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Original Article

Impaired placentomal interferon signaling as the possible cause of retained fetal membrane in parturition-induced cows

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Abstract. Although hormonal induction of parturition in cattle results in the successful delivery of healthy calves, the risk of retained fetal membrane is significantly increased. In a previous study, a combination of the long-acting glucocorticoid, triamcinolone acetonide, with a high dose of betamethasone partially normalized the placentomal gene expression during parturition; however, the incidence of retained fetal membrane remained high. This study further explored placentomal dysfunction and aimed to elucidate the mechanism of retained fetal membrane in parturition-induced cows. In this study, transcriptome analysis revealed that enhanced glucocorticoid exposure normalized the expression of a substantial fraction of genes in the cotyledons. In contrast, a significant reduction in the multiple signaling pathway activities, including interferon signaling, was found in the caruncles during induced parturition. Real-time PCR showed that the expression of interferon-tau in the caruncles, but not interferon-alpha or interferon-gamma, was significantly lower in induced parturition than spontaneous parturition. Interferon-stimulated gene expression was also significantly decreased in the caruncles during induced parturition. These results indicate that interferon signaling could be important for immunological control in placentomes during parturition. Additionally, this suggests that interferon-tau might be a pivotal ligand for interferon receptors in the caruncles. This study revealed that peripheral blood leukocytes in prepartum cows transcribed interferon-tau. Macrophage infiltration in the placentome is known to participate in the detachment of the fetal membrane from the caruncle. Thus, this study raised the possibility that immune cells migrating into the caruncles at parturition may act as a source of ligands that activate interferon signaling.
Key words: Cattle, Interferon, Parturition, Placenta, Retained fetal membrane

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In normal bovine parturition, the fetal membrane is discharged within 6 h of fetal expulsion. The retained fetal membrane (RFM), defined as the fetal membrane still present at least 12 h postpartum, usually occurs in only a small percentage of post-calving cows, but this incidence varies widely between herds [1]. RFM is associated with reduced fertility and milk production [1, 2]. A variety of factors, including abortion, stillbirth, multiple births, dystocia, uterine torsion, heat stress, hydrops allantois, and periparturient hypocalcemia, increase the risk of RFM [3].

The bovine placenta comprises more than 100 placentomes, wherein fetal cotyledonary villi are closely attached to a maternal caruncle. Thus, structural and functional placentome changes are required during parturition for the smooth detachment of the fetal placenta following calf delivery [4, 5]. Placental maturation is thought to be induced by endocrine and immunological changes. Increased prepartum cortisol release from fetal adrenals stimulates prostaglandin (PG) E2 synthesis in the cotyledons [6, 7]. PGE2 secretion may stimulate estrogen synthesis, which is important for birth canal softening and the production of PGF2α in the endometrium and myometrium [6, 8]. Streyl *et al*. [9] reported that the gene expression pattern in the placentomes shifts at parturition to promote apoptosis, extracellular matrix degeneration, and innate immune response. Leukocytes infiltrate into placentomes at parturition and secrete matrix metalloproteinases, which play an essential role in the detachment of the fetal membrane from the caruncle [10–13].

Hormonal induction of parturition results in the successful delivery of healthy calves but significantly increases the risk of RFM. Several studies have attempted to overcome this problem by imitating the gradual rise in cortisol secretion from the fetus during the week prior to parturition [14]. The protracted induction of parturition by dexamethasone administration after presensitization with multiple, low-dose dexamethasone injections induces atrophy and flattening of the caruncle epithelium, but does not influence the RFM incidence [15]. Other studies have reported that long-acting glucocorticoids

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tend to reduce the incidence of RFM with parturition induction [16, 17]. A high-dose (0.5 mg/kg body weight) betamethasone injection in pregnant sheep reduced the average number of trophoblast binucleate cells, which disappeared in matured placentomes during spontaneous parturition [18]. Thus, we previously attempted to improve parturition induction in cows using a combination of the long-acting glucocorticoid, triamcinolone acetonide, with a high dose of betamethasone. This treatment reduced the number of trophoblast binucleate cells in placentomes at parturition [19] and upregulated the expression of placentome genes involved in PGE2 and androstenedione synthesis, but did not adequately promote the C–C motif chemokine ligand (CCL) expression [20, 21]. Thus, although the improved induction method partially normalized the placentome function at parturition, the incidence of RFM remained high (33% to 78%).

The development of bovine parturition induction methods that do not increase the incidence of RFM will help to prevent dystocia and reduce the labor required for management in the future. A comprehensive understanding of placentomal maturation at the point of induced parturition is required to develop methods for smooth detachment and expulsion of the fetal membrane. The aim of this study was to explore abnormalities in placentomal function that cannot be normalized by glucocorticoid administration and to clarify the mechanisms of RFM in parturition-induced cows. Transcriptome analysis by RNA-seq was conducted to compare gene expression profiles in placentomes at parturition between spontaneous parturition (without RFM) and induced parturition with different hormone treatments. Additionally, placentomal gene expression in the hormonal induction of parturition with clinical doses of PGF2α and dexamethasone was compared to that with enhanced glucocorticoid exposure. These analyses revealed signaling pathways with low activity in the caruncle that were common to all parturition induction methods. In particular, a detailed analysis of the interferon (IFN) signaling pathway showed that it plays a central role in initiating immune responses, as the mRNA levels of many genes in the caruncle were low during induced parturition.

Materials and Methods

Animal experiments

All animal experimental procedures were carried out in accordance with the guidelines and ethics approved by the Animal Experiment Committee of the Animal Research Center (Approval Number: 1107, 1212, 1307, 1405, and 1508) and the Animal Care and Use Committee on Ethics of the Tokyo University of Agriculture (Approval Number: 2020085).

Placentome collection

The parturition and tissue sample data used for gene expression analysis in this study were the same as those used in a previous study [20]. Briefly, pregnancy was induced in 24 beef cows (Japanese Black, Angus, and crossbred) by transferring *in vivo-fertilized* Japanese Black embryos. The cows were divided into four groups according to the parturition method: spontaneous (SP group; n = 5), PGF2α-induced (PG group; n = 7), dexamethasone followed by PGF2α and estriol (DEX group; n = 6), and triamcinolone acetonide followed by PGF2α and betamethasone sodium phosphate (TABET group; n = 6). The PG group received 25 mg i.m. dinoprost tromethamine (Pronalgon-F® injection; Pfizer Japan Inc., Tokyo, Japan) [22]. The DEX group received 20 mg intramuscular dexamethasone (Kyoritsu Seiyaku Corporation, Tokyo, Japan), followed by 25 mg intramuscular PGF2α and 20 mg intramuscular estriol (Holin®; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) 24 h later [23]. The TABET group received 0.017 mg i.m./kg body weight triamcinolone acetonide (Kenacort-A®; Bristol-Myers Squibb, Tokyo, Japan), followed by 25 mg i.m. PGF2α and 0.5 mg i.m./kg body weight betamethasone sodium phosphate (Rinderon®; Shionogi & Co., Ltd., Osaka, Japan) 5 days later [19].

Placentomes approximately 10 cm in diameter and in a palpable area in the middle of the uterus were manually collected through the birth canal immediately after parturition. The placental cotyledons and caruncles were manually separated and stored at −80°C prior to RNA extraction. PGF2α was administered at 283 ± 3, 279 ± 1, and 279 ± 1 days of gestation in the PG, DEX, and TABET groups, respectively. The gestation lengths were 292 ± 3, 284 ± 3, 281 ± 1, and 280 ± 3 days in the SP, PG, DEX, and TABET groups, respectively. The RFM incidences in the SP, PG, DEX, and TABET groups were 0, 71, 50, and 33%, respectively.

Blood collection

Seven naturally calved Holstein cows were used in this study. All cows discharged their fetal membranes normally after parturition. Venous blood samples were collected from three cows and twice from four cows during the prepartum period, from 9 to 0 days pre-parturition, with heparinized vacuum tubes. A total of 11 samples were centrifuged at 1,600 × *g* for 15 min at 4°C to recover the buffy coat as peripheral blood leukocytes (PBLs), which were stored at −80°C prior to RNA extraction.

Transcriptome analysis

We performed transcriptome analyses using next-generation sequencing to clarify the differences in placentomal gene expression between spontaneous and induced parturition. Total RNA was isolated from the cotyledons and caruncles using ISOGEN (Nippon Gene, Tokyo, Japan). RNA samples were then treated with an RNase-free DNase Set (QIAGEN GmbH, Hilden, Germany) and purified with RNeasy MinElute Cleanup Kits (QIAGEN). RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Libraries were prepared using a TruSeq RNA Sample Preparation Kit V2 (Illumina Inc., San Diego, CA, USA) and were used to generate clusters on the Illumina Cluster Station. The derived libraries were combined and sequenced in 100-bp single reads on two lanes of a flowcell on an Illumina HiSeq 2500 Sequencing System. Image analysis, base calling, de-multiplexing, and adaptor trimming were performed using Illumina bcl2fastq2 software version 2.17, following the manufacturer's instructions, and approximately 10–20 million reads were obtained individually. The raw reads of RNA-seq in this study are available (DDBJ accession number DRA012400). Quality filtering, alignment to the bovine genome sequence (bosTau8), and comparison of RNA expression between the groups were performed using CLC Genomics Workbench ver.9.5 (QIAGEN). Genes with a value of more than 2.0 or −2.0 fold in the three induced-parturition groups compared to those in the spontaneous parturition group were considered differentially expressed genes (DEGs). The Ingenuity Pathways Analysis (IPA) program (QIAGEN) was used to identify the biofunctional signaling networks associated with the differential changes in gene expression between spontaneous and induced parturition. The analysis was performed with n = 3 in each group. Kal's z-test was used to analyze gene set enrichment in the functional categories.

mRNA expression analysis

The total RNA of the placentomal tissues of all individuals and PBLs were isolated using ISOGEN (Nippon Gene) and then reverse-transcribed using the iScript gDNA Clear cDNA Synthesis

Kit (Bio-Rad, Richmond, CA, USA).

Real-time PCR was performed in duplicate using GeneAce SYBR® qPCR Mix α No ROX (Nippon Gene). Details of the oligonucleotide primers are listed in Supplementary Table 1. The PCR conditions were as follows: 10 min at 95°C, followed by 40 cycles at 95°C for 30 sec and 60°C for 60 sec. Real-time PCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad). The data were normalized to the *GAPDH* mRNA levels.

Conventional reverse transcription-PCR (RT-PCR) for *IFNA*, *IFNG*, and *IFNT2* was performed using the HotStarTaq Plus Master Mix Kit (Qiagen) and specific primers (Supplementary Table 1) used for the real-time PCR. The PCR conditions were as follows: 5 min at 95°C, followed by 40 cycles of 30 sec at 94°C, 30 sec at 60°C, and 60 sec at 72°C.

Statistical analysis

The results are presented as the mean ± standard error. The normality and homogeneity of variance were tested using the Shapiro–Wilk normality test and Levene's test, respectively. Differences among SP, PG, DEX, and TABET were estimated using the Tukey–Kramer test. Cotyledon and caruncle data were analyzed separately. The relationships between the variables were evaluated using Pearson's correlation coefficient. Statistical significance was set at $P < 0.05$. All analyses were performed using R statistical package version 4.0.2.

Results

DEGs between placentomes at spontaneous and induced parturition

We compared the number of DEGs in induced-parturition cows to that in spontaneous parturition cows to clarify the transcriptome characteristics in the placentomes at parturition (Fig. 1). The PG group exhibited the greatest number of DEGs in both the cotyledons and caruncles. The DEGs in the cotyledons were decreased in the DEX and TABET groups treated with glucocorticoids. In particular, the DEGs in the TABET group decreased to 11% in the PG group. Although the DEGs of the DEX group were comparable to those of the PG group in the caruncles, the DEGs of the TABET group decreased slightly to 62% of the PG group. These results suggest that long-acting and high-dose glucocorticoids partly normalized gene expression in the cotyledons during induced parturition. In contrast, a large number of DEGs in the caruncles were a common feature of all three induced-partition groups.

IPA in placentomes at parturition

We investigated the canonical pathways between induced and spontaneous parturition, which changed placentome activity based on z-scores. No canonical pathways were common to all three induced-parturition groups in the cotyledons (Supplementary Tables 2 and 3). In addition, there were no significant upregulated canonical pathways common to all three induced-parturition groups in the caruncles (Supplementary Table 4). In contrast, significant z-score reductions in the caruncles, which were common among all three induced-parturition groups, were found in multiple canonical pathways (Table 1). We focused on IFN signaling in the caruncles because the z-score reductions were the largest in all induced-parturition groups (Supplementary Fig. 1).

Fold changes in gene expression involved in IFN signaling are shown in Table 2. As for IFN ligands, the expression of IFN tau-2 (*IFNT2*) in the PG and TABET groups was significantly lower than that in the SP group. The expression of IFN gamma (*IFNG*) in the induced-parturition groups was comparable to that in the SP group. IFN alpha (*IFNA*), which is an important type I IFN, was not listed in the annotation information for *Bos taurus* (ftp://ftp.ensembl.org/pub/release-87/gff3/bos_taurus). The expression of signal transducers and activator of transcription 1 (*STAT1*) and *STAT2* was significantly lower in the PG and DEX groups than in the SP group. The expression of IFN-stimulated genes (*ISGs*) was significantly lower in all induced-parturition groups, except for four genes in the TABET groups (Table 3).

mRNA expression analysis in placentomes at parturition by real-time PCR

We validated the mRNA expression in all placentomal samples using real-time PCR. The expression of *IFNT2* in the caruncles was significantly lower in the PG group than in the SP group, whereas it was insignificantly lower in the DEX group than in the SP group (Fig. 2A). There was no significant difference in the expression

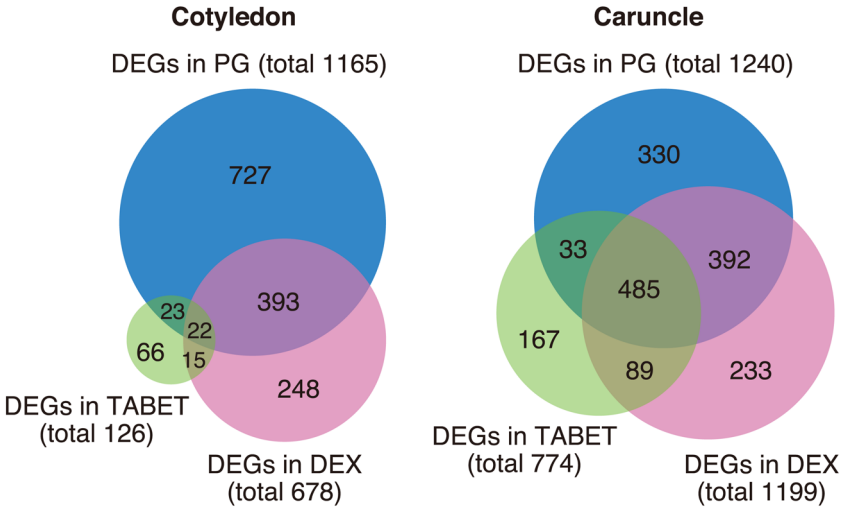

Fig. 1. Differentially expressed genes (DEGs) in cotyledons and caruncles during induced parturition (prostaglandin (PG), dexamethasone (DEX), and TABET groups) compared to those during spontaneous parturition.

Table 1. Top 10 canonical pathways with reduced activity in caruncle tissue during induced parturition

Canonical pathways	z-score	P-value
PG group *vs*. SP group		
Interferon signaling	−3.61	7.94E−10
PTEN signaling	−2.71	0.017
RhoGDI signaling	−2.33	0.370
Antioxidant action of vitamin C	−2.31	0.001
Toll-like receptor signaling	−2.24	0.167
Cell Cycle: G2/M DNA damage checkpoint regulation	−2.12	0.003
Apoptosis signaling	−1.41	0.078
Retinoic acid mediated apoptosis signaling	−1.41	0.011
TREM1 signaling	−1.34	0.324
Role of pattern recognition receptors in recognition of bacteria and viruses	−1.26	0.020
DEX group *vs*. SP group		
Interferon signaling	−3.61	5.37E−10
TREM1 signaling	−2.45	0.158
Cell Cycle: G2/M DNA damage checkpoint regulation	−2.33	0.001
Dendritic cell maturation	−2.32	0.012
PTEN signaling	−2.31	0.005
Role of pattern recognition receptors in recognition of bacteria and viruses	−2.11	0.003
VEGF signaling	−2.00	1.000
VDR/RXR activation	−2.00	0.085
Retinoic acid mediated apoptosis signaling	−1.90	0.001
Toll-like receptor signaling	−1.89	0.028
TABET group *vs*. SP group		
Interferon signaling	−2.65	1.17E−05
Death receptor signaling	−2.45	0.068
PTEN signaling	−2.24	0.167
Retinoic acid mediated apoptosis signaling	−2.24	0.041
VDR/RXR activation	−2.24	0.003
Toll-like receptor signaling	−2.00	0.199
Role of pattern recognition receptors in recognition of bacteria and viruses	−1.67	0.004
Cell Cycle: G2/M DNA damage checkpoint regulation	−1.34	0.018
Coagulation system	−1.34	0.004
Dendritic cell maturation	−1.13	0.379

Table 2. Differential expression of genes involved in interferon signaling in the transcriptome analysis of caruncle tissue

Function	Gene symbol	Entrez ID	Fold change (*vs*. SP group)			FRD P-value		
			PG group	DEX group	TABET group	PG group	DEX group	TABET group
Ligand	*IFNT2*	317698	−24.244	−9.738	−15.587	0.037	0.060	0.039
	IFNG	281237	1.723	1.002	−3.854	1.000	1.000	1.000
Receptor	*IFNAR1*	282257	−1.244	−1.229	−1.211	1.000	1.000	1.000
	IFNAR2	282258	−1.675	−1.284	−1.113	0.268	0.999	1.000
	IFNGR1	508619	−1.088	−1.636	−1.467	1.000	0.576	0.981
	IFNGR2	514889	−1.462	−1.465	−1.353	0.796	0.792	1.000
	IFNLR1	616826	−1.009	1.468	2.278	1.000	1.000	0.643
Signaling factor	*DRIP150* [1)]	529109	1.004	−1.034	1.029	1.000	1.000	1.000
	FITM2	518159	1.221	1.353	1.799	1.000	0.872	0.204
	JAK1	537201	−1.153	−1.109	1.148	1.000	1.000	1.000
	JAK2	525246	1.742	1.147	1.035	0.265	1.000	1.000
	PIAS1	509231	−1.267	−1.226	−1.038	1.000	1.000	1.000
	SOCS1	518795	−2.468	−2.028	−1.014	0.291	0.586	1.000
	STAT1	510814	−2.910	−2.949	−2.126	1.15E−03	1.03E−03	0.079
	STAT2	511023	−2.459	−2.394	−2.058	4.68E−03	7.14E−03	0.064
	TC−PTP [2)]	531009	−1.295	−1.153	1.147	0.989	1.000	1.000

[1)] It is also known as the *MED14*. [2)] It is also known as the *PTPN2*.

Table 3. Differential expression of interferon-stimulated genes in the transcriptome analysis of caruncle tissue

Gene symbol	Entrez ID	Fold change (*vs*. SP group)			FRD P-value		
		PG group	DEX group	TABET group	PG group	DEX group	TABET group
G1P2 [1)]	281871	−33.472	−26.867	−13.186	0	0	8.62E−09
G1P3 [2)]	512913	−11.290	−14.710	−8.749	2.63E−09	2.71E−11	3.90E−07
IFI35	510697	−2.337	−2.825	−2.141	0.028	2.83E−03	0.090
IFIT1	100139670	−25.124	−21.210	−14.266	0	0	3.48E−12
IFIT3	509678	−20.754	−20.195	−8.894	0	0	1.82E−07
IFITM1	353510	−13.446	−5.993	−3.350	2.46E−06	4.11E−03	0.180
IFITM3	777594	−5.914	−4.663	−3.117	1.21E−05	2.86E−04	0.029
IRF1	789216	−2.347	−3.609	−2.300	0.043	2.29E−04	0.072
IRF9	509855	−3.977	−4.818	−3.746	1.97E−06	2.67E−08	1.33E−05
MX1	280872	−23.288	−17.557	−12.238	0	7.35E−13	1.65E−09
OAS1X	347699	−12.123	−12.632	−14.150	2.91E−07	1.62E−07	8.15E−08
PSMB8	282013	−2.764	−3.417	−2.098	0.011	9.24E−04	0.187
TAP1	524959	−3.753	−5.340	−3.519	4.22E−05	4.64E−08	2.44E−04

[1)] It is also known as the *ISG15*. [2)] It is also known as the *IFI6*. RNA-seq reads were not aligned to *IFITM2* (Entrez ID: 615833) in *Bos taurus*.

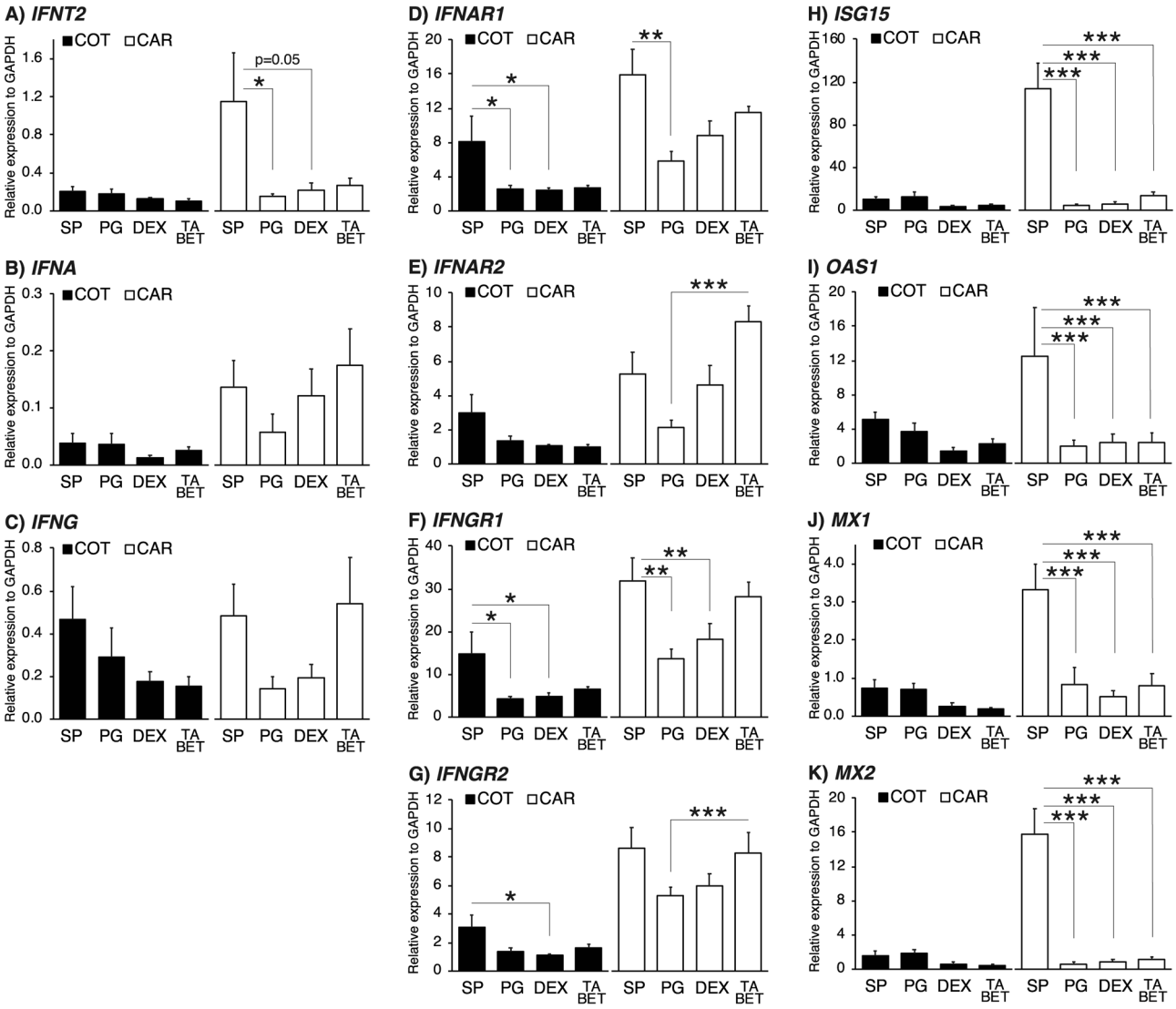

Fig. 2. mRNA expression levels of interferons (*IFNs*), *IFNARs*, *IFNGRs*, and of IFN-stimulated genes (*ISGs*) in the placentome at spontaneous (SP group) and induced (PG, DEX, and TABET groups) parturition. Statistically significant differences in the relative mRNA expression were analyzed in the cotyledon (COT) and caruncle (CAR) tissue. Data are presented as the mean ± standard error. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

of *IFNA* and *IFNG* in any of the groups (Figs. 2B and C). The expression of IFNA receptor 1 (*IFNAR1*) was significantly lower in the DEX group than in the SP group in the cotyledons (Fig. 2D) and significantly lower in the PG group than in the SP group in the cotyledons and caruncles (Fig. 2D). IFNG receptor 1 (IFNGR1) expression was significantly lower in the PG and DEX groups than in the SP group in both the cotyledons and caruncles (Fig. 2F). The expression of *IFNAR2* and *IFNGR2* in the caruncles was significantly higher in the TABET group than in the PG group (Figs. 2E and G). We analyzed the mRNA expression of typical ISGs in the IFNT. The expression levels of *ISG15*, *OAS1*, *MX1*, and *MX2* in the caruncles were significantly lower in all induced-parturition groups than in the SP group (Figs. 2H–K). These results suggest that *IFNT2* and *IFNR* expression is involved in the regulation of IFN signaling and increased *ISG* expression in the caruncles during parturition.

IFN expression in peripheral blood leukocytes during prepartum period

We analyzed *IFN* expression in PBLs to examine the specificity of *IFNT2* expression in the placentomes at parturition and systemic IFN expression during the prepartum period. Expression of *IFNA*, *IFNG*, and *IFNT2* was detected in PBL (Fig. 3A). A strong positive correlation between *IFNT2* and *IFNA* expression was observed in the prepartum period (Fig. 3B). There were no correlations between *IFNT2*/*IFNA* and *IFNG* expression during the prepartum period (Figs. 3C and D). These results suggest that type I IFN expression is regulated independently of type II IFN in PBL during the prepartum period.

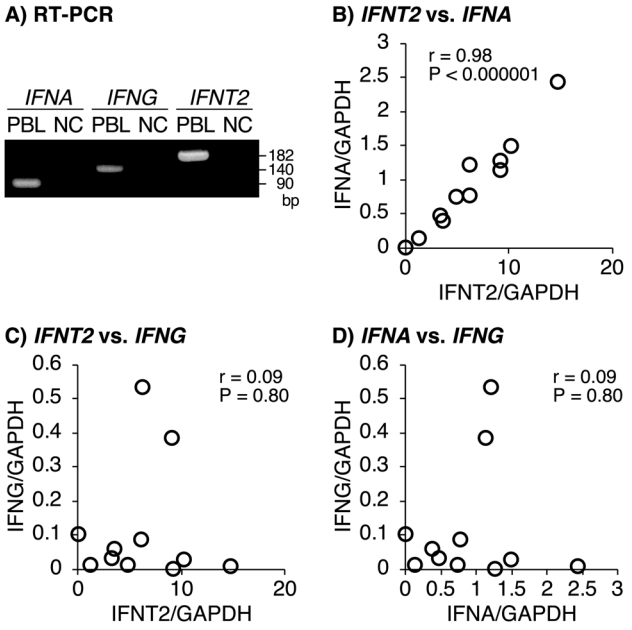

Fig. 3. *IFN* expression in peripheral blood leukocytes (PBLs) during the prepartum period. *IFNA*, *IFNG*, and *IFNT2* expression levels in PBLs were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) (A). Panels B–D indicate the correlation between the expression levels of each IFN analyzed by real-time PCR. NC: negative control.

Discussion

In a previous study, we reported that parturition induction with a combination of the long-acting glucocorticoid, triamcinolone acetonide, with a high dose of betamethasone may normalize PGE2 and androstenedione synthesis in the cotyledons at parturition [20]. In this study, we revealed that enhanced glucocorticoid exposure in the TABET group normalized the expression of a substantial fraction of genes in the cotyledons. In contrast, this induction method did not upregulate *CCL* expression in the caruncles, suggesting that an inadequate inflammatory response may be an RFM factor in the TABET group [21]. Accordingly, the current study identified 774 DEGs in the caruncles of the TABET group. Aberrant gene expression in the caruncles was attributed to the immaturity of the placenta, as parturition was induced before the expected delivery date and there were no external signs at the time of hormonal treatment was initiated. No RFM occurred in the SP group, and it is unlikely that inadequate management was the cause of RFM in the induced-parturition cows. These results suggest that the analysis of the impaired expression of genes in the caruncles during induced parturition could help in understanding the etiology of RFM.

IPA demonstrated that the IFN signaling pathway activity was quite low in the caruncles during induced parturition, regardless of glucocorticoid administration. Furthermore, reduction in Toll-like receptor (TLR) signaling activity and role of pattern-recognition receptors in recognition of bacteria and virus activity was common to all induced-parturition groups. The stimulation of TLR by cell-free fetal DNA, which is released from senescent fetal membrane cells at term, might induce proinflammatory events leading to spontaneous parturition [24]. These pattern-recognition receptor-mediated signaling pathways trigger innate immune responses and induce type I IFN in response to antiviral responses [25]. Therefore, we conclude that activation of the IFN signaling pathway is essential to stimulate an innate immune response in the caruncles during parturition in cows.

Interestingly, mRNA expression analysis by real-time PCR revealed that the differentially expressed IFN ligand in the caruncles was *IFNT2*, not *IFNA* and *IFNG*, which are major cytokines produced by mammalian immune cells. The low *IFNT2* and IFN receptor (*IFNAR1* and *IFNGR1*) expression levels in the caruncles during induced parturition may be the cause of the significantly low *ISG* expression. Enhanced glucocorticoid exposure in the TABET group had little effect on the IFN signaling pathway. Although *IFNAR2* and *IFNGR2* expression in the TABET group increased, the mechanism was unclear. The expression of these receptors is thought to be ubiquitous in most nucleated cells, but can be modulated. Distinct localization of IFNAR1 and IFNAR2 is found in the bovine endometrial and glandular epithelia during the estrous cycle and early pregnancy [26, 27]. These *IFNARs* were expressed in bovine caruncles during early pregnancy and showed time-dependent changes [27]. In addition, murine proinflammatory (classical) macrophages produce larger amounts of Ifnar1 than anti-inflammatory (non-classical) macrophages, and both murine classic and non-classic macrophages also produce Ifnar2, Ifngr1, and Ifngr2 [28]. These findings suggest that the endometrial epithelium functions and infiltration of macrophages may be involved in the modulation of *IFNAR* and *IFNGR* expression in the caruncles during parturition.

IFNT, a type I IFN even as IFNA, is a well-known signaling molecule for maternal recognition of pregnancy in ruminants [29, 30]. It has been demonstrated that IFNT expression is spatially and temporally limited *in utero*. IFNT secretion from trophectoderm cells during peri-implantation conceptuses peaks from days 19 to 20 after the onset of estrus and is no longer detectable by day 25 [31–34]. Therefore, it was our understanding that the remaining cotyledonary villi in the interdigitated area might be a source of IFNT2 in the caruncles. However, we could not determine the tissue

localization of IFNT2 in the placentomes by immunohistochemistry (data not shown). We then examined *IFN* expression in PBLs to determine whether *IFNT2* expression was specific to the placentomes at parturition. Surprisingly, *IFNT2* was expressed even as *IFNA* and *IFNG* in prepartum PBL. In addition, strong positive correlations were found between *IFNT2* and *IFNA* expression levels in PBL, but not in *IFNG*. The bovine CD4+ T-cell subset is the major producer of IFNG in peripheral blood mononuclear cells during the peripartum period [35, 36]. However, type I IFN production in the maternal circulation during the peripartum period in cows has not been studied. This study revealed for the first time that *IFNT* is expressed in bovine PBLs during the prepartum period. Large-scale studies of both beef and dairy breeds are needed to determine the types of IFNT-producing PBLs.

We performed direct PCR amplicon sequencing for *IFNT2* to validate the specificity of the PCR primers (data not shown). The sequence identity of 182 bp amplicons to *IFNT2* (NM_001015511.3) was 99.5%. Type I IFN genes are found together in a cluster on chromosome 8 in cattle. Although the exact number of *IFNT* genes is uncertain, at least six different *IFNT* variants are expressed from multiple loci in individual bovine conceptuses [37]. Phylogenetic analysis showed relatively low IFNT identities compared to other type I IFNs (IFN omega, ~70% amino acid identity; IFNA, ~50% identity; and IFN beta, ~25% identity), whereas these IFNs possessed a similar secondary and tertiary structure. These findings indicate that the type I IFN signaling pathway in the caruncles at parturition may be primarily activated by IFNT variants.

Miyoshi *et al*. [38] revealed that numerous CD14-positive macrophages were found in placentomes collected within 1 h of normal parturition in cows. In contrast, Nelli *et al*. [39] reported a lower number of macrophage populations in the caruncles of cows with RFM. They detected *IFNT2* expression in the caruncles, but the expression level in the RFM group was comparable to that in the normal expulsion group. The contradictory results in this study might be due to the differences in the tissue collection procedures: Nelli *et al*. [39] collected placentomes between 3 and 24 h postpartum, but we collected placentomes immediately after parturition. The expression levels of several genes in the placentome change considerably in a brief period of 6 h postpartum [13]. Type I IFNs are produced by various immune cells, including macrophages [40]. Although IFNT2-expressing cells in the caruncles were not identified in this study, we speculate that migrated macrophages from the maternal circulation into the caruncles might be a pivotal source of ligands that activate the IFN signaling pathway. This is consistent with our previous study showing that *CD11B* expression, a macrophage surface antigen, was increased in the caruncles during spontaneous parturition compared to induced parturition [21]. In addition, *IFNA* expression in this study, which is coordinately regulated with *IFNT2* in PBL, did not increase in the placentomes during spontaneous parturition. Thus, the specific increase in *IFNT2* expression in the caruncles might be due to the type of infiltrated leukocytes. Identification of IFNT-producing cells in placentomes is a key question for future research.

Inflammatory cells synthesize matrix metalloproteinases that degrade the extracellular matrix proteins. Immune cells infiltrating the placentomes during normal parturition promote detachment of the fetal membrane by degrading collagen fibers [5]. The activation of the IFN signaling pathway by these immune cells may further enhance the inflammatory response in the placentomes. This study proposed that inadequate activation of the IFN signaling pathway is an important factor in RFM occurrence in induced-parturition cows, suggesting that IFNT2 may be involved in the promotion of placentomal maturation at parturition by inducing inflammation.

Conflict of interests: The authors declare that there are no conflicts of interest related to this study.

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References

1. **Laven RA, Peters AR.** Bovine retained placenta: aetiology, pathogenesis and economic loss. *Vet Rec* 1996; **139**: 465–471. [Medline] [CrossRef]
2. **Beagley JC, Whitman KJ, Baptiste KE, Scherzer J.** Physiology and treatment of retained fetal membranes in cattle. *J Vet Intern Med* 2010; **24**: 261–268. [Medline] [CrossRef]
3. **Gilbert RO.** Management of reproductive disease in dairy cows. *Vet Clin North Am Food Anim Pract* 2016; **32**: 387–410. [Medline] [CrossRef]
4. **Holm LW, Salvatore C, Zeek-Minning P.** The histology of the postterm bovine placenta. *Am J Obstet Gynecol* 1964; **88**: 479–489. [Medline] [CrossRef]
5. **McNaughton AP, Murray RD.** Structure and function of the bovine fetomaternal unit in relation to the causes of retained fetal membranes. *Vet Rec* 2009; **165**: 615–622. [Medline] [CrossRef]
6. **Schuler G, Fürbass R, Klisch K.** Placental contribution to the endocrinology of gestation and parturition. *Anim Reprod* 2018; **15**: 822–842. [CrossRef]
7. **Shenavai S, Preissing S, Hoffmann B, Dilly M, Pfarrer C, Özalp GR, Caliskan C, Seyrek-Intas K, Schuler G.** Investigations into the mechanisms controlling parturition in cattle. *Reproduction* 2012; **144**: 279–292. [Medline] [CrossRef]
8. **Whittle WL, Holloway AC, Lye SJ, Gibb W, Challis JR.** Prostaglandin production at the onset of ovine parturition is regulated by both estrogen-independent and estrogen-dependent pathways. *Endocrinology* 2000; **141**: 3783–3791. [Medline] [CrossRef]
9. **Streyl D, Kenngott R, Herbach N, Wanke R, Blum H, Sinowatz F, Wolf E, Zerbe H, Bauersachs S.** Gene expression profiling of bovine peripartal placentomes: detection of molecular pathways potentially involved in the release of foetal membranes. *Reproduction* 2012; **143**: 85–105. [Medline] [CrossRef]
10. **Chakrabarti S, Patel KD.** Matrix metalloproteinase-2 (MMP-2) and MMP-9 in pulmonary pathology. *Exp Lung Res* 2005; **31**: 599–621. [Medline] [CrossRef]
11. **Miyoshi M, Sawamukai Y.** Specific localization of macrophages in pregnant bovine caruncles. *Reprod Domest Anim* 2004; **39**: 125–128. [Medline] [CrossRef]
12. **Walter I, Boos A.** Matrix metalloproteinases (MMP-2 and MMP-9) and tissue inhibitor-2 of matrix metalloproteinases (TIMP-2) in the placenta and interplacental uterine wall in normal cows and in cattle with retention of fetal membranes. *Placenta* 2001; **22**: 473–483. [Medline] [CrossRef]
13. **Takagi M, Yamamoto D, Ohtani M, Miyamoto A.** Quantitative analysis of messenger RNA expression of matrix metalloproteinases (MMP-2 and MMP-9), tissue inhibitor-2 of matrix metalloproteinases (TIMP-2), and steroidogenic enzymes in bovine placentomes during gestation and postpartum. *Mol Reprod Dev* 2007; **74**: 801–807. [Medline] [CrossRef]
14. **Comline RS, Hall LW, Lavelle RB, Nathanielsz PW, Silver M.** Parturition in the cow: endocrine changes in animals with chronically implanted catheters in the foetal and maternal circulations. *J Endocrinol* 1974; **63**: 451–472. [Medline] [CrossRef]
15. **Hartmann D, Bollwein H, Honnens A, Niemann H, Rath D, Pfarrer C.** Protracted induction of parturition enhances placental maturation, but does not influence incidence of placental retention in cows. *Theriogenology* 2013; **80**: 185–192. [Medline] [CrossRef]
16. **Nasser LF, Bo GA, Barth AD, Mapletoft RJ.** Induction of parturition in cattle: effect of triamcinolone pretreatment on the incidence of retained placenta. *Can Vet J* 1994; **35**: 491–496. [Medline]
17. **Bo GA, Fernandez M, Barth AD, Mapletoft RJ.** Reduced incidence of retained placenta with induction of parturition in the cow. *Theriogenology* 1992; **38**: 45–61. [Medline] [CrossRef]
18. **Braun T, Li S, Moss TJM, Newnham JP, Challis JRG, Gluckman PD, Sloboda DM.** Maternal betamethasone administration reduces binucleate cell number and placental lactogen in sheep. *J Endocrinol* 2007; **194**: 337–347. [Medline] [CrossRef]
19. **Hirayama H, Koyama K, Sawai K, Fujii T, Naito A, Fukuda S, Kageyama S.** Localization of TGF-β and TGF-β receptor in bovine term placentome and expression differences between spontaneous and induced parturition. *Placenta* 2015; **36**: 1239–1245. [Medline] [CrossRef]

20. **Yasuhara T, Koyama K, Sakumoto R, Fujii T, Naito A, Moriyasu S, Kageyama S, Hirayama H.** Enhanced glucocorticoid exposure facilitates the expression of genes involved in prostaglandin and estrogen syntheses in bovine placentomes at induced parturition. *Theriogenology* 2019; **139**: 1–7. [Medline] [CrossRef]
21. **Hirayama H, Sakumoto R, Koyama K, Yasuhara T, Hasegawa T, Inaba R, Fujii T, Naito A, Moriyasu S, Kageyama S.** Expression of C-C motif chemokines and their receptors in bovine placentomes at spontaneous and induced parturition. *J Reprod Dev* 2020; **66**: 49–55. [Medline] [CrossRef]
22. **Claydon RK.** Induction of parturition in cattle during the later stages of pregnancy: a comparison of three treatments. *Vet Rec* 1984; **114**: 113–114. [Medline] [CrossRef]
23. **Goto Y, Kaneyama K, Kobayashi S, Imai K, Shin-Noh M, Tsujino T, Nakano T, Matsuda S, Nakane S, Kojima T.** Birth of cloned calves derived from cultured oviductal epithelial cells of a dairy cow. *Nihon Chikusan Gakkaiho* 1999; **70**: 243–245. [CrossRef]
24. **Phillippe M.** Cell-free fetal DNA, telomeres, and the spontaneous onset of parturition. *Reprod Sci* 2015; **22**: 1186–1201. [Medline] [CrossRef]
25. **Uematsu S, Akira S.** Toll-like receptors and Type I interferons. *J Biol Chem* 2007; **282**: 15319–15323. [Medline] [CrossRef]
26. **Shirozu T, Iwano H, Ogiso T, Suzuki T, Balboula AZ, Bai H, Kawahara M, Kimura K, Takahashi H, Rulan B, Kim S-W, Yanagawa Y, Nagano M, Imakawa K, Takahashi M.** Estrous cycle stage-dependent manner of type I interferon-stimulated genes induction in the bovine endometrium. *J Reprod Dev* 2017; **63**: 211–220. [Medline] [CrossRef]
27. **Wang W, Liu R, Liang X, Zhao Q, Qu P, Yao K, Jiang M, Luo Y, Zhang W, Qing S.** Expression of IFNAR1 and IFNAR2 in cattle placenta during early pregnancy. *Reprod Domest Anim* 2018; **53**: 385–392. [Medline] [CrossRef]
28. **Han S, Zhuang H, Lee PY, Li M, Yang L, Nigrovic PA, Reeves WH.** Differential responsiveness of monocyte and macrophage subsets to interferon. *Arthritis Rheumatol* 2020; **72**: 100–113. [Medline] [CrossRef]
29. **Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM.** Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. *Nature* 1987; **330**: 377–379. [Medline] [CrossRef]
30. **Bazer FW.** Pregnancy recognition signaling mechanisms in ruminants and pigs. *J Anim Sci Biotechnol* 2013; **4**: 23. [Medline] [CrossRef]
31. **Ealy AD, Yang QE.** Control of interferon-tau expression during early pregnancy in ruminants. *Am J Reprod Immunol* 2009; **61**: 95–106. [Medline] [CrossRef]
32. **Hirayama H, Moriyasu S, Kageyama S, Sawai K, Takahashi H, Geshi M, Fujii T, Koyama T, Koyama K, Miyamoto A, Matsui M, Minamihashi A.** Enhancement of maternal recognition of pregnancy with parthenogenetic embryos in bovine embryo transfer. *Theriogenology* 2014; **81**: 1108–1115. [Medline] [CrossRef]
33. **Helmer SD, Hansen PJ, Anthony RV, Thatcher WW, Bazer FW, Roberts RM.** Identification of bovine trophoblast protein-1, a secretory protein immunologically related to ovine trophoblast protein-1. *J Reprod Fertil* 1987; **79**: 83–91. [Medline] [CrossRef]
34. **Kazemi M, Malathy PV, Keisler DH, Roberts RM.** Ovine trophoblast protein-1 and bovine trophoblast protein-1 are present as specific components of uterine flushings of pregnant ewes and cows. *Biol Reprod* 1988; **39**: 457–463. [Medline] [CrossRef]
35. **Ishikawa H, Shirahata T, Hasegawa K.** Interferon-gamma production of mitogen stimulated peripheral lymphocytes in perinatal cows. *J Vet Med Sci* 1994; **56**: 735–738. [Medline] [CrossRef]
36. **Shafer-Weaver KA, Corl CM, Sordillo LM.** Shifts in bovine CD4+ subpopulations increase T-helper-2 compared with T-helper-1 effector cells during the postpartum period. *J Dairy Sci* 1999; **82**: 1696–1706. [Medline] [CrossRef]
37. **Ealy AD, Wooldridge LK.** The evolution of interferon-tau. *Reproduction* 2017; **154**: F1–F10. [Medline] [CrossRef]
38. **Miyoshi M, Sawamukai Y, Iwanaga T.** Reduced phagocytotic activity of macrophages in the bovine retained placenta. *Reprod Domest Anim* 2002; **37**: 53–56. [Medline] [CrossRef]
39. **Nelli RK, De Koster J, Roberts JN, de Souza J, Lock AL, Raphael W, Agnew D, Contreras GA.** Impact of uterine macrophage phenotype on placental retention in dairy cows. *Theriogenology* 2019; **127**: 145–152. [Medline] [CrossRef]
40. **Kumagai Y, Takeuchi O, Kato H, Kumar H, Matsui K, Morii E, Aozasa K, Kawai T, Akira S.** Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. *Immunity* 2007; **27**: 240–252. [Medline] [CrossRef]