dilution; GE Healthcare UK Ltd, Little Chalfont, UK) or anti-mouse (1:10 000 dilution; Rockland Immunochemicals, Inc., Limerick, PA, USA) IgG antibodies for 1 h at room temperature and washed three times with TBS with 0.5% Tween-20. The signals were detected using an ECL Western Blotting Detection System (GE Healthcare UK Ltd). The optical densities of the immunospecific bands were quantified using an NIH image computer-assisted analysis system.

**Detection of neutrophils and macrophages in the CL using the periodic acid–Schiff reaction**

Formalin-fixed sections (5 μm) of the luteal tissue samples were stained with periodic acid–Schiff (PAS) reagent (Sigma) for 10 min and then counterstained with haematoxylin as previously described (Al-Zaib et al. 2002, Jiemtaweebon et al. 2011). In general, it is recognised that PAS staining is a useful method to detect granulophilic leukocytes. However, red blood cells and other immune cells, such as macrophages, are stained in the luteal tissue. Therefore, in addition to positive cells by PAS staining, we checked the shape of the nucleus of the cells and assessed segmented granulocytes as PMNs. Thus, we could distinguish PMNs, especially neutrophils, from other PAS-stained-positive cells, such as red blood cells and macrophages. To detect macrophages within the CL, sections were deparaffinised in xylene and rehydrated using decreasing concentrations of ethanol. The sections were incubated in Tris buffer (pH 10) at 98 °C for 10 min and immersed in 0.3% H2O2 in methanol for 1 h to block endogenous peroxidase activity. Sections were then rinsed with TBS, incubated with 4% Block ACE in TBS to reduce non-specific reactions and then incubated with MABs of human macrophage surface antigens (diluted 1:100; AM-3K, TransGenic, Inc., Hyogo, Japan) at 4 °C overnight. As a negative control, the sections were incubated with a mouse IgG as a control for murine primary antibodies (Vector Laboratories, Burlingame, CA, USA) overnight at 4 °C. Thereafter, sections were rinsed three times for 5 min each in TBS and incubated with biotinylated goat anti-mouse IgG at room temperature for 1 h. Sections were then incubated with avidin–biotin reagent (1:2) for 30 min, and positive signals were visualised using 0.02% 3,3’-diaminobenzidine tetrahydrochloride in 50 mM Tris–HCl (pH 7.4) containing 0.02% H2O2, followed by nuclear staining with haematoxylin.

These stains were used on each tissue block, and five fields per section were examined at ×400 magnification. The quantification of the number of PMNs and macrophages were performed independently by three observers. The results were expressed as means ± S.E.M. per unit area.

**Hormone determination**

The measurement of P4 concentration in the culture medium was performed using a direct enzyme immunoassay (EIA; Miyamoto et al. 1992). The EIAs for P4 were performed according to previous reports. Within-assay and between-assay coefficients of variation for P4 were 4.7 and 6.5% respectively. The ranges of the standard curves for these assays were 0.05–50 ng/ml, and the effective dose ED50 was 2.4 ng/ml for P4.

**Preparation of IFNT**

Recombinant bovine IFNT was produced by *Escherichia coli*. Briefly, bovine IFNT cDNA (bTP-509A, gifted by Dr R M Roberts, University of Missouri, Columbia, MO, USA) was
inserted into pET-21a (Invitrogen Corp.) E. coli expression vectors (Imakawa et al. 1987). After purification of crude IFNT by HPLC, we confirmed low levels of endotoxin using the LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ, USA). The activity of IFNT was then determined by a viral resistance assay using Madin-Darby bovine kidney cells and was found to be 59 050 IU/ml (5.95 × 10^5 IU/mg at 456 μM).

**Isolation of PMNs**

PMNs were isolated from whole blood collected via jugular venipuncture on days 8–12 of the oestrous cycle (day of ovulation = day 1) as described previously (Jiemtaweeboon et al. 2011). Blood samples were centrifuged at 1000 g for 30 min at 10 °C over Polymorphoprep (Axis-Shield, Oslo, Norway) according to the manufacturer's method. To remove red blood cells, ACK lysing buffer (Life Technologies) was added to PMNs and centrifuged at 500 g for 10 min at 10 °C. This lysis procedure was repeated twice on the cell pellet. Isolated PMNs were resuspended at a concentration of 2 × 10^6 cell/ml in RPMI-1640 (Invitrogen Corp.) containing 0.1% fasting blood sugar (FBS), gentamicin solution and amphotericin B solution. The purity of the PMNs, determined before using in the experiment, was >95%, and these cells resulted in nearly pure granulocyte populations as determined by flow cytometric evaluation (Beckman Coulter, Inc., City of Hope, CA, USA; Jiemtaweeboon et al. 2011). Additionally, we observed gimsa-stained PMNs by microscope, and these cells appeared clear granule and segmented nuclear.

**Transmigntation assay of PMNs**

PMN chemotaxis was evaluated using a ten-well microchemotaxis chamber (Neuro Probe, Gaithersburg, MD, USA) as described in our previous study (Jiemtaweeboon et al. 2011). In this instrument, test solutions in the bottom chamber are separated from leukocytes in the upper chamber by an 8-μm pore size filter (Neuro Probe). The following solutions (300 μl) were pipetted on the bottom chamber: RPMI-1640 medium alone as a control, IFNT (0.1 or 1 ng/ml), recombinant bovine IL8 (2.5 or 5 ng/ml; Kingfisher Biotech, Inc.) and recombinant ISG15 (0.1 or 1 ng/ml; Aviva Systems Biology, San Diego, CA, USA). Previously, it has been reported that bovine IL8 of 2.5 or 5 ng/ml stimulated steroidogenesis on granulosa cells without adverse effect of cell viability (Shimizu et al. 2012). In addition, similar doses of ISG15 (0.1–10 ng/ml) stimulate neutrophil chemotaxis in humans (Owashi et al. 2003); 5 ng/ml of IL8 was found to stimulate neutrophil migration (data not shown). After assembling the instrument, PMNs were added to the upper chamber (250 μl/well, 2 × 10^6 cell/ml). After incubation at 37 °C in 5% CO_2 for 3 h, migrated cells in the bottom chamber were counted using flow cytometry. At least four experiments were performed, with each concentration of agents tested with two replicates per experiments.

**Activation of PMNs by IFNT**

To investigate the effect of IFNT on PMN function, PMNs were incubated with or without IFNT (0.1 or 1 ng/ml) for 4 h. Subsequently, PMNs were washed with PBS twice to remove IFNT and used for a co-culture experiment with LCs or a migration assay with or without IL8.

**Steroidogenic LCs culture**

The CLs at the mid luteal phase were collected at a local slaughterhouse in Obihiro, Japan, and dispersed using collagenase IV (Sigma). The luteal stages were estimated as mid (days 8–12) by macroscopic observation of the ovary and uterus as described previously (Miyamoto et al. 1992). LCs were used in the present study using the method described by Klipper et al. (2004). Briefly, to isolate LCs, endothelial cells within the CL were removed from the bovine mid CL (days 8–12 of the oestrous cycle) using magnetic tosyl-activated beads coated with BS-1 lectin (binds glycoproteins on the bovine endothelial cells), indicating BS-1 positive cells are endothelial cells. Therefore, in the present study, BS-1 negative cells were assessed as steroidogenic LCs, as described previously (Klipper et al. 2004). LCs were cultured in DMEM/F12 medium (Invitrogen Corp.) containing 5% FBS (Invitrogen Corp.), 2.2% NaHCO₃, gentamicin solution (50 mg/l; Sigma) and amphotericin B solution (2.5 mg/l; Sigma).

LCs were cultured for 24 h after isolation, rinsed twice with PBS and stimulated with medium only (control), IFNT (0.1 or 1 ng/ml), IFN beta (IFNB, other type 1 IFN, 0.1 or 1 ng/ml; Kingfisher Biotech, Inc.), IFN gamma (IFNG, type 2 IFN, 0.1 or 1 ng/ml; Kingfisher Biotech, Inc.) or IL8 (2.5 or 5 ng/ml) for 24 h at 37 °C in DMEM/F12 medium containing 0.1% FBS, NaHCO₃, gentamicin solution and amphotericin B solution. At the end of the treatment period, culture media were collected for the determination of the P₄ concentration, and the cells were collected and stored at −80 °C until the mRGA expression was analysed. At least three experiments were performed, with each concentration of agents tested with two replicates per experiments.

**PMNs–LCs co-culture**

LCs of the mid luteal phase were plated as described above. PMNs were isolated the day after LCs were isolated. PMNs (2 × 10^6 cells/ml) were added to LCs under FBS-free conditions. The medium was collected 4 h after co-culture for P₄ analysis.

**Statistical analysis**

All data are presented as means ± S.E.M. The statistical significance of differences was assessed by a Student’s t-test or one-way ANOVA followed by Bonferroni's multiple comparison test. Probabilities <5% (P<0.05) were considered significant.

**Results**

**Change of plasma P₄ concentration and immune-related factors within the CL in pregnant or non-pregnant cows**

Plasma samples were collected on days 10 and 16 from pregnant and non-pregnant animals, and P₄...
CXCL6, another neutrophilic chemoattractant, did not differ between pregnant and non-pregnant cows (Fig. 1E). Although mRNA expression of CCL2 (a major chemokine of macrophages) was at the same level within both groups, mRNA expression of TNFz (inflammatory cytokine secreted mainly from macrophages) was significantly lower in the CL of pregnant animals than in non-pregnant animals (Fig. 1G).

**Change of PMNs and macrophages within the CL in pregnant or non-pregnant cows**

Figure 2 shows PMNs (Fig. 2A) and macrophages (Fig. 2B) within the CL during pregnancy and non-pregnancy, as detected by PAS staining or anti-macrophage staining respectively. The number of PMNs within the CL was significantly higher (P<0.05) during pregnancy compared with non-pregnancy. The number of macrophages within the CL did not differ between pregnancy and non-pregnancy.

**Effects of IFNT on secretion of P4 and expression of ISG15 and IL8 mRNA on LCs**

IFNT did not change P4 secretion from LCs (Fig. 3A). ISG15 mRNA expression increased in a dose-dependent manner on treatment with IFNT, confirming that LCs can respond with IFNT treatment in vitro (Fig. 3B). Both IFNG (type 2 IFN) and IFNB (type 1 IFN) also increased ISG15 mRNA expression at 1 ng/ml treatment (Fig. 3B). Additionally, IFNT at 1 ng/ml clearly stimulated concentrations were determined as shown in Fig. 1A. Although plasma P4 concentration did not differ on day 10 between pregnant and non-pregnant animals, plasma P4 was significantly higher in pregnant animals compared with non-pregnant animals on day 16, suggesting that the non-pregnant animals on day 16 were undergoing luteolysis. Previous studies demonstrated that ISG15 was upregulated in the CL by IFNT during the maternal recognition period in cows (Gifford et al. 2007, Bott et al. 2010). Similarly, the present study demonstrated that the mRNA expression of ISG15 tended to increase (P<0.1) in the CL of the pregnant animals compared with non-pregnant animals (Fig. 1B). The mRNA expression of OAS1, another marker of IFN response, clearly increased within the CL of the pregnant animals, which was not observed in non-pregnant animals (data not shown), thereby confirming the previous hypothesis that IFNT may influence the CL via local or peripheral circulation. The CL of pregnant animals had a higher expression of IL8 mRNA (Fig. 1C) and its protein (Fig. 1D) compared with the CL of non-pregnant animals. However, mRNA expression of
expression of IL8 mRNA in LCs (Fig. 3C). On the other hand, IFNG, but not IFNB, also stimulated IL8 mRNA expression in LCs (Fig. 3C), suggesting that the stimulatory effect of IFNT for ISG15 and IL8 mRNA was greater compared with IFNG or IFNB but that this effect was not specific to IFNT in LCs.

**Effects of IFNT, IL8 and ISG15 on PMN migration**

To clarify the mechanism of PMN recruitment within the CL during the maternal recognition period, we investigated the effect of candidate factors such as IFNT, IL8 and ISG15 on PMN migration in vitro. Although the capacity of PMN migration did not change with IFNT treatment (Fig. 4A), PMN migration was significantly stimulated by IL8 treatment in a dose-dependent manner (Fig. 4B). Interestingly, recombinant ISG15 also increased PMN migration in vitro (Fig. 4C).

**Change of PMN capacity by IFNT**

The data of Fig. 4A, showing that IFNT did not affect PMN migration, led us to hypothesise that IFNT changes PMN capacity or function. At first, we investigated the migratory capacity of PMNs treated with IFNT compared to PMNs without IFNT. PMNs were treated with IFNT at 1 ng/ml for 4 h before the assay. Subsequently, we determined migratory capacities induced by IL8 (Fig. 5A). IL8 (2.5 and 5 ng/ml) significantly stimulated PMN migration in both IFNT-treated and non-treated groups, revealing higher migration levels in IFNT-treated PMNs than in non-treated PMNs. Next, we investigated the PMN function on P4 secretion from LCs using direct co-culture systems (Fig. 5B). PMNs were isolated from peripheral blood during the mid

luteal phase in cows and then activated with or without IFNT (0.1 or 1 ng/ml) for 4 h before use in the co-culture experiment. Although normal PMNs (non-treated with IFNT, concentration of 2 × 10⁶ cells/ml) did not affect P4 secretion from LCs, IFNT-activated PMNs marginally but significantly increased P4 secretion from LCs compared with the control group (without PMNs). Finally, we investigated the direct effect of IL8 on luteal function in vitro and showed that IL8 clearly increased P4 secretion from LCs dose dependently (Fig. 5C).

**Discussion**

The present study investigated changes of the CL during the maternal recognition period (day 16) in cows. Our novel finding was the increase in neutrophils and IL8 within the CL during pregnancy, suggesting the involvement of the IFNT function within the CL in preparation for pregnancy in cows. Although IFNT did not affect P4 secretion and neutrophil migration directly, IFNT stimulated IL8 mRNA expression on LCs and changed neutrophilic ability, resulting in an increased migration in IFNT-activated neutrophils. Moreover, both IFNT-activated neutrophils and IL8 increased P4 secretion from LCs in vitro. Taken together, the present results suggest IFNT upregulates neutrophil numbers and function via IL8 on LCs in the CL of the early pregnant animals, and both neutrophils and IL8, stimulated by IFNT, are associated with the increase in P4 concentrations during the maternal recognition period in cows.

PMNs are the most important immune cells as the first line of cellular defence against acute inflammatory reaction (Paape et al. 2003). PMNs have been detected in the CL in cows, humans and rats during the oestrous (menstrual in humans) cycle and early pregnancy
showed the increase in mRNA expression of ISG15 and IL8 by IFNT treatment in LCs. These findings led us to hypothesise that IFNT regulates the luteal function and induces migration of PMNs within the CL during the maternal recognition period in cows.

In the next step, we focused on the mechanisms enabling PMNs to infiltrate the CL of pregnant animals during the maternal recognition period. Although it was thought that IFNT had the potential to induce PMNs migration directly, IFNT did not induce PMN migration in the in vitro migration assay. As expected, IL8 clearly stimulated PMN migration in a dose-dependent manner. Importantly, ISG15 protein also induced PMN migration. Therefore, it is suggested as a possible mechanism that the IFNT signal regulates the expression of IL8 as well as ISG15, thereby allowing PMNs to infiltrate the CL during pregnancy by the chemotactic effects of IL8 and ISG15 during the maternal period. On the other hand, Bott et al. (2010) previously reported that infusion of IFNT into the uterine vein increased ISG15 mRNA expression in the endometrium in ewes. In addition, Ashley et al. (2010) investigated the reproductive phenotype in ISG15−/− mice and demonstrated that up to 50% of the embryos die between 7.5 and 12.5 days post-coitum in ISG15−/− mothers when mated to ISG15−/− fathers. These findings suggest some essential roles of ISG15 for pregnancy, and further studies are required to clarify the detailed function of ISG15, not only as an IFNT marker but also as a key regulatory factor for pregnancy in cows.

P4 released from the CL is a central hormone to establish pregnancy. In the present study, we discarded our expected hypothesis that IFNT directly stimulates the secretion of P4 from mid-LCs. Our findings confirm recent findings by Talbott et al. (2014) in which normal-PMNs (no activation by IFNT) did not affect P4 secretion from mid-LCs in vitro. Interestingly, IFNT-activated PMNs significantly increased P4 secretion levels, suggesting that a high number of PMNs (maybe activated by IFNT) within the CL could be one of the reasons for the increase in plasma P4 levels at day 16 of pregnancy (Fig. 1A). Moreover, IFNT pretreatment changed the migratory sensitivity of PMNs for IL8. Actually, type 1 IFN signalling expands the neutrophil lifespan and regulates the expression of anti-apoptotic factors (Taylor et al. 2011). These findings suggest that IFNT regulates the neutrophil capacity including migration, lifespan and apoptosis. Therefore, neutrophils activated by IFNT might stimulate P4 secretion. However, we could not demonstrate the detailed mechanism of the increase in P4 secretion by IFNT-activated PMNs in the present study, and further studies are required. In addition, IL8, stimulated by IFNT, has a potential to upregulate P4 secretion, as is shown in both our current and recent studies (Shimizu et al. 2012). In contrast, Talbott et al. (2014) showed that IL8 did not alter P4 secretion in purified luteal steroidogenic cells. This is considered a possible reason that may have led to the differences in the outcomes of the experiments, which
may account for differential cellular responses in P₄ secretion, involving the isolation of LCs and culture medium. Thus, further investigations are required to elucidate the precise mechanisms underlying the effects of IL8 on luteal function.

Macrophages are one of the regulatory immune cells controlling immunotolerance by secreting cytokines, differentiating M1 or M2 macrophages and modulating inflammation (Sica et al. 2008, Coffelt et al. 2009). Macrophages play a fundamental role in establishing pregnancy because, as Care et al. (2013) demonstrated, macrophage depletion after conception causes embryo implantation arrest associated with diminished plasma P₄ concentrations, because macrophage depletion results in the disruption of the luteal vascular network. In our results, there were no differences in the number of macrophages within the CL between non-pregnancy and pregnancy in cows. On the other hand, mRNA expression of TNFα (inflammatory cytokine secreted mainly by macrophages) was present in significantly lower levels in the CL of pregnant animals than that of non-pregnant animals, suggesting the qualitative change of macrophages within the CL. For instance, macrophages activated by pro-inflammatory cytokines (TNFα) differentiate M1-type macrophages and promote inflammatory responses, whereas M2-type macrophages produce anti-inflammatory cytokines, including IL10 and PGE2 (Sica et al. 2008, Coffelt et al. 2009). Therefore, we hypothesised that the characteristics of these macrophages are assumed to differ depending on each luteal environment. However, future investigation is required to clarify the importance of macrophages within the CL during pregnancy in cows.

In summary, the present results demonstrate that the immune microenvironment in the bovine CL during the maternal recognition period of pregnant animals was different from the CL of non-pregnant animals. The present study found an increase in neutrophils and IL8 within the CL during pregnancy. In addition, IFN systems (IFNT and ISG15) have the potential to regulate luteal function. Therefore, authors have speculated that IFNT is a maternal recognition signal that induces up-regulation of regulatory cytokines and down-regulation of adhesion genes: implication for regulation by IL-1β. 

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References


Jientaweeboon S, Shirasuna K, Nitta A, Kobayashi A, Schubert HJ, Shimizu T & Miyamoto A 2011 Evidence that polymorphonuclear...


