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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 $PGF_{2\alpha}$. 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 as compared with the CL of non-pregnant animals. Although IFNT did not affect progesterone (P_4) 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 P_4 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 P_4 concentrations during the maternal recognition period in cows.

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Introduction

The corpus luteum (CL) is formed from the ovulated follicle and grows rapidly to secrete progesterone (P_4), 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 $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$ 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

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CL, where macrophage depletion after conception caused embryo implantation arrest, associated with diminished plasma P_4 concentration and poor uterine receptivity in mice. In addition, a considerable number of neutrophils and high concentrations of interleukin 8 (IL8, neutrophil-specific chemoattractant) during the early luteal phase in the CL of cows (Jiemtaweeboon *et al.* 2011) have been reported. On the other hand, Poole & Pate (2012) reported an increase in the CD8αβ+ and γδCD8αβ+ T cell population within the CL during early pregnancy (day 18 after insemination) compared to the CL of non-pregnant animals. However, at present, there are few data concerning immune cell population within the CL during the maternal recognition period in 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 P_4 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 of 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 $PGF_{2\alpha}$ and then GnRH at 48 h after $PGF_{2\alpha}$. 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 effeminator (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.* 2008*a*). 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 Chomczynski & 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*. 2008*b*). 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*. 2008*b*). 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′-GGTATGATGCGAGCT-GAAGCACTT-3′ and reverse, 5′-ACCTCCCTGCTGTCAAGGT-3′ for ISG15 (accession no. NM_174366); forward, 5′-TAGGC-CTGGAACATCAGGTC-3′ and reverse, 5′-TTTGGTCTGGCT-GGATTACC-3′ for 2′-5′-oligoadenylate synthetase 1 (OAS1; accession no. NM_001040606); forward, 5′-CCTCTT-GTTCAATATGACTTCCA-3′ and reverse, 5′-GGCCCACTCT-CAATAACTCTC-3′ for *IL8* (accession no. NM_173925); forward, 5′-CTGGCCCACTGAAGACAA-3′ and reverse, 5′-AAATACGGAGCAGGAAATAATA-3′ for *CXCL6* (accession no. AF_149249); forward, 5′-AAGACCATCCTGGGCAAGGA-3′ and reverse, 5′-TGGGTTGTGGAGTGAGTGCTC-3′ for CCL2 (accession no. NM_281043); forward, 5′-TGACGGGCTT-TACCTCATCT-3′ and reverse, 5′-TGATGGCAGACAGGAT-GTTG-3′ for *TNFα* (accession no. AF_348421) and forward, 5′-CCAAGGCCAACCGTGAGAAAAT-3′ and reverse, 5′-CCA-CATTCCGTGAGGATCTTCA-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 Na_2EDTA 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-15 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., Limerich, 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-Zi'abi *et al*. 2002, Jiemtaweeboon *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% H_2O_2 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% H_2O_2, 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 P_4 concentration in the culture medium was performed using a direct enzyme immunoassay (EIA; Miyamoto *et al*. 1992). The EIAs for P_4 were performed according to previous reports. Within-assay and between-assay coefficients of variation for P_4 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 ED_{50} was 2.4 ng/ml for P_4.

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 manufacture'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 giemsa-stained PMNs by microscope, and these cells appeared clear granule and segmented nuclear.

Transmigration 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 onto 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 (Owhashi *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 replications 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% $NaHCO_3$, 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, $NaHCO_3$, gentamicin solution and amphotericin B solution. At the end of the treatment period, culture media were collected for the determination of the P_4 concentration, and the cells were collected and stored at −80 °C until the mRNA 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 P_4 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 P_4 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 P_4

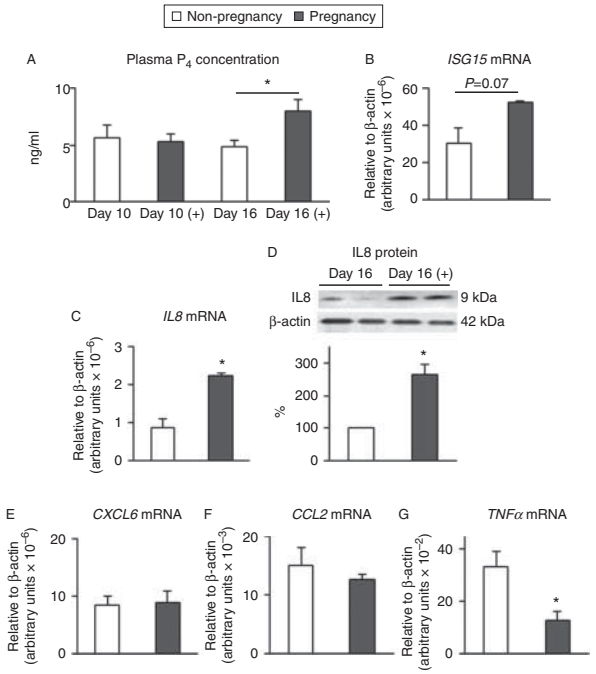

Figure 1 Plasma P_4 and immune-related factors in the bovine CL during maternal recognition period. (A) The plasma P_4 concentration at days 10 and 16 during oestrous cycle (white bars) or pregnancy (black bars) respectively ($n=10$ respectively). (B, C, D, E, F and G) The expressions of *ISG15* mRNA (B), IL8 mRNA (C), IL8 protein (D), *CXCL6* mRNA (E), *CCL2* mRNA (F) and *TNFα* mRNA (G) in the CL at day 16 during the oestrous cycle or pregnancy respectively (non-pregnancy, $n=4$ and pregnancy, $n=5$). White bars indicate non-pregnant cows and black bars indicate pregnant cows. All values are shown as the mean±S.E.M. (relative to β-actin mRNA levels). Significant differences (*$P<0.05$) as determined by Student's *t*-test.

concentrations were determined as shown in Fig. 1A. Although plasma P_4 concentration did not differ on day 10 between pregnant and non-pregnant animals, plasma P_4 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 *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 TNFα (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 P_4 and expression of ISG15 and IL8 mRNA on LCs

IFNT did not change P_4 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

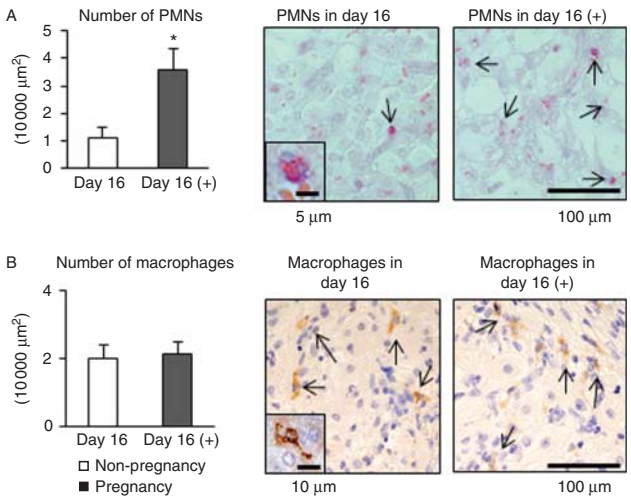

Figure 2 PMN and macrophages in the CL. (A) The number of PMNs at day 16 during the oestrous cycle (white bars) or pregnancy (black bars), and the typical images of PMNs within the CL are shown respectively. Black arrows show PMNs in the CL. (B) The number of macrophages at day 16 during the oestrous cycle or pregnancy, and the typical images of macrophages within the CL are shown respectively. White bars indicate non-pregnant cow and black bars indicate pregnant cow. All values are shown as the mean±S.E.M. Significant differences (*$P<0.05$) as determined by Student's *t*-test.

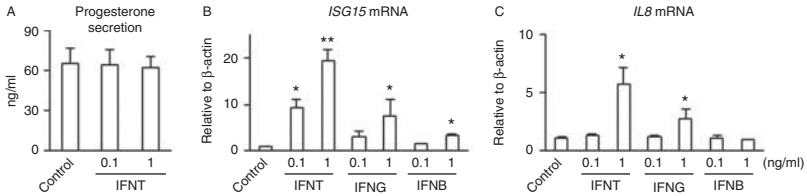

Figure 3 Regulation of mRNA expression of *ISG15* and *IL8* and secretion of P_4 by IFNT on luteal cells (LCs). P_4 secretion in culture medium (A), mRNA expression of *ISG15* (B) and mRNA expression of *IL8* (C) on LCs after IFNT, IFNG or IFNB treatment are shown. All values are shown as the mean±S.E.M. (relative to β-actin mRNA levels). Different superscript letters indicate significant difference ($P<0.05$ or $^{**}P<0.01$) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

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 P_4 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 2×10^6 cells/ml) did not affect P_4 secretion from LCs, IFNT-activated PMNs marginally but significantly increased P_4 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 P_4 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 P_4 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 P_4 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 P_4 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

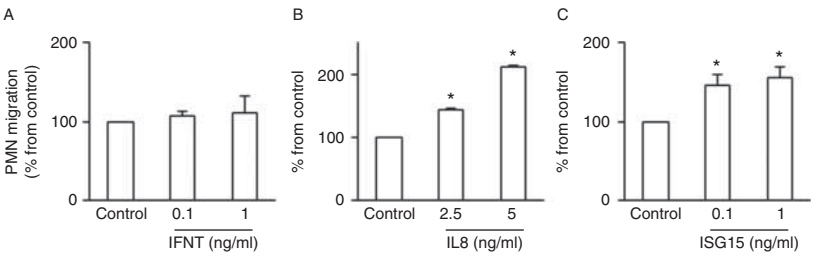

Figure 4 Transmigration assay of PMNs by IFNT, IL8 and ISG15. The number of PMNs migration by IFNT (A), IL8 (B) and ISG15 (C) are shown. All values are shown as the mean±S.E.M. Significant differences ($^*P<0.05$) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

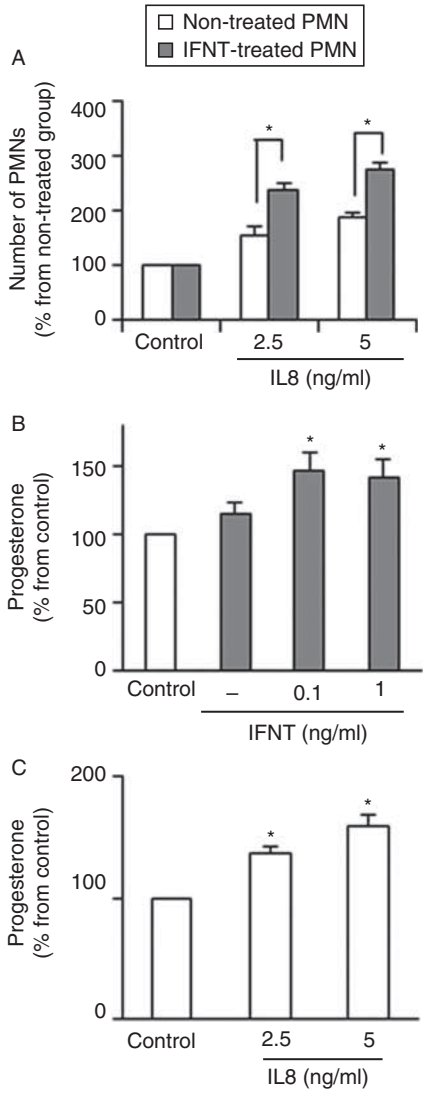

Figure 5 Effect of IFNT on PMN function and effect of IL8 on luteal function. (A) The number of PMNs that were stimulated by migration, with or without IFNT treatment before migration assay. White bars indicate non-IFNT-treated PMNs and black bars indicate IFNT-treated PMNs. (B) P_4 secretion from luteal cells (LCs) co-cultured with or without PMNs. Black bars indicate co-cultured experimental groups between LCs and PMNs. (C) P_4 secretion from LCs treated with IL8. All values are shown as the mean ± S.E.M. Significant differences (*$P<0.05$) as determined by one-way ANOVA followed by Bonferroni's multiple comparison test.

(Brannstrom *et al.* 1994, Best *et al.* 1996, Brannstrom & Friden 1997, Jiemtaweeboon *et al.* 2011). Especially, it has been recently demonstrated that PMNs have multiple functions within the CL, such as luteal development, angiogenesis and luteolysis induced by inflammation in cows (Jiemtaweeboon *et al.* 2011). Interestingly, a large number of PMNs, together with high levels of IL8, were observed within the CL during the maternal recognition period, suggesting that these changes within the CL are the direct result of the function of IFNT during pregnancy. Indeed, the present study 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.

P_4 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 P_4 from mid-LCs. Our findings confirm recent findings by Talbott *et al.* (2014) in which normal-PMNs (no activation by IFNT) did not affect P_4 secretion from mid-LCs *in vitro*. Interestingly, IFNT-activated PMNs significantly increased P_4 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 P_4 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 P_4 secretion. However, we could not demonstrate the detailed mechanism of the increase in P_4 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 P_4 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 P_4 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 P_4 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 P_4 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 and neutrophilic functions. Taken together, we suggest that IFNT is a maternal recognition signal that induces dramatic changes not only in the uterus but also in the CL during the maternal recognition period (day 16) in cows.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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