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—Original Article—

Upregulation of Interferon-stimulated Genes and Interleukin-10 in Peripheral Blood Immune Cells During Early Pregnancy in Dairy Cows

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Abstract. In cows, interferon-tau (IFNT) regulates maternal recognition around days 15–19 after artificial insemination (AI). The present study hypothesized that if key target genes of IFNT are clearly upregulated in earlier stages of pregnancy, these genes could be use as indices of future pregnancy in cows. Therefore, we determined the expression of these genes in peripheral blood mononuclear leukocytes (PBMCs) and polymorphonuclear granulocytes (PMNs) during the maternal recognition period (MRP). Twenty multiparous Holstein cows were subjected to AI on day 0 and categorized into the following groups: pregnancy (Preg, n = 9), embryonic death (ED, n = 5) and non-pregnancy (NP, n = 6). Progesterone levels in the Preg group were higher than those in the NP group on days 12–21. ISG15 and OAS-1 (IFN-stimulated genes: ISGs) mRNA in PBMCs on day 8 was higher in the Preg group than in the NP group, and these mRNAs in PMNs was higher in the Preg group on day 5 than in the NP and ED groups. Interleukin-10 (IL-10, Th2 cytokine) mRNA expression increased on day 8 in the PBMCs of pregnant cows. Tumor necrosis factor α (TNFα, Th1 cytokine) mRNA expression was stable in all groups. In an *in vitro* cell culture experiment, IFNT stimulated mRNA expression of ISGs in both PBMCs and PMNs. IFNT stimulated IL-10 mRNA expression in PBMCs, whereas IFNT increased TNFα mRNA levels in PBMCs *in vitro*. The results suggest that ISGs and IL-10 could be responsive to IFNT before the MRP in peripheral blood immune cells and may be useful target genes for reliable indices of pregnancy before the MRP.

Key words: Cow, Immune cells, Interferon tau, Pregnancy

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During the past 5 decades, milk production per cow has dramatically increased because of improved management, nutrition, and genetic selection [1, 2]. In contrast, the decreases in fertility and conception rates of the modern high-producing dairy cow are the major causes of economic loss for dairy producers [2, 3]. After establishment of pregnancy in domestic ruminants, the conceptus secretes interferon-tau (IFNT) as a maternal recognition factor [4]. IFNT acts in the uterus around day 16 after insemination and prevents luteolysis by inhibiting prostaglandin $F_{2\alpha}$ release, resulting in the maintenance of corpus luteum function; therefore, this period is termed the maternal recognition period (MRP) [5]. IFNT induces the synthesis and secretion of IFN-stimulated genes (ISGs) such as ISG15, 2′,5′-oligoadenylate synthetase (OAS-1), IFN regulatory factor 1, Mx1 and Mx2 not only in the uterus but also in blood cells in ewes and cows [6–10]. In fact, in bovine peripheral blood leukocytes, ISG15 mRNA levels were higher in pregnant cows than in nonpregnant cows on days 18 and 20 after artificial insemination (AI) [7–9]. Technology for the early detection of pregnancy is needed to identify nonpregnant cows and to synchronize and artificially inseminate these cows prior to the next ovulation, and many studies have focused on identifying easy-to-use markers of pregnancy using blood samples. These previous studies investigated possible marker genes using whole-blood leukocytes and peripheral blood mononuclear cells (PBMCs) because leukocytes and PBMCs contain T lymphocytes, which are required to induce an immune response. However, leukocytes comprise different types of cells such as T lymphocytes, monocytes, neutrophils and eosinophils. Thus, in the present study, we investigated the gene expression of ISGs in PBMCs (lymphocytes and monocytes) and polymorphonuclear granulocytes (PMNs: neutrophils and eosinophils) separately following AI.

Peak levels of IFNT are observed around days 15–19 of pregnancy in cows. However, Green *et al.* [9] reported that Mx2 mRNA levels in leukocytes increased on day 14 of pregnancy following AI compared with the levels in nonpregnant cows. Additionally, IFNT mRNA is first expressed in 16-cell stage *in vitro* fertilized embryos on day 4 [11], and IFNT protein was detected in the trophectoderm of nonhatched blastocysts on day 7 using confocal microscopy [12]. These findings suggest that IFNT signals are transmitted before

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the MRP (around days 15–19) in cows. On the other hand, general alterations occur in the maternal inflammatory and immune system during pregnancy. A shift from T-helper 1 (Th1) to T-helper 2 (Th2) cytokines is considered responsible for this immunomodulation during pregnancy. For example, inflammatory cytokines such as tumor necrosis factor α (TNFα) and IFNγ can terminate normal pregnancy upon injection during early gestation [13]. In contrast, anti-inflammatory cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGFβ) play crucial roles in preventing fetal loss [13, 14]. Therefore, we hypothesized that IFNT can potentially regulate ISGs and the Th1/Th2 balance in immune cells during the early phase of pregnancy in cows because IFNT prevents fetal demise in pregnant mice [13].

The aim of the present study was to examine the time-dependent changes in the mRNA expression of ISGs and Th1/Th2-related factors in PBMCs and PMNs after AI *in vivo* and the effect of IFNT treatment *in vitro* on PBMCs and PMNs obtained on different days of the estrous cycle.

Materials and Methods

In vivo study

The blood collection experiments were conducted at the Field Center of Animal Science and Agriculture, Obihiro University, and all experimental procedures complied with the Guidelines for the Care and Use of Agricultural Animals of Obihiro University. Between September 2010 and December 2010, 20 multiparous Holstein cows were artificially inseminated on day 0. Blood was collected on days 1–2, 5, 8, 12, 15, 18, and 21 of pregnancy following AI to detect plasma progesterone (P4) concentrations and separate immune cells. In addition, blood was continuously collected on days 24, 27, 30, 35, and 40 of pregnancy to detect plasma P4 concentrations. Pregnancy was confirmed by ultrasonography on day 40. Plasma P4 concentrations were determined by direct enzyme immunoassays [15].

Whole blood (20 ml) was mixed with an equal volume of PBS. The suspension was layered onto Ficoll-Paque solution (Lymphoprep, Axis-Shield, Oslo, Norway) and then centrifuged at 1000 × *g* at 10 C for 30 min as described previous study [16–18]. The plasma and buffy coat (population of mononuclear cells) were separated as PBMCs layer. After removing PBMCs, contaminating red blood cells were washed in hypotonic distilled water for approximately 10 sec. Isotonicity was restored by the addition of twice-concentrated PBS. PMNs were centrifuged at 500 × *g* at 10 C for 10 min, and then the cell pellet was washed twice with PBS. Isolated PBMCs and PMNs were resuspended at a concentration of 2×10^6 cells/ml in RPMI 1640 (Invitrogen Japan, Tokyo, Japan) with 0.1% fetal bovine serum (FBS, Invitrogen Japan). The viability of PBMCs and PMNs was 99% as assessed by the Trypan blue staining. To check the purity of PBMCs and PMNs before use in the experiment, the PBMCs and PMNs were subjected to a flow cytometric evaluation (Beckman Coulter, Brea, CA, UAS), and they were found to be >98% and >95% pure, respectively (Fig. 2) [16–18]. To analyze mRNA expression, PMNs and PBMCs were placed into 1.5-ml microcentrifuge tubes with 400 µl of TRIzol reagent and stored at −80 C until analysis.

In vitro study

PBMCs and PMNs were isolated from whole blood collected via jugular venipuncture on days 4–5, 7–8 and 10–12 of the estrous cycle (day of ovulation = day 1; non-AI cows, n = 4) as described previously [17, 18]. Isolated PBMCs and PMNs were resuspended at a concentration of 5×10^6 cell/ml in RPMI 1640 medium containing 0.1% FBS, gentamicin (50 mg/l) and amphotericin B (2.5 mg/l) purchased from Sigma (St. Louis, MO, USA). Cells were incubated in the same medium with the following treatments: control (no further addition) or IFNT (0.1, 1 or 10 ng/ml; recombinant bovine IFNT was purchased from Pestka Biomedical Laboratories, Piscataway, NJ, USA. Specific activity as determined in a viral resistance assay using bovine kidney MDBK cells was 1×10^6 IU/ml). Each treatment was performed in 48-well plates in duplicate. Incubation was performed for 4 h at 37 C. At the end of the treatment period, cells were stored at −80 C until mRNA expression was analyzed.

RNA isolation and real-time PCR

Total RNA was extracted following the protocol of Chomczynski and Sacchi using TRIzol reagent [19] as in our previous study [20]. The extracted total RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at –80 C until use for cDNA production. RNA samples were treated with DNase using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) as in our previous study [20]. The synthesized cDNA was stored at –30 C.

The mRNA expression of ISG15, OAS-1, IL-10, TNFα, and β-actin was quantified by real-time PCR with a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) as described in our previous study [20]. The primers used for real-time PCR were as follows: 5′-ggtatgatgcgagctgaagcactt-3′, forward, and 5′-acctccctgctgtcaaggt-3′, reverse, for ISG15 (Accession No. NM_174366); 5′-taggcctggaacatcaggtc-3′, forward, and 5′-tttggtctggctggattacc-3′, reverse, for OAS-1 (Accession No. MN_001040606); 5′-ttctgccctgcgaaaacaa-3′, forward, and 5′-tctcttggagctcactgaagactct-3′, reverse, for IL-10 (Accession No. NM_174088); 5′-tgacgggctttacctcatct-3′, forward, and 5′-tgatggcagacaggatgttg-3′, reverse, for TNFα (Accession No. AF_348421); and 5′-ccaaggccaaccgtgagaaaat-3′, forward, and 5′-ccacattccgtgaggatcttca-3′, reverse, for β-actin (Accession No. MN_173979.3). The PCR products were subjected to electrophoresis, and the target band was excised and purified using a DNA purification kit (SUPRECTM-01; Takara Bio, Otsu, Japan). The expression of each gene was normalized using β-actin as the internal standard.

Statistical analysis

All data are presented as means ± SEM. The statistical significance of differences was assessed by time-dependent repeated measures ANOVA followed by Fisher's multiple comparison test. Probabilities less than 5% ($P<0.05$) were considered significant.

Results

Change in plasma P4 concentration after AI

On the basis of the results of transrectal ultrasound on day 40 and plasma P4 levels after AI, we categorized the cows into the following groups: pregnancy (Preg, n = 9, detected embryo on day

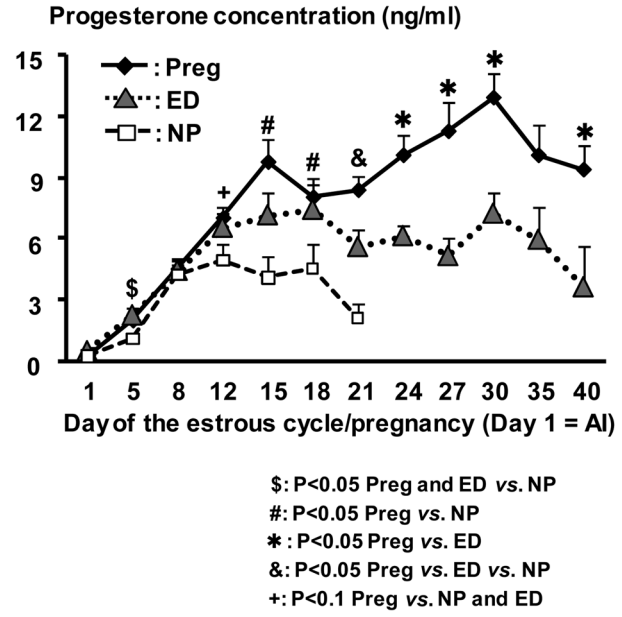

Fig. 1. Changes in plasma P4 concentrations following AI. Changes in plasma P4 concentrations following AI are shown. Black lozenges, gray triangles and white squares indicate the pregnancy (n = 9), embryonic death (n = 5), and nonpregnancy groups (n = 6), respectively.

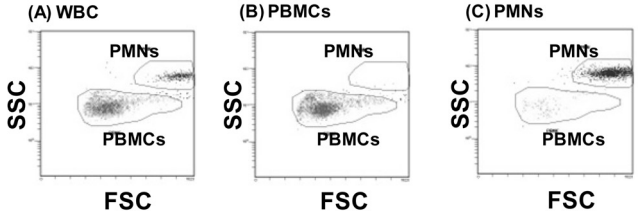

Fig. 2. Separation of PBMCs and PMNs from peripheral white blood cells. Before the analysis and experiment, we evaluated the purity of PBMCs and PMNs separated from white blood cells using flow cytometry. Fig. 2A indicates the plotting data of peripheral white blood cells (not separate between PMNs and PBMCs). PBMCs were separated using Lymphoprep, and the purity of the PBMCs was >98% (Fig. 2B). After removing the PBMC layer using Lymphoprep, we observed clear separation of the PMN population (Fig. 2C), and the purity of the PMNs was >95%. These cells resulted in nearly pure granulocyte populations as determined by flow cytometric evaluation.

40 and high P4 levels), embryonic death (ED, n = 5, high P4 levels but no embryo detected on day 40) and nonpregnancy (NP, n = 6, return of the estrous cycle around day 21) (Fig. 1). On day 5 after AI, plasma P4 levels were higher in the Preg and ED groups than in the NP group. Additionally, P4 levels in the Preg group were continuously higher than those in the NP group on days 12–21. P4 concentrations in the ED group were similar to those in the NP group on days 8–18 and remained lower than those in the Preg group after the MRP.

Purity assessment of PBMCs and PMNs from the bovine white blood cells

Before analysis of mRNA expression in *in vivo* and *in vitro* experiments, we evaluated the purity of PBMCs and PMNs separated from white blood cells using flow cytometry. Fig. 2A represents the plotting data of peripheral white blood cells (not separated between PBMCs and PMNs). After removing the PBMC layer using Lymphoprep, clear separation of the PBMC (Fig. 2B) and PMN populations was confirmed (Fig. 2C). The PBMCs and PMNs were >98% and >95% pure, respectively.

Expression of ISG15, OAS-1, IL-10 and TNFα mRNA between PBMCs and PMNs after AI

In the PBMCs of the Preg group, ISG15 mRNA expression was higher on days 8 ($P<0.05$) and 12 ($P<0.1$) compared with the NP and ED groups after AI (Fig. 3A). In the PMNs of the Preg group, ISG15 mRNA expression was already higher on days 5 ($P<0.05$), 8 and 12 ($P<0.1$) compared with the NP and ED groups after AI (Fig. 3B). Similar to the ISG15 mRNA expression, OAS-1 mRNA was increased on days 8 (vs. NP) and 12 (vs. NP and ED) in the PBMCs of Preg group. On day 21, OSA-1 mRNA was higher in the PBMCs of the Preg and ED groups compared with the NP group (Fig. 3C). Moreover, in the PMNs of Preg group, OAS-1 mRNA expression was already higher on days 5 ($P<0.1$), 8 and 12 ($P<0.05$) compared with the NP and/or ED group. On day 18, OAS-1 mRNA in PMNs in the Preg group was significantly higher than in the NP group, and OAS-1 mRNA increased on day 21 in the Preg and ED groups than in the NP group (Fig. 3D). In the PBMCs of Preg group, IL-10 mRNA expression was continuously higher from days 8 to 21 after AI compared with the NP and ED groups (Fig. 3E). IL-10 mRNA in PMNs did not significantly change during the experimental period (Fig. 3F). On the other hand, TNFα mRNA expression did not differ due to pregnancy status in both PBMCs and PMNs (Fig. 3G and H).

Effect of IFNT on mRNA expression of ISG15, OAS-1, IL-10 and TNFα in PBMCs and PMNs

In PBMCs, IFNT at 1 and 10 ng/ml stimulated mRNA expression of ISG15 and OAS-1 on all experimental days of the estrous cycle (Fig. 4A and C). In PMNs, IFNT at 0.1 ng/ml clearly increased mRNA expression of ISG15 and OAS-1 in all stages of the estrous cycle (Fig. 4B and D). The increase ratio of ISG15 and OAS-1 mRNA in PMNs was about double that of PBMCs. Although IFNT stimulated IL-10 mRNA expression in PBMCs, IL-10 mRNA was decreased by IFNT treatment in PMNs on days 10–12 of the estrous cycle (Fig. 4E and F). TNFα mRNA expression was upregulated by IFNT treatment (1 and 10 ng/ml) in PBMCs on days 7–8 and 10–12 of the estrous cycle. Also, mRNA expression of TNFα tended to be increased by IFNT at 10 ng/ml in PMNs on days 10–12 of the estrous cycle.

Discussion

In the present study, we categorized the cows into 3 groups: pregnancy (Preg, detected embryo on day 40 and high P4 levels), embryonic death (ED, high P4 levels but no embryo detected on

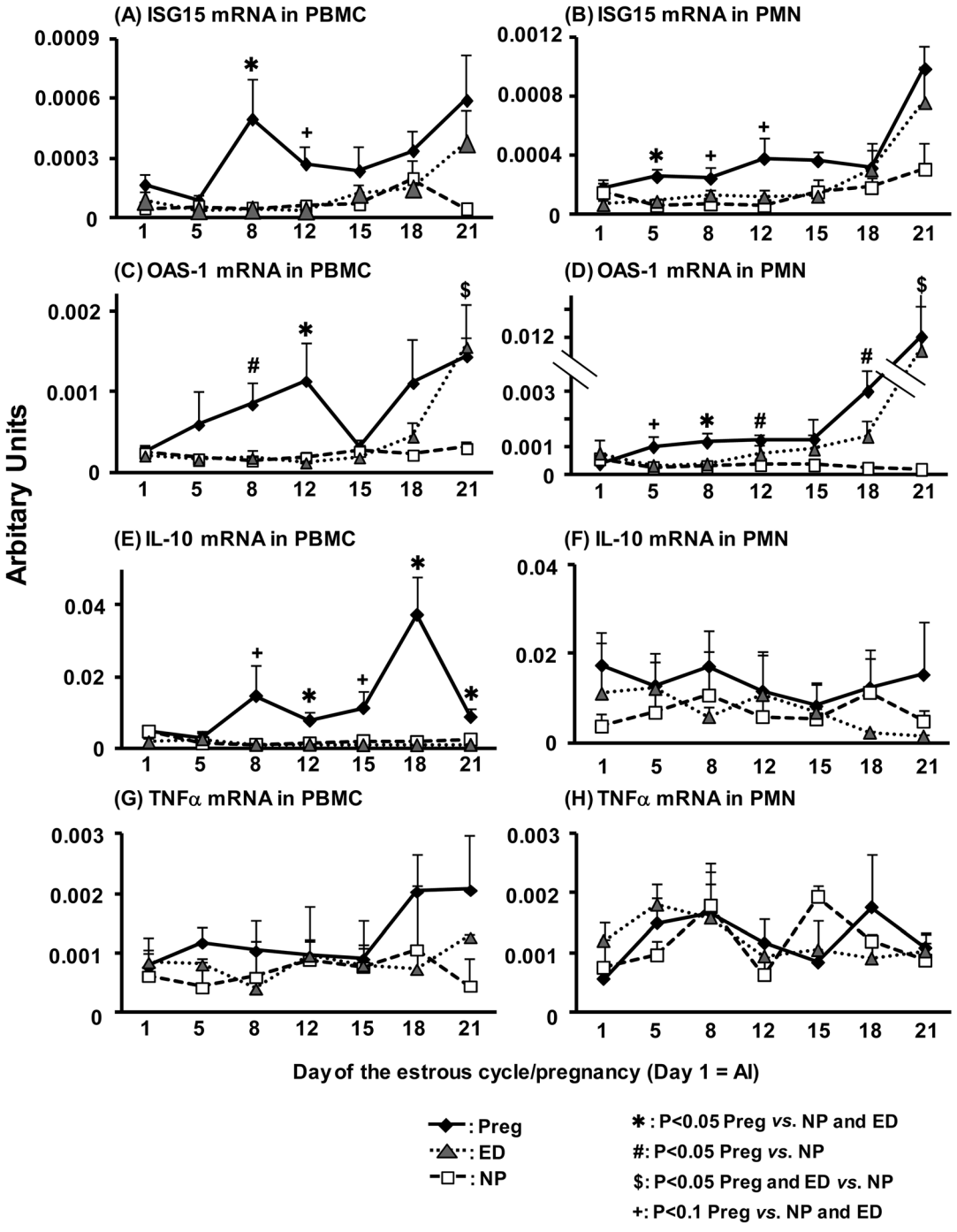

Fig. 3. Changes in the mRNA expression of ISG15, OAS-1, IL10 and TNFα in PBMCs and PMNs following AI. Changes in the mRNA expression of ISG15, OAS-1, IL10 and TNFα in PBMCs and PMNs following AI are shown. Black lozenges, gray triangles and white squares indicate the pregnancy (n = 9), embryonic death (n = 5) and nonpregnancy groups (n = 6), respectively.

day 40) and nonpregnancy (NP, return of the estrous cycle around day 21) as shown in Fig. 1. It has been reported that P4 concentrations are lower in artificially inseminated cows in which pregnancy fails than in cows in which pregnancy is established successfully [21–24]. In support of these previous findings, on day 5 after AI, plasma P4 levels were higher in the Preg and ED groups than in the NP group. Indeed, Carter *et al.* [25] reported that implantation of P4 on day 3 of pregnancy elevated P4 concentrations until day

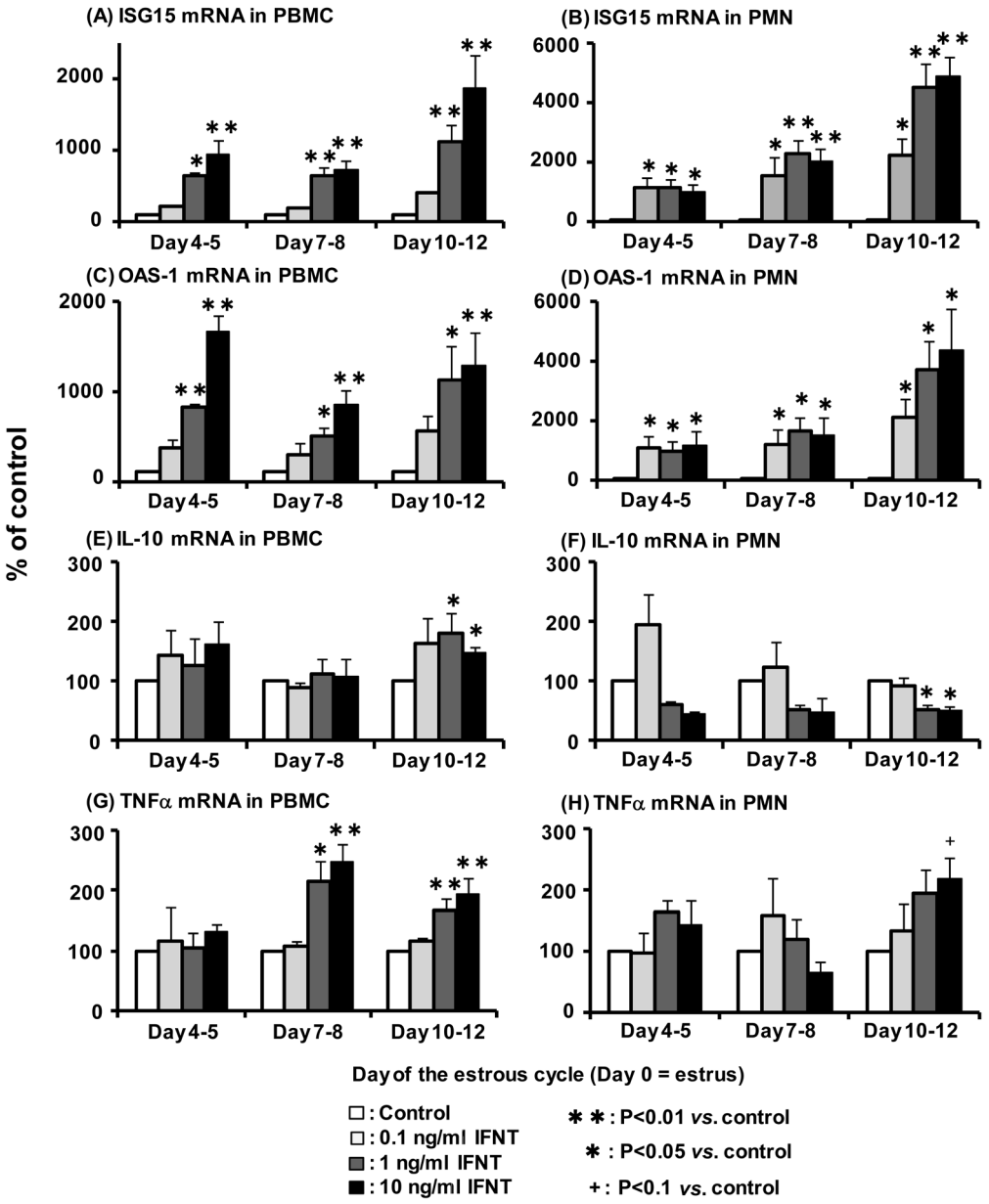

Fig. 4. Effects of IFNT on the mRNA expression of ISG15, OAS-1, IL10 and TNFα in PBMCs and PMNs. Effects of IFNT on the mRNA expression of ISG15, OAS-1, IL10 and TNFα in PBMCs and PMNs are shown. White bars, thin gray bars, deep gray bars and black bars indicate control and 0.1, 1 and 10 ng/ml IFNT treatment groups, respectively. Data are shown as percentages of the control value (n = 4 for each day of the estrous cycle).

8 and that this was associated with a larger conceptus recovered on day 16. On the other hand, P4 either *in vivo* [25] or *in vitro* [26] does not directly affect the ability of embryos to develop to the blastocyst stage in cattle. Thus, these findings suggest that P4-induced changes in the uterine environment are responsible for the advancement in conceptus elongation [25, 27]. Interestingly, the P4 concentrations in the ED group were similar to those in the Preg group on days 1–18, but the P4 levels remained lower than those in the Preg group after the MRP, resulting in loss of pregnancy. These results indicate that detection of P4 levels only is not enough to distinguish whether pregnancy occurs successfully.

We investigated the difference in mRNA expression levels in immune cells between the Preg, ED and NP groups. ISG15 and OAS-1 mRNA expression in PBMCs was higher in the Preg group than in the NP group on day 8 (Fig. 3A and C). Surprisingly, the mRNA expression of these genes in PMNs was higher in the Preg

group than in the NP and ED groups on day 5 (Fig. 3B and D), suggesting that PMNs have higher sensitivity than PBMCs and are potential marker cells for detection of early pregnancy. Indeed, the expression of OAS-1 mRNA was 31.7- and 5.7-fold higher in PMNs and PBMCs, respectively, on day 21 compared with the levels on day 1 in the present study. On the other hand, the increase in ISGs in the ED group delayed in proportion as Preg group, but the ISGs levels in ED group gradually increased around the MRP. The above findings suggest that the earlier response of immune cells to a conceptus and/or IFNT might be essential to establishment of pregnancy in cows. However, the present data did not clearly distinguish pregnancy from nonpregnancy on days 15 and 18 in multiparous cows using ISG15 and OAS-1 in immune cells despite high expression period of IFNT from the conceptus. Although ISGs are recognized as useful markers to distinguish pregnancy from nonpregnancy, Green *et al.* [9] concluded that multiparous cows were less likely to be accurately diagnosed than primiparous cows with the ISG test on day 18. They hypothesized one of the reasons for the difference in response of ISGs is that primiparous heifers (younger) had larger embryos than multiparous (older) cows during the MRP [9]. However, we have no clear answer for why these ISGs in the Preg group did not increase during the MRP compared with the other groups despite the prediction of high levels of IFNT secretion from embryo. Further investigation is required to clarify this.

Similar to the changes in ISG expression, IL-10 mRNA expression started to increase on day 8 in PBMCs but not in PMNs in pregnant cows before the MRP in the present study (Fig. 3E and F). TNFα mRNA expression was stable in all groups (Fig. 3G and H), indicating Th2 predominance in early pregnancy in cows. Importantly, Dixit and Parvizi [28] reported significantly high secretion of adrenocorticotropin (ACTH) and nitric oxide (NO) from peripheral blood lymphocytes on day 7 of pregnancy compared with that in cyclic cows. In addition, both ACTH and NO cause a shift toward Th2 by increasing the levels of Th2 cytokines and decreasing the levels of Th1 cytokines [29, 30].

In the present study, the expression of ISGs and/or IL-10 started to change on day 5 in PMNs and day 8 in PBMCs. IFNT mRNA was expressed in *in vitro* fertilized embryos on day 4 [11], and IFNT protein was detected in the trophectoderm of blastocysts on day 7 [12], suggesting that these early changes of maternal response are induced by local IFNT signals. Using a mathematical model, Shorten *et al.* [31] demonstrated that if IFNT signaling occurs later than day 13 of the cycle, pregnancy is not recognized irrespective of the IFNT concentration. Other candidate markers are early pregnancy factor (EPF) and EPF-like substances because EPF and EPF-like substances are detected in the serum of pregnant cows and are extraordinarily produced by embryos within 24–48 h after fertilization [32]. Ito *et al.* [32] suggested that these EPFs regulate the maternal immune response to prevent rejection of the allograft fetus. Additionally, the preimplantation embryo produces TGFβ and insulin-like growth factors I and II in cows [33]. Therefore, these conceptus-derived factors can potentially contribute to early changes in maternal immune cells during pregnancy.

Oliveria *et al.* [34] demonstrated greater antiviral activity in uterine vein blood from Day15 pregnant sheep. Additionally, Bott *et al.* [35] showed that preadsorption of blood by IFNT antibody reduced this antiviral activity, suggesting the release of IFNT into the uterine vein during the MRP. To verify whether IFNT affects immune cells before the MRP, we investigated the *in vitro* effect of IFNT treatment (0.1–10 ng/ml) on PBMCs and PMNs obtained at different periods of the estrous cycle. As might have been expected, IFNT obviously stimulated ISG15 and OAS-1 mRNA expression in both PBMCs and PMNs on all examined days of the estrous cycle (Fig. 4A–D). In addition, IFNT at 0.1 ng/ml increased ISG expression only in PMNs, and the ratio of stimulation was higher in PMNs than in PBMCs, demonstrating that PMNs are more susceptible to IFNT activity.

In the present study, IFNT stimulated IL-10 mRNA expression in PBMCs, but IL-10 mRNA expression decreased after IFNT treatment in PMNs on days 10–12 of the estrous cycle (Fig. 4E and F). Although there was no change in TNFα mRNA in immune cells *in vivo*, IFNT increased TNFα mRNA levels in PBMCs on days 7–8 and 10–12 (Fig. 4G), suggesting that the IFNT response *in vitro* may not have been of a sufficient magnitude to be detectable under the conditions of this *in vivo* study. On the other hand, it has been reported that IFNT upregulated not only Th2 cytokines (IL-10 and IL-4 mRNA) but also Th1 cytokines (IFNγ) in bovine T cell lines [36]. The above findings suggest that IFNT may be involved in the regulation of Th1/Th2 cytokines as well as induction of maternal recognition. Further investigation is required to determine whether IFNT actually regulates the function of immune cells and its significance *in vivo*.

Recently, Forde *et al*. [37] reported no differences in the expression of endometrial genes between pregnant and cyclic heifers prior to Day 16 (Day 5, 7 and 13), and these data are inconsistent with our present results. However, they discussed that while IFNT early in development may elicit a local effect on gene expression in the endometrium, it may occur in a cell-specific manner [37]. Additionally, while IFNT (100 or 1000 ng/ml) treatment for 24 h stimulated USP18 gene (IFNT-induced gene) and ISG20 (IFNT-regulated gene) by 500 and 1500%, respectively, in endometrial cells [37], IFNT treatment at 1 ng/ml for 4 h stimulated ISG genes by 5000% in PMNs in the present study. These results suggest that the action of IFNT may differ in dose- and cell-specific manner and that therefore we could detect the increase in ISGs in immune cells due to the high response for IFNT.

In conclusion, the mRNA expression of ISGs and IL-10 in PBMCs increased on day 8 of pregnancy after AI in pregnant cows. In addition, the mRNA expression of ISGs in PMNs was higher on day 5 in pregnant cows, suggesting that PMNs are the more sensitive immune cell population. IFNT treatment regulated the mRNA expression of ISGs, IL-10 and TNFα in PBMCs and PMNs *in vitro*. Therefore, changes in the maternal immune system may occur before the MRP, and IFNT might be related with these changes *via* ISG responses and regulation of Th1/Th2 cytokines in cows. Thus, ISGs in both PBMCs and PMNs and IL-10 in PBMCs could be useful as target genes for reliable indices of pregnancy before the MRP in the cow but need further investigation.

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