Activation of lysosomal cathepsins in pregnant bovine leukocytes

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Abstract

In ruminants, interferon-tau (IFNT)-mediated expression of interferon-stimulated genes in peripheral blood leukocytes (PBLs) can indicate pregnancy. Recently, type 1 IFN-mediated activation of lysosomes and lysosomal cathepsins (CTSs) was observed in immune cells. This study investigated the status of lysosomal CTSs and lysosomes in PBLs collected from pregnant (P) and non-pregnant (NP) dairy cows, and conducted *in vitro* IFNT stimulation of NP blood leukocytes. Blood samples were collected 0, 7, 14 and 18 days post-artificial insemination, and the peripheral blood mononuclear cells (PBMCs) and polymorphonuclear granulocytes (PMNs) separated. The fluorescent activity of CTSS and CTSK in PMNs significantly increased with the progress of pregnancy, especially on day 18. *In vitro* supplementation of IFNT significantly increased the activities of CTSS and CTSK in NP PBMCs and PMNs. CTSS expression was significantly higher in PBMCs and PMNs collected from P day-18 cows than from NP cows, whereas there was no difference in CTSK expression. IFNT increased CTSS expression but did not affect CTSK expression. Immunodetection showed an increase of CTSS in P day-18 PBMCs and PMNs. *In vitro* stimulation of IFNT increased CTSS in NP PBMCs and PMNs. Lysosomal acidification showed a significant increase in P day-18 PBMCs and PMNs. IFNT also stimulated lysosomal acidification. Expressions of lysosome-associated membrane protein (LAMP) 1 and LAMP2 were significantly higher in P day-18 PBMCs and PMNs. The results suggest that pregnancy-specific activation of lysosomal functions by CTS activation in blood leukocytes is highly associated with IFNT during maternal and fetal recognition of pregnancy.

Reproduction (2018) **155** 515–528

Introduction

There have been many trials to establish pregnancy diagnosis by direct and indirect methods such as the measurement of serum and/or milk progesterone levels (Nakao *et al.* 1983, Morton *et al.* 2010, Okumu *et al.* 2010), the detection of pregnancy-associated glycoproteins (PAGs) (Zoli *et al.* 1992), the observation of estrous behavior and the rectal palpation (RP) or ultrasonic examination of the conceptus (Fricke 2002, Romano *et al.* 2007). RP is a very old and still widely practiced method in large dairy animals and requires a great deal of skill and an experienced technician. During RP, manipulation of the uterus and fetal membranes increases the risk of iatrogenic embryonic mortality and can lead to abortion (Abbitt *et al.* 1978, Vaillancourt *et al.* 1979). Currently, ultrasonography is the most widely implemented direct method of early pregnancy

diagnosis in cattle, but also requires appropriate equipment and a skilled operator. Although these methods are effective, the time of detection is rather late, that is, after the maternal and fetal recognition of pregnancy, which occurs approximately during the critical period of luteolysis (Bazer *et al.* 1991, Thatcher *et al.* 1995). Therefore, a suitable indicator for early pregnancy detection is necessary.

Interferon tau (IFNT), a type 1 interferon, is a unique pregnancy recognition signal in ruminants (Bazer 2013). In cattle, mRNA transcripts of IFNT are detectable at the blastocyst stage (Rizos *et al.* 2003) followed by an increase of protein secretion from trophoblast cells of the conceptus around days 14–16 after fertilization and reaches a peak of secretion at days 18–19 (Thatcher *et al.* 1995, Mann *et al.* 1999, Roberts *et al.* 1999). The secreted IFNT alters tissues and cells to activate the signals for recognition of

pregnancy and implantation. These IFNT-mediated signals could be possible markers for pregnancy evaluation and detection.

IFNT binds to the type 1 interferon receptor subunits (IFNAR1 and IFNAR2) and induces cell signaling via the Janus activated kinases (JAKs) and tyrosine kinase 2 (TYK2) pathways (Darnel *et al.* 1994, Der *et al.* 1998, Platanias 2005).

At the time of pregnancy recognition, IFNT affects uterine tissues by activating pregnancy-specific signaling pathways such as increases of the interferon-stimulated genes (ISGs) *ISG15*, *MX1*, *MX2* and so forth. (Ott *et al.* 1998, Hicks *et al.* 2003). ISG expression is clearly up-regulated in blood leukocytes in the earlier stages of pregnancy (Han *et al.* 2006, Gifford *et al.* 2007, Oliveira *et al.* 2008). These reports suggest that the circulating blood leukocytes are highly affected by type 1 IFN (IFNT) at the time of maternal recognition. However, since the changes in *MX2* mRNA from day 0 to day 18 after artificial insemination (AI) have high variation, (Stevenson *et al.* 2007), it is still necessary to increase the reliability of these pregnancy predictions. Other ISGs such as 2'-5'-oligoadenylate synthetase 1 (*OAS1*) have been found to be a suitable pregnancy diagnosis on day 18 after AI in heifers, but not in cows (Green *et al.* 2010). Therefore, discovery and evaluation of novel pregnancy-specific extra uterine markers in the peripheral blood cells is still required. Besides, in addition to IFN-specific genes, it is necessary to develop new approaches to investigate pregnancy-specific responses in the blood cells.

Cathepsins (CTSs) are lysosomal proteases which are well known for intra/extracellular protein degradation for amino acid recruitment, and to participate in many biological processes (Turk *et al.* 2000) such as the bulk degradation of proteins within lysosomes. In humans, several CTSs have been identified and are categorized into three distinct groups based on the amino acid found in the active site; serine (A and G), aspartic (D and E) and cysteine (B, F, H, K, L, O, S, V, X and W) CTSs are known (Turk *et al.* 2001, Rossi *et al.* 2004). The aspartic CTSD and some of the cysteine CTSs, including CTSs B, L, and H, are ubiquitous and among the most abundant lysosomal proteases (Rossi *et al.* 2004). In the relationship between pregnancy and CTSs, available evidence supports the idea that a variety of proteases regulate well-defined events of trophoblast invasion in many species during implantation and placentation (Ishida *et al.* 2004, Walter *et al.* 2006). In rodents, endometrial CTSB and CTSL serve to regulate normal uterine development during implantation and placentation (Afonso *et al.* 1997). CTSL proteolytic activity has been found in the uteri of cats (Verhage *et al.* 1989, Li *et al.* 1991), pigs (Geisert *et al.* 1997) and mice (Hamilton *et al.* 1991, Afonso *et al.* 1997). Increase of CTS gene expressions and localization and activation of CTSL on days 14–18 of pregnant ovine uterus are regulated by IFNT (Song *et al.*

2005). However, no earlier data are currently available on the pregnancy-dependent expression patterns in CTSs mRNA in peripheral blood leukocytes (PBLs), although their presence in unspecified ovary mRNA samples has been reported previously (Petanceska & Devi 1992, Bromme & Okamoto 1995, Rantakokko *et al.* 1996, Kirscheke *et al.* 1998, Soderstrom *et al.* 1999).

Recently, an *in vitro* study has demonstrated that lysosomal proteases are activated by cytokines such as type 1 IFN (IFNB) in cultured mouse macrophages (Creasy *et al.* 2011). Type 1 IFN-stimulated pathways may be involved in several types of cells including blood leukocytes, because they exist in many types of somatic cell. Lysosomes contain a considerable number of proteases, the best known of which are the CTSs which are active in an acidic environment (Kirschke *et al.* 1998). Activation of lysosomes is correlated with activation of CTSs in the immune cells (Creasy *et al.* 2011).

In cattle, lysosomal activity during early pregnancy, including the gene expression patterns of lysosomal membranes and lysosomal proteases (CTSB and CTSK), and the presence of CTSB proteins in blood leukocytes remain unknown. Thus, the current investigation was undertaken to investigate the status of lysosomal CTSs and lysosomes in bovine leukocytes during early pregnancy, as well as to investigate the effects of IFNT.

Materials and methods

Animals

The animals used in this study were the multiparous Holstein Friesian cows which belong to the Hokkaido University Dairy Farm. All animal handling procedures were approved by the Hokkaido University Guidelines for the Care and Use of Experimental Animals (16-0019).

Collection of blood samples and isolation of peripheral blood mononuclear cells (PBMCs) and polymorphonuclear granulocytes (PMNs)

Healthy lactating dairy cows showing natural heat were used for AI without estrous synchronization program. After AI, blood samples were collected from the jugular vein into tubes containing heparin on day (d) 0, d7, d14 and d18. Pregnancy was confirmed by transrectal ultrasonography 40–60 days after AI. For the isolation of PBMCs, whole blood was mixed with an equal volume of phosphate buffer solution (PBS). Three milliliters of Lymphocyte Separation Medium 1077 (PromoCell, GmbH Sickingenstr, Heidelberg, Germany) was added into a separate tube. The blood cell suspension containing tube was centrifuged at 440g for 40 min at room temperature. After centrifugation, the supernatant was carefully removed and the layer in the interphase containing mononuclear cells was carefully aspirated and transferred into another tube. The mononuclear cell suspension was washed with PBS by centrifugation at 440g. After washing, PBMCs were transferred

into Roswell Park Memorial Institute (RPMI) 1640 medium containing 5% fetal bovine serum (FBS) for further analysis.

For isolation of the PMNs, 10 mL of RBC lyses buffer (The buffer solution containing NH_4Cl :87.06%, NaHCO_3 :12.54% and EDTA2Na :0.40% w/v) was added into the same tube and pipetted carefully to rupturing the erythrocytes. After centrifugation, the supernatant was removed. This step was repeated until white precipitation, a layer of granulocytes (PMNs), was observed. The PMN precipitate was washed with PBS and the separated PBMCs and PMNs samples were lysed in ISOGEN-II (Nippon Gene, Toyama, Japan) and stored at -80°C for further RNA extraction. The cells were then used for measuring CTSB and CTSK activity, gene expression analysis, immunostaining and lysosomal activity.

In vitro culture and stimulation of PBMCs and PMNs by IFNT

PBMCs and PMNs were isolated from whole blood collected from the jugular vein of healthy lactating non-AI cows at mid-late estrous stage. The cells were cultured in the RPMI 1640 containing 5% FBS with or without recombinant IFNT (rbIFNT) which was produced by *Escherichia coli* using cDNA (bTP-509A, gifted by Dr RM Roberts, University of Missouri, Columbia, MO, USA) and expression vector (Imakawa *et al.* 1987). Antiviral activity determined by MDBK cells was 8×10^6 IU/mL. Final concentration of 1000 IU/mL IFNT was designed based on the antiviral activity of plasma collected from 15 days of pregnant ovine uterine vein (500–1000 U/mL) that might be a possible stimulating concentration to leukocytes locally (Oliveira *et al.* 2008). The cells were then cultured for 3, 6 and 12 h at 38.5°C in a CO_2 incubator. At the end of each treatment, cells were collected and centrifuged at $10\,000g$ for 10 min, followed by washing twice with PBS. For measuring CTSs activity, $10\,\mu\text{L}$ cell suspensions were removed from whole cell suspension and $200\times$ diluted CTSs substrates were added and gently mixed. Cells were cultured for 40 min at 38.5°C in a CO_2 incubator. After incubation, the samples were washed twice with PBS solution. The cells were then used for measuring CTSB and CTSK activity, gene expression analysis and immunostaining of CTSB.

RNA extraction, cDNA synthesis and qPCR

Total RNA of PBMCs and PMNs from *in vivo* collected and *in vitro* -cultured samples was separately extracted and stored at -80°C until analysis. After standardizing the RNA quantity using a NanoDrop spectrophotometer (Thermo Fisher Scientific), cDNA was synthesized with a ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The synthesized cDNA was stored at -30°C . To assess the gene expression, quantitative PCR (qPCR) was performed using the KAPA SYBR FAST PCR Kit optimized for Roche Light Cycler nano (Roche Diagnostics GmbH). Primers for *ISG15* as a pregnancy-positive marker, CTSs (*B* and *K*) and lysosomal-associated membrane protein (*LAMP*) 1, 2 were designed and commercially synthesized based on reported sequences.

The reactions were carried out in a normal qPCR 8 strip tube, in a total volume of $10\,\mu\text{L}$ containing $1\,\mu\text{L}$ ($0.5\,\mu\text{L}$ reverse and $0.5\,\mu\text{L}$ forward) of each primer, $5\,\mu\text{L}$ KAPA SYBR FAST qPCR Kit and $4\,\mu\text{L}$ of cDNA, and subjected to the following cycling condition: a denaturation step at 95°C for 30 s, an amplification step of 45 cycles at 95°C for 10 s, 55°C for 15 s and 72°C for 30 s. The expression level of each gene was normalized using glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) and *ACTB* as the internal standard. The qPCR primer sequences used are presented in Table 1.

Detection of activities of CTSB and CTSK in PBMCs and PMNs

Detection of CTSs activity was performed with fluorescent substrates. Cell suspensions ($10\,\mu\text{L}$) of both PBMCs and PMNs were used. Magic Red CTSs (*B* and *K*) assay kits (MR-RR₂ and MR-LR₂) (Immunochemistry Technologies, LLC, Bloomington, MN, USA) were prepared by diluting the Magic Red stock solutions to $200\times$ with RPMI 1640 containing 5% FBS. To stain the nuclei, $1\,\mu\text{L}$ of Hoechst33342 was added, and the tubes were incubated for 40 min in a CO_2 incubator at 38.5°C . After incubation, the samples were washed twice with PBS solution. A $10\,\mu\text{L}$ sample was dropped onto a slide glass with a cover slip ($18 \times 18\text{ mm}$) and immediately placed under a fluorescence microscope (Keyence, Osaka, Japan) using a 550-nm excitation filter at $400\times$. All images in both PBMCs and PMNs were captured in the same exposure conditions.

Table 1 List of primer and primer sequence used for RT-qPCR.

Primer name		Primer sequence (5'-3')	Tm (°C)	Product length (bp)	GenBank accession number
<i>ISG15</i>	FWD:	TGAGGGACTCCATGACGGTA	59.67	72	AC_000173.1
	REV:	GCTGGAAGCAGGCACATTG	60.39		
<i>CTSB</i>	FWD:	CACTTGGAAGGCTGGACACA	66.30	141	AC_000180.1
	REV:	GCATCGAAGCTTTCAGGCAG	67.20		
<i>CTSK</i>	FWD:	TCCGGCCACATTATCCACAC	67.9	99	AC_000174.1
	REV:	ACCACAGGCAGCAGTAGAAC	61.9		
<i>LAMP1</i>	FWD:	GTGAAGAATGGCAACGGAC	66.6	250	AC_000169.1
	REV:	GCATCAGCTGGACCTCGTAA	65.2		
<i>LAMP2</i>	FWD:	AAGAGCAGACCGTTTCCGTG	66.5	110	AC_000187.1
	REV:	CGAACACTCTTGGGCAGTAG	62.4		
<i>GAPDH</i>	FWD:	CACCCTCAAGATTGTCAGCA	63.90	103	AC_000162.1
	REV:	GGTCATAAGTCCCTCCACGA	63.80		
<i>ACTB</i>	FWD:	TGGACTTCGAGCAGGAGATG	65.5	221	AC_000182.1
	REV:	GTAGAGGTCCTTGCGGATGT	63.0		

The fluorescence intensity was measured by ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA).

Immunodetection of CTSSB in *in vivo* collected and *in vitro* cultured PBMCs and PMNs

Separated PBMCs and PMNs collected from day 18 pregnant and NP cows, and from samples after 6 h of *in vitro* stimulation by IFNT, were fixed in 4% paraformaldehyde (PFA) in 0.5 mL tubes for 24 h at 4°C and processed using standard procedures. Cells were washed and then permeabilized with PBS containing 0.2% TritonX 100 (PBST) for 10 min. Then, the cells were treated for blocking with PBST containing 1% BSA for 1 h at room temperature. After blocking, incubation with the primary CTSSB polyclonal antibody (anti-rabbit IgG, 1:1000) (Calbiochem, EMD Millipore Corporation) was performed for 2 h at room temperature. The cells were also incubated with the fluorescence in conjugated secondary antibody (donkey anti-rabbit IgG, 1:1000) (GE Healthcare Bio Sciences AB) for 1 h at room temperature in dark conditions. A negative control was set by substituting the primary antibody with PBST+1% BSA at the respective dilution. Between each step, cells were washed three times in PBS for 5 min. After washing with PBS, the cell suspension was dropped onto a glass slide and covered with a cover glass (18×18 mm). All images were captured with a fluorescent microscope (Leica Microsystems) in 1.5 s exposures time using a 550-nm excitation filter at 400×. The fluorescence intensity was measured by ImageJ analysis software (National Institutes of Health).

Assessment of lysosomal activity in PBMCs and PMNs collected from pregnant and non-pregnant day 18 cows

Separated PBMCs and PMNs from day 18 pregnant and NP cows or from samples after 6 h of *in vitro* stimulation by IFNT were incubated in pre-warmed RPMI 1640 medium with 1-μM solution of DMSO diluted with LysoSensor Green DND 189 (L7535, Invitrogen by Thermo Fisher Scientific, Life Technologies) for 15 min at 38.5°C in a CO₂ incubator. Then, Hoechst 33342 (Sigma-Aldrich) was added to stain the nuclei. After incubation, samples were washed twice with PBS solution. After washing, 10 μL of cell suspension was put onto a glass slide, covered with cover glass (18×18 mm) and observed under the fluorescence microscope (Leica Microsystems) using a 590-nm excitation filter at 400×. The fluorescence images were captured with similar exposure conditions and analyzed by ImageJ Software (National Institutes of Health).

Statistical analysis

Data were analyzed using GraphPad prism version 5 for Windows (GraphPad Software, www.graphpad.com) and were presented as the mean ± S.E.M. One-way ANOVAs and Student's *t* tests were performed. *P* values less than 0.05 (*P* < 0.05) were considered to represent statistically significant differences.

Results

Activities of CTSSB and CTSSK in PBMCs and PMNs in pregnant and non-pregnant cows

Fluorescence intensity of CTSSB and CTSSK activity were detected in both PBMCs and PMNs (Fig. 1A and B). The fluorescence intensity of CTSSB activity was higher in PBMCs (*P* < 0.05) and PMNs (*P* < 0.01) after d14 when compared to d0 and d7 of pregnancy, but was not different for cells from NP cows (Fig. 2A and B). Similar to CTSSB, strong fluorescence from CTSSK activity was detected in the PBMCs and PMNs of pregnant cows (Fig. 1B). The fluorescence intensity of CTSSK in PMNs was higher (*P* < 0.05) on d14 and d18 of pregnancy than on d0 and d7. The fluorescence intensity of CTSSK was

Figure 1 Activities of CTSSB and CTSSK in PBMCs and PMNs collected from d18 pregnant and non-pregnant cows. Photos show the fluorescence images of CTSSB (A) and CTSSK (B) in PBMCs and PMNs on d18 non-pregnant (NP) and pregnant (P) cows. After the cells were incubated with fluorescence substrate for CTSSB and CTSSK, cells were washed, and the fluorescence activities were detected by a fluorescent microscope using a 550-nm excitation filter at 400×. The scale bar represents 50 μm.

Figure 2 Changes in the activities of CTSA and CTSC in PBMCs and PMNs collected from pregnant and non-pregnant cows. Relative activities of CTSA in PBMCs (A) and PMNs (B), and of CTSC in PBMCs (C) and PMNs (D) on d 0–18 after insemination in dairy cows that were later diagnosed as pregnant (black bars) or not pregnant (gray bars). Ten cows (5 for pregnant and 5 for non-pregnant) were used for detecting the activities of CTSA and CTSC in PBMCs and PMNs. All data are shown as the mean $\pm$ S.E.M. Different letters indicate significant differences ($P < 0.05$) among the days in pregnant (P) cows according to ANOVA followed by Fisher's as a multiple-comparison test. Asterisks indicate differences ($**P < 0.0001$, Student's *t* test) between non-pregnant (NP) and pregnant (P) cows on the same days after AI.

higher ($P < 0.001$) in the pregnant d18 PMNs than it was in NP d18 cells (Fig. 2D).

Effect of IFNT stimulation on activity of CTSA and CTSC in PBMCs and PMNs obtained from non-AI cows

An increase in CTSA activity was observed both in PBMCs and PMNs after the addition of IFNT (Fig. 3A). Increases of fluorescence intensity were observed in PBMCs ($P < 0.05$) after 12 h and in PMNs ($P < 0.001$) 6 h after stimulation with IFNT (Fig. 3B and C). In addition,

IFNT increased CTSC activity in both PBMCs and PMNs (Fig. 4A). Fluorescence intensity was increased in PBMCs after 6 h ($P < 0.05$) and in PMNs after 3 h ($P < 0.05$) of IFNT treatment (Fig. 4B and C).

Gene expression of CTSA and CTSC in PBMCs and PMNs from non-pregnant and pregnant cows

To confirm that the leukocytes of pregnant cows were affected by IFNT stimulation, expression of *ISG15*, a positive marker gene of pregnancy, was detected. In

Figure 3 CTSB activity of non-pregnant PBMCs and PMNs after *in vitro* stimulation by IFNT. Photos show the fluorescence images of CTSB in PBMCs and PMNs after 3, 6 and 12 h of IFNT stimulation (A). Relative fluorescence intensity of CTSB activity of PBMCs (B) and PMNs (C). After IFNT stimulation, cells were incubated with Magic Red treatment. The CTSB activity was detected using a 550-nm excitation filter at 400 $\times$. The scale bar represents 50 μ m. Gray bars show the fluorescence intensity of the non-treated group and black bars the IFNT-treated group. All data are shown as the mean $\pm$ S.E.M. Different letters indicate difference ($P < 0.05$) among IFNT treatment times according to ANOVA followed by Fisher's as a multiple-comparison test. Experiment was repeated 4 times. Asterisks indicate differences ($*P < 0.05$, $**P < 0.01$, $***P < 0.0001$, Student's *t* test) between groups with the same incubation time.

Figure 4 CTSK activity of non-pregnant PBMCs and PMNs after *in vitro* stimulation of IFNT. Photos show the fluorescence images of CTSK in PBMCs and PMNs after 3, 6 and 12 h of IFNT stimulation (A). Relative fluorescence intensity of CTSK activity of PBMCs (B) and PMNs (C). After IFNT stimulation, cells were incubated with Magic Red treatment. The CTSK activity was detected using a 550-nm excitation filter at 400 $\times$. The scale bar represents 50 μ m. Gray bars show the fluorescence intensity of the non-treated group and black bars the IFNT-treated group. All data are shown as the mean $\pm$ S.E.M. Different letters indicate difference ($P < 0.05$) among the IFNT treatment times according to ANOVA followed by Fisher's as a multiple-comparison test. Experiment was repeated 3 times. Asterisks indicate differences (** $P < 0.01$, *** $P < 0.0001$, Student's *t* test.) between groups with the same incubation time.

Figure 5 Expression of *CTSB* and *CTSK* mRNA in PBMCs and PMNs of pregnant and non-pregnant cows after AI. The graphs show the expression levels of *CTSB* in PBMCs (A) and PMNs (B), and *CTSK* in PBMCs (C) and PMNs (D), standardized by *ACTB*. Gray bars show

PBMCs, *ISG15* mRNA expression levels were higher ($P < 0.05$) on d18 in pregnant cows than they were in other stages (Supplementary Fig. 1A, see section on supplementary data given at the end of this article). In PMNs, expression of *ISG15* mRNA was higher ($P < 0.05$) on d18 than it was at other stages (Supplementary Fig. 1B).

Expression of the *CTSB* gene in PBMCs was higher on d14 ($P < 0.05$) and d18 ($P < 0.01$) in pregnant cows than it was in NP cows, respectively (Fig. 5A). *CTSB* expression was also higher ($P < 0.05$) in PMNs on d18 of pregnancy than in those collected from NP cows (Fig. 5B). *CTSK* mRNA expression levels were not affected in either PBMCs or PMNs (Fig. 5C and D).

Effect of IFNT stimulation on the expression of *CTSB* and *CTSK* in PBMCs and PMNs obtained from non-AI cows

Expression of *CTSB* mRNA in PBMCs and PMNs was higher ($P < 0.05$) after 6 h of IFNT stimulation (Fig. 6A and B). *CTSK* mRNA level was not affected by IFNT treatment (Fig. 6C and D). *ISG15* mRNA expression was increased in both PBMCs ($P < 0.0001$) and PMNs ($P < 0.01$) by IFNT treatment ($P < 0.01$) (Supplementary Fig. 2C and D).

Immunohistochemistry of *CTSB* protein in PBMCs and PMNs

After immunostaining, *CTSB* was clearly detected in *in vivo* collected PBMCs and PMNs (Fig. 7A). Image analysis showed that fluorescent intensities of *CTSB* were higher in pregnant PBMCs ($P < 0.001$) and PMNs ($P < 0.05$), respectively, than those in NP cows (Fig. 7B and C). *In vitro* stimulation with IFNT also showed an increase of fluorescence in PBMCs ($P < 0.05$) and PMNs ($P < 0.01$), respectively, compared with non-stimulated control cells (Fig. 7D, E and F).

Lysosomal activity in PBMCs and PMNs

Clear fluorescence indicating the acidification status of lysosomes was observed in both PBMCs and PMNs (Fig. 8A). The fluorescence intensities of PBMCs and PMNs from pregnant cows were higher ($P < 0.05$) than those from NP cows (Fig. 8B and C). *In vitro* stimulation

the gene expression levels of non-pregnant (NP) cows, and black bars show the pregnant (P) cows. Twelve cows (6 for pregnant and 6 for non-pregnant) were used for detecting the activities of *CTSB* and *CTSK* in PBMCs and PMNs. All data are shown as the mean $\pm$ S.E.M. Different letters indicate differences ($P < 0.05$) among the days in pregnant (P) cows according to ANOVA followed by Fisher's as a multiple-comparison test. Asterisks indicate differences (* $P < 0.05$, ** $P < 0.01$, Student's *t* test) between non-pregnant (NP) and pregnant (P) cows on the same days after AI.

Figure 6 Effect of IFNT stimulation on the expression of CTSCB and CTSC in PBMCs and PMNs obtained from non-AI cows. Expression levels of CTSCB in PBMCs (A) and PMNs (B), and CTSC in PBMCs (C) and PMNs (D), using RT-qPCR standardized with the reference gene

of both cells by IFNT showed increases ($P < 0.01$) in fluorescence intensity compared with non-stimulated control cells (Fig. 8D, E and F).

Gene expression of LAMP1 and LAMP2 in PBMCs and PMNs from d18 of non-pregnant and pregnant cows

Expression of *LAMP1* was higher ($P < 0.05$) in both PBMCs and PMNs on d18 in pregnant cows (Fig. 9A and B). Similarly, expression of *LAMP2* was higher in PBMCs ($P < 0.05$) and PMNs ($P < 0.01$) of d18 pregnant cows, respectively (Fig. 9C and D).

Discussion

Although many methods are used for the diagnosis of pregnancy, generally, all these techniques are only applicable three weeks after fertilization (Romano *et al.* 2007, Morton *et al.* 2010, Okumu *et al.* 2010). Exploring new candidate genes which are induced by type 1 interferon (that is, IFNT) and their potential use as markers of pregnancy through immune cells increases the possibility of early diagnosis of pregnancy in cattle. In the present study, we investigated the pregnancy-specific lysosomal functions associated with CTSCs, lysosomal proteases and their regulations by IFNT in PBMCs and PMNs using *in vivo* and *in vitro* experiments.

In the present study, *in vivo* analysis revealed a significant increase in the activity of CTSCs B and K in PMNs in pregnant cows on d18 compared with that of cells collected from d0 post-AI cows. To confirm whether the pregnancy-related CTSCs activation is caused by IFNT, an *in vitro* study was conducted. As expected, the fluorescence intensity of these CTSCs enzyme activity was increased by IFNT stimulation both in PBMCs and PMNs. These results strongly suggest that the activation of the CTSCs of immune cells could be stimulated by IFNT.

To confirm that the increase of CTSC activity is pregnancy-specific in blood leukocytes, *ISG15* was also analyzed as a potential positive marker of pregnancy *in vivo*. Its response to *in vitro* stimulation using rIFNT, which has been well established as a suitable early pregnancy detection marker in many studies (Green *et al.* 2010, Shirasuna *et al.* 2012, Puglisi *et al.* 2014), was also assessed. Our results showed high *ISG15* expression levels, indicating that the PBMCs and PMNs were affected by IFNT stimulation both in *in vivo* and *in vitro* (Supplementary Figs 1 and 2). Therefore, the increase of CTSC activities on d18 was very possibly due to IFNT-mediated stimulation, as its secretion is maximal at this stage.

ACTB. IFNT stimulation was performed for 3, 6 and 12 h. Experiment was repeated 7 times for CTSCB and 5 times for CTSC, respectively. All data are shown as the mean $\pm$ S.E.M. Different letters indicate differences ($P < 0.05$) among the stimulation times by ANOVA followed by Fisher's PLSD procedure as a multiple-comparison test.

Figure 7 Immunostaining and detection of CTSB protein in *in-vivo*-collected pregnant/ non-pregnant and IFNT-stimulated of non-pregnant PBMCs and PMNs. Immunohistochemical localization of the CTSB protein in PBMCs and PMNs collected from d18 pregnant (P) and non-pregnant (NP) cows (A), relative fluorescent levels of CTSB in PBMCs (B) and PMNs (C). Immunohistochemical localization of the CTSB protein in control and IFNT-treated PBMCs and PMNs, relative fluorescent levels of CTSB in PBMCs (E) and PMNs (F). The images were captured with a fluorescence microscope using a 590-nm excitation filter at 400 $\times$. The scale bar represents 50 μ m. Six cows (3 for pregnant and 3 for non-pregnant) were used for the analysis. *In vitro* stimulation was repeated 3 times. Asterisks (***) P <0.0001, (*) P <0.01, (*) P <0.05, Student's *t* test indicate differences in non-pregnant (NP) vs pregnant (P) or control vs IFNT stimulation.

CTSB and CTSK are lysosomal proteases capable of digesting matrix proteins and activating other proteases involved in matrix degradation (Kirschke *et al.* 1998). CTSs participate in the bulk degradation of proteins within the lysosomes, but many CTSs have also been shown to be critically involved in distinct physiological processes, including bone remodeling, protein processing, antigen presentation, degradation of extracellular matrix and initiation of cell death (Reiser *et al.* 2010, Turk *et al.* 2012). CTSs are predominantly endopeptidases and are relatively nonspecific enzymes. Lysosomal proteases such as CTS activity are regulated by certain cytokines. Fiebiger *et al.* (2001) reported that CTSs S and B activity increased in human dendritic cells (DCs) in response to cytokines such as TNF alpha and IL1 beta, leading to increased class 2 MHC dimer formations and T cell recognition. Altering endosomal pH and lysosomal protease activity can be modulated by cytokines such as IL6 and IL10 (Drakesmith *et al.* 1998, Fiebiger *et al.* 2001).

The present study indicated that type 1 interferon (that is, IFNT) increased the activity of CTSs. IFNT might have affected CTSs by different mechanisms than TNF alpha and IL1 beta. Sendide *et al.* (2005) showed that CTS activity, enhanced by IL10 and IL6, leads to improved presentation and recognition of T cell epitopes in IL-treated DCs.

The differences in the expression of CTSB and CTSK genes in pregnant and NP cows suggest functional diversity in the immune cell mechanisms responsible for the expression of CTSs genes that support implantation and placentation during the establishment and maintenance of pregnancy. The results of the present study support the hypothesis that CTSs are dynamically expressed in pregnant bovine PBLs and that they play important roles in the implantation, placentation and growth of the conceptus. In the present study, CTSB activity and gene expression were increased both in pregnant d18 and IFN-induced cultured PBMCs and PMNs. This indicates the IFNT-induced up-regulation

Figure 8 Lysosomal activity in *in-vivo*-collected pregnant/non-pregnant and IFNT-stimulated PBMCs and PMNs. Fluorescence image of lysosomal acidification in PBMCs and PMNs collected from d18 pregnant (P) and non-pregnant (NP) cows (A), relative fluorescent levels of lysosomal activity in PBMCs (B) and PMNs (C). Fluorescence image of lysosomal acidification in control and IFNT-treated PBMCs and PMNs *in vitro* (D), relative fluorescent levels of lysosomal activity in PBMCs (E) and PMNs (F). The lysosomal acidifications were detected with the lysosensor using a 590-nm excitation filter at 400 $\times$. The scale bar represents 50 μ m. Six cows (3 for pregnant and 3 for non-pregnant) were used for the analysis. *In vitro* stimulation was repeated 3 times. All data are shown as the mean $\pm$ standard error of the mean (S.E.M.). Asterisks (** $P < 0.05$, Student's *t* test) indicate difference between the control and IFNT treatment groups.

pathway of the *CTSB* gene followed by an increase of the protein, as well as *CTSB* activity and gene expression. However, despite high activity of *CTSK* in PBMCs and PMNs both in pregnant d18 and IFN-stimulated cells, gene expression was not clearly affected by pregnancy or IFN stimulation. The different expression patterns of *CTSB* B, K, L and S are reported in osteoclasts (Ishibashi *et al.* 2001) even though *CTSB* and *CTSK* belong to same cysteine protease. *In vitro*, *CTSB* and *CTSK* mRNA levels in PBMCs increased significantly after 3 and 6 h of IFNT stimulation, compared to the control group. Also, in PMNs, these mRNA levels significantly increased at 3-, 6- and 12-h time intervals in the IFNT-stimulated group compared to the control group. Emond *et al.* (2000) showed that, *in vitro*, IFNT stimulated the independent gene expression pattern of a granulocyte macrophage colony stimulating factor in bovine leukocytes and endometrial stromal cells independently.

Cysteine *CTSB*s are optimally active in a slightly acidic pH, becoming mostly unstable at a neutral pH, and different *CTSB*s have different activation conditions (Turk *et al.* 2012). Similar intracellular processing of

CTSK is also reported (Dodds *et al.* 2001). Therefore, the different gene expression patterns of *CTSK* and *CTSB* may not correlate with enzymatic activities. In addition, detection of fluorescent activity was achieved with different fluorescent substrates for *CTSB* and *CTSK*. Thus, another possible explanation for the non-correlation with *CTSK* mRNA levels and fluorescence generated by digestion of fluorescent substrate is the fluorescence intensity caused by excitation strength or the amount of conjugated fluorescent dyes. In the case of *CTSB* activity, protein synthesis and gene expression were increased in the PBMCs and PMNs on d18 of pregnancy *in vivo* and after IFNT stimulation *in vitro*. *CTSB* could be a potential marker for pregnancy detection in the blood cells.

Interestingly, in the present study, clear activation of lysosomes by increasing acidification was detected, as well as the increase of gene expression of *LAMP1* and *LAMP2* in pregnant PBLs. Lysosomes are membrane-bound dynamic organelles that are indispensable for endocytosis, phagocytosis and autophagy (Eskelinen *et al.* 2003). Lysosomes contain enzymes that breakdown unused intracellular materials, such as acid hydrolases

Figure 9 Expression of *LAMP1* and *LAMP2* mRNA in pregnant and non-pregnant PBMCs and PMNs. The graphs show the expression levels of *LAMP1* in PBMCs (A) and PMNs (B), and of *LAMP2* in PBMCs (C) and PMNs (D) collected from pregnant and non-pregnant d18 cows using RT-qPCR, standardized with the reference gene *ACTB*. Asterisks indicate differences for *LAMP1* (* $P < 0.05$) and for *LAMP2* (* $P < 0.05$, ** $P < 0.01$) between non-pregnant (NP) and pregnant (P) cows after AI, determined by Student's *t* test.

synthesized in the endoplasmic reticulum and packaged in the Golgi complex into so-called primary lysosomes. These lysosomes fuse with endosomes to form endolysosomes or secondary lysosomes; hydrogen ions then create an acidic environment and activate the enzymes. Thus, mature and active lysosomes are characterized by a highly acidic pH and activated hydrolytic enzyme are formed (Singh 2011). Lysosomes contain many different types of hydrolytic enzymes, including CTSs, expressing their maximum enzymatic activity at low pH maintained by active proton pumps. The acidic condition of lysosomes is maintained by a vacuolar ATPase that pumps protons from the cytosol into the lysosomal lumen (Luzio *et al.* 2007). *LAMP1* and *LAMP2* are major lysosomal transmembrane proteins which regulate lysosomal functions including acidification (Saftig & Klumperman 2009). Therefore, the increase of lysosomal acidification as well as CTS activity caused by low pH can be explained by an increase of *LAMP1* and *LAMP2*, followed by an increase of functional lysosomal membrane activation. Stimulation of lysosomal function by IFNB as well as activation of CTSs in mouse bone marrow-derived macrophages has been reported (Creasy *et al.* 2011).

However, there are currently no reports discussing the direct effect of IFNT on lysosomal activation mechanisms associated with the roles of lysosomal membrane proteins. *MxA*, a type 1 IFN-stimulated gene (protein), co-localizes with *LAMP1* in lysosomes of *MxA* expressing cells (Stertz *et al.* 2006). This indicates that lysosomal activation by lysosomal membrane proteins may be functional with ISG via a type 1 IFN pathway in

pregnant bovine leukocytes. It is still necessary to clarify the mechanisms.

In conclusion, the present study first showed the activation of lysosomes and CTS enzymes in PBLs at the time of maternal and fetal recognition of pregnancy and that their activation is mediated by IFNT. These findings suggest a possible marker for potential early pregnancy detection.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0078>.

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.

Funding

This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (KAKENHI, 15H04579) and by the Cooperative Research Program for Agriculture Science & Technology Development (RDAPJ01029305).

Acknowledgements

We are grateful to all members and technical staff of Hokkaido University Dairy Farm for continuous support for blood sampling. We would like to thank Editage (www.editage.jp) for English language editing.

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Received 15 February 2018

First decision 20 March 2018

Revised manuscript received 27 March 2018

Accepted 6 April 2018