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Title	Pregnancy specific regulation of lysosomal cathepsins in bovine blood leukocytes
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Citation	北海道大学. 博士(農学) 甲第13322号
Issue Date	2018-09-25
DOI	10.14943/doctoral.k13322
Doc URL	http://hdl.handle.net/2115/90535
Type	theses (doctoral)
File Information	Md._Abdus_Shabur_Talkder.pdf

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Pregnancy specific regulation of lysosomal cathepsins in bovine blood leukocytes

（ウシ末梢白血球におけるリソソームカテプシンの妊娠特異的応答機構に関する研究）

Doctor of Philosophy

in Division of Agrobiology,

Graduate School of Agriculture

at

Hokkaido University

JAPAN

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Abstract

In ruminants, interferon tau (IFNT) is an important pregnancy recognition signal which is secreted from the trophectoderm of the conceptus at the blastocyst stage. IFNT-mediated expression of interferon stimulated genes (ISGs) 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 was conducted to explore the dynamics of lysosomes and lysosomal CTSs in PBLs collected from pregnant and non-pregnant dairy cows, and conducted *in vitro* IFNT-stimulation of blood leukocytes from cyclic cows. Multiparous Holstein Friesian cows were subjected to artificial insemination (AI) after estrus detection. Leukocytes collected from peripheral bloods on d18 followed by confirmation of pregnant and non-pregnant cows were separated and used for the measurement of lysosomal acidification, activities of CTSs B and K, expression of *LAMP-1, -2* and CTSs (*B*, *K*). Lysosomal activity and CTSs (B, K) activities were increased significantly in the pregnant leukocytes. Expression levels of *LAMP-1, -2* a major lysosomal membrane proteins and genes, and *CTSs* (*B*, *K*) genes showed higher expression in leukocytes from pregnant cows than non-pregnant leukocytes. Besides, immunodetection showed a significant increase of CTSK and LAMP-1 in the leukocytes of pregnant cows. To observe the cell specific activity and expression difference, the next experiment was conducted with separated peripheral blood mononuclear cells (PBMCs) and polymorphonuclear granulocytes (PMNs) from leukocytes collected on d0, 7, 14 and 18 post AI. Activities of CTSs B, K and L in PBMCs and PMNs were increased significantly in the progress of pregnancy. Expression levels of *CTSs* (*B*, *K*) genes were significantly

increased at d14 and peaked at d18 either in PBMCs or PMNs in pregnant cows compared with non-pregnant cows. Assessment of lysosomal activity was also showed higher and increased significantly in PBMCs and PMNs in pregnant cows. Expression levels of *LAMP-1, -2* were increased significantly in pregnant cows compared to non-pregnant cows. Immunodetection analysis revealed that, CTSB protein was detected and significantly increased in PBMCs and PMNs in pregnant cows compared to non-pregnant cows. To confirm the pregnancy dependent activation of lysosome and lysosomal CTSs as well as gene expressions are triggered by IFNT secreted from conceptus, *in vitro* experiment with recombinant IFNT (rbIFNT) was performed. Treatment of IFNT significantly increased CTSs (B, K and L) activity in PBMCs and PMNs respectively. These results suggest that PMNs have higher sensitivity than PBMCs and are suitable for potential marker cells for detection of early pregnancy. In *in vitro*, expression levels of *CTSs* (*B*, *K*) were increased significantly both in PBMCs and PMNs by IFNT treatment. Assessment of lysosomal activity showed the higher and increased significantly in PBMCs and PMNs by IFNT treatment. Immunodetection analysis revealed that CTSB protein was detected and significantly increased in PBMCs and PMNs after IFNT treatment. These results suggest that, CTSs activity is up-regulated by the IFNT stimulation in relation to the lysosomal function. The findings of these study demonstrated that, lysosomal CTSs could be reactive to IFNT in PBLs during early pregnancy and may be useful target genes for reliable directory around the maternal and fetal recognition of pregnancy.

The pregnancy-specific lysosomal activation of blood leukocytes is highly possible by IFNT. However, IFNT stimulation on PBLs mechanism is unclear. Therefore, the next

experiment was conducted to investigate the involvement of type 1 IFN signal transduction pathway by IFNT-mediated lysosomal activation. Lysosomal activity and expression of *LAMP-1*, *-2* as wells as activities of CTSs (B, K) were significantly increased. In contrast, addition of AZD1480, a Janus activated kinase (JAK) inhibitor, significantly decreased the lysosomal and CTSK activity, expression levels of *LAMP-1*, *-2* and *CTSK* in the presence of IFNT. Immunodetection also showed a significant increase of CTSK and LAMP-1 after IFNT stimulation and it has decreased significantly after treatment of AZD1480 in the presence of IFNT. These results suggest that lysosomal activity, expressions of CTSs and LAMP genes as well as protein synthesis in leukocytes are regulated by IFNT through a type 1 IFN mediated pathway at the time of pregnancy recognition.

It is concluded that, the lysosomes and lysosomal CTSs activities, expression and protein levels could be responsive to IFNT during maternal-fetal recognition period of pregnancy in PBLs via type 1 IFN signal transduction pathway and also lysosome and lysosomal CTSs potentially be useful biomarkers for early pregnancy detection.

ACKNOWLEDGEMENTS

Above all, the author takes an opportunity to express his deepest sense of gratefulness to the almighty Allah (SWT) who empowers the author to complete the research work as well as to submit the thesis for the degree of Doctor of Philosophy (PhD) in Animal Reproduction.

Distinctly, the author acknowledges his deepest gratitude, profound appreciation and indebtedness to his honorable Sensei and research supervisor Professor Dr. Masashi Takahashi, Laboratory of Animal Genetics and Reproduction, Department of Animal Science, Research Faculty of Agriculture, Hokkaido University, Japan for his scholastic guidance, constant encouragement, ingenious suggestions and sincere supervision during the entire period of research work and also in writing the thesis successfully.

The author sincerely expresses his heartfelt gratitude to honorable Associate Prof. Dr. Manabu Kawahara and Assistant Prof. Dr. Hanako Bai, Laboratory of Animal Genetics and Reproduction, Department of Animal Science, Research Faculty of Agriculture, Hokkaido University, Japan for their valuable advices during the research work.

The author gives special thanks to his former MS supervisor Prof. Dr. M.A.M. Yahia Khandoker, Department of Animal Breeding and Genetics, Bangladesh Agricultural University for his encouragement of higher education.

The author gives special thanks to Prof. Yasuo Kobayashi and Associate Prof. Satoshi Koike in the Laboratory of Animal Function and Nutrition for give me opportunity to sharing the machineries at the beginning stage of this research.

The author also would like to give special thanks Associate Prof. Nagano Masashi, Laboratory of Theriogenology, Graduate School of Veterinary Medicine, Hokkaido

University for his kind cooperation.

The author also would like to give heartfelt thanks to Assistant Prof. Mitani Tomohiro, Field Science Center for Northern Biosphere for his kind assistance during sample collection period.

The author also gives cordial thanks to Dr. Balboula Zaky Ahmed, Department of Theriogenology, Mansoura University, Egypt for his valuable suggestions and advice during research time.

The author would like to give thanks to all the members and office staffs of Hokkaido University Dairy farm for their kind assistance during the sampling time throughout the research period.

The author would like to give cordial thanks to all laboratory members and gives special thanks to Hiroki Iwano for his kind cooperation.

The author is also grateful to the Ministry of Science and Technology, Government of the Peoples Republic of Bangladesh for selecting me as a fellow of "Bangobandhu Science and Technology Fellowship Trust" providing financial support to continue the research work.

The author would like to give indebted thanks to his wife Dr. Shirin Akter, Associate Professor, Department of Parasitology, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh, Bangladesh for her personal support, encouragement, sacrifices and great patience at all times.

I would like to give special thanks to my daughter Marzia Tabassum Rida, who is the reason to come me in Japan with my family.

Last, but by no means least, the author expresses his deepest sense of gratitude and feelings to his beloved father (departed), mother, younger brother, sister and relatives for their heartiest blessings during the entire period of my academic life.

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Abbreviations

mRNA	: Messenger ribonucleic acid
EDTA	: Ethylene diamine tetra-acetate
NCBI	: National Center for Biotechnology Information
RT-qPCR	: Real time quantitative polymerase chain reaction
bp	: Base pair
cDNA	: Complementary DNA
PBLs	: Peripheral blood leukocytes
ECM	: Extra cellular matrix
PBS	: Phosphate buffered saline
PBMCs	: Peripheral blood mononuclear cells
PMNs	: Polymorphonuclear granulocytes
DMSO	: Dimethyl sulfoxide
RPMI	: Roswell park memorial institute
NH4Cl	: Ammonium chloride
NaHCO3	: Sodium bi carbonate
EDTA2Na	: Ethylenediaminetetraacetic acid disodium salt
W/V	: Weight by volume
CTSs	: Cathepsins
IFNT	: Interferon tau
PFA	: Paraformaldehyde
V-ATP	: Vacuolar adenosine tri phosphate
TNF	: Tumor necrosis factor
IL	: Interleukin
MHC	: Major histocompatability complex
DCs	: Dendritic cells
JAK	: Janus activated kinase
TYK	: Tyrosine kinase
GAPDH	: Glyceraldehyde -3- phosphate dehydrogenase

Unit measures abbreviations

%	: Percentage
n	: Number
min	: Minute
ml	: Milliliter
ng	: Nanogram
s	: Second
× *g*	: Times gravity
°C	: Degree Celsius
µM	: Micro molar
µg	: Microgram
µl	: Microliter
nM	: Nano molar
IU	: International unit

Chapter I

General Introduction

In most mammalian species, an embryo secretes signals to specify its presence to the mother for successful establishment of pregnancy. The interferon tau (IFNT), a key pregnancy recognition signal in ruminants, is secreted from the trophectoderm of the conceptus at the blastocyst stage and its level increases with the elongation of the conceptus (Bazer FW, 2013). The IFNT during the maternal recognition period (MRP) is important for successful establishment of pregnancy in ruminants.

During the pregnancy recognition period, IFNT acts to uterine tissues to silence the expression of estrogen receptor (ESR) alpha and oxytocin receptor (OXTR), which prevents the oxytocin-dependent release of luteolytic pulses of prostaglandin F2α (PGF2α) secretion by uterine tissue. Therefore, the corpus luteum (CL) continues to produce progesterone required for maintenance of pregnancy (Bazer, 2013, Wolf *et al*. 2003, Thatcher *et al*. 2001). IFNT may act other organs and cells than that the uterine tissue. Recently, it has reported that pregnancy induced up-regulation of interferon stimulated gene 15 (*ISG15*) and myxovirus resistance 1 (*MX1*) genes in bovine liver on d18 (Mayerholz *et al*. 2016) and peripheral blood leukocytes (PBLs) (Gifford *et al*. 2007, Han *et al*. 2006).

Nowadays, many studies have focused on identifying pregnancy-specific markers in blood samples such as PBLs or peripheral blood mononuclear cells (PBMCs) which contain T lymphocytes required to induce the immune response. Recent transcriptome analysis studies have shown that PBLs are excellent sources of biological makers of physiological conditions in humans and other animals (Galvao *et al*. 2012, Huck *et al*. 2005,

Ledderose *et al*. 2001). Microarray analysis further indicated that many genes induced IFN stimulated are up-regulated in neutrophils during early pregnancy (Kizaki *et al*. 2013). Because PBLs reflect the physiological changes occurred in early pregnancy period, the gene expression profile of PBLs is might be a potential source of pregnancy-prediction biomarkers (Ott and Gifford, 2010).

Pregnancy diagnosis is an essential factor of dairy industry for good management, optimizing production and maximizing the reproductive efficiency. Early identification of non-pregnant cows after artificial insemination (AI) improves the reproductive efficiency and pregnancy rate by shortening the interval between AI services and increasing AI service rate (Fricke, 2010). However, unfortunately none of the methods fulfill all of the attributes of the ideal early pregnancy detection due to limitation of accuracy, later stage of applicability, requirement of elaborate instruments and laboratory setup. For pregnancy diagnosis, various direct and indirect methods are employed in cows, such as the measurement of serum and/or milk progesterone levels (Morton *et al*. 2010, Okumu *et al*. 2010, Nakao *et al*. 1983), detection of pregnancy associated glycoproteins (PAGs) (Zoli *et al*. 1992), observation of estrus behavior, rectal palpation (RP) or ultrasonic examination of the conceptus (Romano *et al*. 2007, Fricke, 2002). RP is a traditional and widely practiced method, in large animals even today which requires a great deal of skill and experienced technician. However, on the other hand, manipulation of the uterus and fetal membranes increases the risk of iatrogenic embryonic mortality and can lead to abortion (Vaillancourt *et al*. 1979, Abbitt *et al*. 1978). Currently, ultrasonography is mostly implemented direct method of early pregnancy diagnosis in cattle, but it requires appropriate equipment by a

skilled operator. Pregnancy detection using both methods are relatively late following the maternal recognition of pregnancy (day 16-20), which occurs at approximately the critical period of luteolysis (Thatcher *et al*. 1995, Bazer *et al*. 1991). Therefore, an alternate method of early pregnancy detection is necessary.

Levels of early pregnancy factor (EPF) and pregnancy specific protein-B (PSP-B) in blood were used as indicators for pregnancy diagnosis in cows (Austin *et al*. 1999). Blood assessments of pregnancy specific protein-B (PSP-B) have been developed and displayed similar accuracy as trans-rectal ultrasonography in cattle (Romano and Larson, 2010), however, PSP-B tests are accurate only after day 28, approximately a week after the expected time of return to estrus. All these methods are generally applicable 3-4 weeks after fertilization. However, it is still essential to develop a method to detect pregnancy at earlier time before next estrus around d18 after first insemination, a time when ultrasonography cannot reliably detect pregnancy.

It has been suggested that pregnancy or non-pregnancy status must be determined in dairy cows as soon as possible after insemination with less stress to avoid the subsequent embryonic mortality (Melrose, 1979; Studer, 1969). In ruminants, the research of early pregnancy diagnosis is significant due to relatively high incidence of pregnancy loss occur during the periimplantation period of pregnancy. Diskin *et al*. (2006) reported that in cattle, fertilization rate is 90% with an average calving rate of about 55%, including an embryonic fetal mortality of about 35%.

Apart from uterine endometrium, increased expression of ISGs (*ISG15*, *MX1* and *MX2*) was also found outside uterus, such as in the corpus luteum and PBLs during

pregnancy (Gifford *et al*. 2008, Oliveira *et al*. 2008). The question is whether ISGs can be used for early and non-invasive detection of pregnancy away from uterine tissues. Yankey *et al*. (2001) reported a significant increase in *MX1* and *MX2* mRNA levels on d15 after insemination in pregnant ewes. Subsequent studies have confirmed these results in dairy cows (Kose *et al*. 2014, Green *et al*. 2010, Gifford *et al*. 2007). Moreover, exogenous administration of IFNT up-regulated ISGs expression in the endometrium, CL and PBLs *in vivo* (Matsuyama *et al*. 2012, Spencer *et al*. 1999). The idea is that expression pattern of ISGs in PBLs could be a good indicator of conceptus development as emphasized by many scientists (Matsuyama *et al*. 2012, Han *et al*. 2006). The systemic effect of the conceptus has also been investigated with regard to IFNT and the expression of *ISG* in PBLs (Oliveira *et al*. 2008) and (Ott and Gifford, 2010), gene expression of *ISGs* is also induced in bovine PBLs (Pugliesi *et al*. 2014, Green *et al*. 2010) on pregnant d18. These reports also suggested that ISGs expression in PBLs could be evaluated to determine cow pregnancy status (Forde and Lonergan, 2012).

During pregnancy recognition period, IFNT affects uterine tissues with activating pregnancy-specific signal such as increase of several ISGs (*ISG15*, *MX1*, *MX2* and *OAS1*). ISGs in PBLs are found as a pregnancy specific responding factor and also as a potential pregnancy marker. The advancement of molecular techniques has opened new avenues for exploring a unique molecule as pregnancy markers in ruminants through studies on IFNT induce ISGs. However, ISGs expression varies according to the stage of estrus cycle and individual variation of the cows, which make it to difficult to determine the threshold level of positive pregnancy. Therefore, it is necessary to discovery other novel markers which

have the ability to detect pregnancy at earlier period with easy and clear manner.

Review of Literature

In early pregnancy, maternal recognition and implantation of the conceptus are critical for successful establishment of pregnancy. IFNT plays a unique role in the establishment of pregnancy and changes the gene expressions not only in the uterus but also in the PBLs in early pregnancy.

Recent researches have demonstrated that portion of the IFNT is secreted into the uterine cavity, then it possibly enter into the peripheral blood circulation through uterine vein (Bott *et al*. 2010, Oliveira *et al*. 2008) and also up-regulates some ISGs such as- (*ISG15*, *MX1*, *MX2* and *OAS1*) in blood leukocytes (Oliveira *et al*. 2008). Based on this up-regulation, this study aimed to explore novel pregnancy markers in immune cells which are stimulated by type 1 IFN. IFNT stimulates transcription of ISGs that appear to play roles in conceptus implantation during early pregnancy (Spencer and Bazer, 2004).

After discovery of the role of human chorionic gonadotropin (hCG) from the human conceptus, numerous investigations were initiated to determine if similar actions are involved in pregnancy recognition in ruminants. It is not clear whether the increase of IFNT in the blood stream is due to its escape from the uterus or it is an indirect effect of IFNT stimulating immune cells trafficking through the uterus to produce a substance with antiviral activity. However, recent reports have emerged and demonstrated that IFNT is produced by the ruminant conceptus and also acts systematically (Gifford *et al*. 2007, Han *et al*. 2006, Yankey *et al*. 2001). Oliveira *et al*. (2008) have reported that IFNT moved from the uterus and increased 500-1,000 fold antiviral activity in the venous blood to the uterine artery blood in early pregnancy in ewes. These results raise the low, but detectable levels of

IFNT and antiviral activity in the blood (Roberts *et al*. 1992). Bott *et al*. (2010) reported that increase of antiviral activity in the plasma collected from uterine vein is highly possible caused by IFNT. These important findings definitely demonstrated that IFNT potentially has a direct systemic effect.

Interestingly, Tuo *et al*. (1998) reported that exogenous IFNT has dramatic effects on leukocytes recirculation and redistribution in lambs by reducing $CD4^+$, $CD5^+$ and gamma delta plus T cells in the peripheral circulation without changing the numbers of $CD8^+$ T cells. The existence and distinct possibility that IFNT present in the peripheral circulation during the very earliest stages of pregnancy recognition signaling and can apparently bind and alter the function of circulating immune cells.

In addition, systemic gene expression changes during early pregnancy may aid in establishing pregnancy. Nakayama *et al*. (2002) supported the idea of promoting effects of immune cells on early embryo implantation by showing that PBMCs isolated from pregnant human enhanced murine embryo spreading and invasion *in vitro*. Kosake *et al*. (2003) reported that PBMCs enhanced the attachment of BeWo-cell spheroids to endometrial cells derived from human uteri in the late proliferative and early secretary phase, indicating that PBMCs may be able to induce endometrial cells to become 'receptive' to an embryo. Conceptus-induced signals are also believed to stimulate the systemic maternal immune pathway through the lymph and blood circulation, particularly via PBMCs, platelets and cell free DNA in serum (Yang *et al*. 2014, Shirasuna *et al*. 2012).

The utility of ISGs for pregnancy detection in dairy cattle has been demonstrated recently. Several ISGs in cattle (Gifford *et al*. 2007) and in sheep (Oliveira *et al*. 2008,

Yankey *et al*. 2001) have been tested as early pregnancy diagnosis markers, but have shown variable effectiveness. A pregnant d18 evaluation of *ISG15* mRNA yields in dairy cows revealed less accuracy in pregnancy detection although accuracy was enhanced with serial blood collection from d17 to d25 (Han *et al*. 2006). The use of fold changes in steady-state levels of *MX2* mRNA from d0 to d18 after AI was deemed an unreliable method of pregnancy diagnosis in dairy heifers because of low sensitivity and negative predictive value (Stevenson *et al*. 2007). 2′-5′-oligoadenylate synthetase1 (*OAS1*) has been found to be a suitable pregnancy test on d18 after AI in heifers but not in cows (Green *et al*. 2010).

Recently, it has been reported that lysosomal cysteine proteases are activated by type 1 IFN (IFN-β) in *in vitro* culture of mouse macrophages (Creasy *et al*. 2011). Type 1 IFN stimulated pathway may be involved in several types of cell like blood leukocytes because type 1 IFN pathway exists in many types of somatic cells. The ISGs may be activated in lysosomal CTSs through type 1 IFN cell signaling pathway in PBLs and possibly to play important roles during early pregnancy in ruminants. Interestingly, up-regulation of *ISGs* expression has been identified in circulating immune cells during implantation, making these factors a potential source of non-invasive biomarkers for early pregnancy.

IFNT is a member of the type 1 interferon (IFN) family along with interferon alpha (IFNA), beta (IFNB), delta (IFND), and omega (IFNW). IFNT was first named as protein X or trophoblastin (Bazer *et al.* 1996) and also termed ovine trophoblast protein-1 (oTP-1) as the first primary protein secreted by the ovine conceptus (Godkin *et al.* 1982). After cDNA cloning and amino acid sequencing, it was designated as ovine IFNT (Imakawa *et al.* 1987).

IFNT arose from IFNW in the Ruminantia about 36 million years ago (Demmers *et al.* 2001), sharing 70% homology, but are also quite similar to the IFNA and IFNB (~50% and ~25%) homology respectively (Roberts *et al*. 1997). IFNT has high amino acid sequence homology across ruminant species, but to some extent it differs other species. Bovine IFNT is glycosylated with N-linked oligosaccharides while ovine IFNT is not glycosylated and caprine IFNT proteins exist as both glycosylated and non-glycosylated forms (Demmers *et al.* 2001). Similar to other type 1 IFNs, IFNT has antiviral (Pontzer *et al.* 1990), antiproliferative and immunomodulatory (Bazer and Johnson, 1991, Roberts, 1989), antitumor (Pontzer *et al.* 1990), and therapeutic (Bekisz *et al.* 2004, Martal *et al.* 1998) properties.

As a member of the type 1 IFN family, IFNT stimulates transcription of ISGs through the type 1 IFNs JAK-STAT cell signaling pathway (Spencer and Bazer, 2004). Some ISGs such as *ISG15, MX1, MX2, OAS1, CTSL* and *CST3* are expressed in response to IFNT activation of an alternate cell signaling pathway (Spencer *et al*. 2007).

1.1 Interferon stimulated genes (ISGs) and establishment of pregnancy

ISGs thought to be play important role in implantation and development of the conceptus. Selected some ISGs expressed in the PBLs are described here with their functional implications in implantation and establishment of pregnancy.

ISG15 was first identified in mouse Ehrlich ascites tumor cells as being highly induced by IFN treatment (Farrell *et al.* 1979). ISG15, also called ubiquitin cross-reactive protein (UCRP) due to its cross reactivity with ubiquitin-specific antibodies (Haas *et al.*

1987), is synthesized as a 17 kDa precursor termed ISG17 and processed to a 15 kDa protein by a specific protease which is critical for conjugation to target proteins (Kim and Zhang, 2003, Johnson *et al.* 1999 & 1998, Austin *et al.* 1996). Upon interferon treatment, ISG15 can be detected in cells in both free and conjugated forms. *ISG15* mRNA in PBLs was increased in pregnant dairy cows compared to non-pregnant cows (Gifford *et al*. 2007). This report suggests that ISG15 is not only part of an antiviral response, but also critical factor at the fetal-maternal interface during conceptus development and implantation.

MX is induced by Type 1 IFNs and it is a potent inhibitor of viral replication (Horisberger and Gunst, 1991). Expression of MX is induced in all cells that possess type 1 IFN receptors and has been used as an indicator of viral infection (Haller *et al.* 1998). It has reported that significant increases in *MX1* and *MX2* mRNA levels in PBLs on d15 after insemination in pregnant ewes (Yankey *et al*. 2001) and in dairy cows (Kose *et al*. 2014, Green *et al*. 2010, Gifford *et al*. 2007).

1.2 Lysosomes

Lysosomes are membrane-bound intracellular organelles containing acid hydrolases and responsible for intracellular digestion, and are found in virtually all eukaryotic cells (Saftig and Klumperman, 2009). In 1950, lysosomes originally described by Christian de Duve (de Duve, 1959, Appelmans *et al.* 1955), it is involved in many cellular processes and considered as crucial regulators of cell homeostasis. Lysosomes are limited by a single 7-10 nm phospholipid-bilayer (Saftig *et al.* 2010). Lysosomal membrane proteins are generally heavily glycosylated at their luminal domain and form a glycocalyx, which is suggested to

protect the membrane from the action of the hydrolytic enzymes contained within this organelle (Granger *et al.* 1990). One crucial role of the membrane limiting lysosomes is to separate the potent activities of lysosomal acid hydrolases from other cellular constituents, thereby preventing uncontrolled proteolytic activity (Saftig *et al.* 2010). The lysosomal membrane also facilitates interaction and fusion with other cellular compartments, including endosomes, autophagosomes and the plasma membrane (Schröder *et al.* 2010). Lysosomal membrane proteins mediate a number of essential functions for this organelle, including acidification of the lysosomal lumen, import of protein from the cytosol and transport of degradation end products out of the lysosome. The lysosomal associated membrane protein (LAMP-1, -2) have diverse biological functions, but precise role has not been defined yet. Interestingly, it has been reported that LAMP-1 is a differentiation marker for mouse mammary epithelial HC11 cells (Cella *et al*. 1996). Hence, the cell surface expression of LAMP-1 on tumor cells (Saitoh *et al*. 1992, Sarafian *et al*. 1998) and on peripheral blood lymphocytes. Kanan *et al*. (1996) has suggested that these glycoproteins may play a role as adhesion of molecules. Recently, it has been shown that LAMP-1 and LAMP-2 were expressed on the surface of keratinocytes treated with ionomycin in order to increase the intracellular calcium concentration (Jans *et al.* 2004). LAMP-1 mice deficient are viable and demonstrated a mild phenotype with normal lysosomal morphology and function (Andrejewski *et al.* 1999). Deficiency of LAMP-2 induced a more severe phenotype with extensive accumulation of autophagic vacuoles in many tissues, and degradation of long lived proteins is severely impaired (Tanaka *et al.* 2000).

1.3 Cathepsins (CTSs) family

Lysosome contains considerable number of proteases. Among them the best known are CTSs family of proteases. Several CTSs have been identified in human and are categorized into three distinct groups based on the amino acid found in the active site; serine (A and G), cysteine (B, C, F, H, K, L, O, S, V, X and W) and aspartic CTSs (D and E) (Turk *et al.* 2012). The aspartic CTSD and some of the cysteine CTSs, including CTSs B, L, C and H are ubiquitous among the most abundant lysosomal proteases (Rossi *et al.* 2004). All CTSs are member of papain family and present in all living organisms in mammals. CTSs are essential parts of the proteolytic machinery, besides their fundamental functions of catabolism and protein processing, cysteine CTSs perform diverse functions (Vasiljeva *et al*. 2007, Chapman *et al*. 1997). The main function of CTSs is to participate in degradation and recycling of proteins within the lysosomes, but many CTSs have also been shown to be critically involved in distinct physiological processes, including intracellular protein turnover, immune response, bone remodeling, proprotein processing, antigen presentation, degradation of extra cellular matrix (ECM) and initiation of cell death (Turk *et al.* 2012, Reiser *et al.* 2010).

CTSB is the most abundant and widely expressed in this family and its role appears to be reflected by the house keeping nature of its promoter (Kirschke *et al*. 1995). Intracellular protein catabolism is one of the functions of CTSB which have entered the lysosomal system from outside the cell via endocytosis or phagocytosis or from other compartments within the cell (autophagy) (Bohley and Seglen, 1992). CTSB action and localization in lysosomes imply that it is important in protein degradation.

CTSK is abundantly expressed in osteoclasts and also exists in immune cells. It has been implicated in the activation of dentritic cells (DCs) and in contributing to autoimmune inflammatory processes (Takayanagi H, 2010). The enzyme has a broad pH activity optimum between pH 5-8. At neutral pH, it is more stable than other CTSs. It plays an important role in other pathological diseases including bone and cartilage turnover.

In the ovine uterus, CTSL is up-regulated by IFNT and progesterone during early pregnancy (Song *et al.* 2006 & 2005). CTSL proteolytic activity has been found in the uteri of cats (Li *et al*. 1991, Verhage *et al*. 1989), pigs (Geisert *et al*. 1997), and mice (Hamilton *et al*. 1991). Therefore, regulation of proteases by IFNT may affect endometrial remodeling and regulation of invasive actions of ovine conceptuses.

Recent evidences support the idea that a variety of proteases regulate well defined events of trophoblast cells invade into the endometrial epithelium in many species during implantation and placentation (Walter *et al*. 2006, Ishida *et al*. 2004). In mice, CTSB and CTSL are necessary for embryo development and decidualization during the periimplantation period (Afonso *et al.* 1997).

The expansion of the knowledge about proteolytic enzymes and their role in proteolysis is one of crucial mechanisms for precise cellular control of reproductive processes under normal and/or pathological conditions in all living organisms. Therefore, this study attempted to address the role of IFNT on lysosomal CTSs in the diverse aspect function of immune cells which is important for the establishment of pregnancy.

Chapter II

Pregnancy specific response of lysosomal cathepsins in bovine leukocytes

2.1 Introduction

The IFNT is a unique pregnancy recognition signal in ruminants (Bazer FW, 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 conceptus around days 14-16 after fertilization and reaches to peak of secretion at days 18-19 after artificial insemination (AI) (Mann *et al*. 1999, Roberts *et al*. 1999, Thatcher *et al*. 1995). IFNT binds to the type 1 interferon receptor (IFNAR1 and IFNAR2), whose function is essential for the generation of the biological activities of all the different type 1 IFNs. IFNAR1 and IFNAR2 induce cell signaling via the Janus activated kinases (JAKs) and Tyrosine kinase 2 (TYK2) pathways, respectively (Platanias 2005, Darnell *et al*. 1994, Der *et al*. 1998). Activation of JAKs that are associated with type 1 IFNs receptors results in tyrosine phosphorylation of STAT (signal transducer and activator of transcription) 1 and STAT2, that leads to the formation of STAT1-STAT2-IFR (IFN regulatory factor) 9 complexes, which are known as ISGF (IFN stimulated gene factor) 3 complexes. These complexes translocate to the nucleus which binds and activates interferon stimulated response elements (ISREs) in DNA to initiate amplify the effects of type 1 IFNs (Choi *et al*. 2001, Spencer *et al*. 1998). During pregnancy recognition period, IFNT affects uterine tissues with activating pregnancy-specific signal pathway such as increase of interferon stimulated genes *ISG15*, *MX1*, and *MX2* etc. (Oliveira *et al*. 2008, Hicks *et al*. 2003, Ott *et*

al. 1998). Besides, ISGs expression is clearly up-regulated in blood leukocytes in earlier stage of pregnancy (Gifford *et al*. 2007, Han *et al*. 2006).

Recently, it has been reported that lysosomal cysteine proteases are activated by type 1 IFN (IFN-β) in *in vitro* culture of mouse macrophages (Creasy *et al*. 2011). Type 1 IFN-stimulated pathway may be involved in several types of cell like blood leukocytes because type 1 IFN pathway exists in many types of somatic cells.

Lysosomes are membrane bound organelles containing hydrolytic enzymes and control the intracellular turnover of macromolecules (Luzio *et al*. 2007), by the action of numerous soluble catalytic enzymes, which is collectively known as acid hydrolases. Lysosome associated membrane proteins (LAMPs) are the major protein constituents of the lysosomal membrane. Several highly glycosylated lysosomal membrane proteins have been identified (Hunziker *et al*. 1996, Peters and Von Figura, 1994), but their specific functions still remain unknown. The most abundant lysosomal associated membrane proteins are LAMP-1 and LAMP-2. They have been estimated to constitute 50% of lysosomal membrane proteins (Saftig *et al.* 2009). These proteins play an important role to protect the membrane against the hydrolytic activity of the lysosomal enzymes and it prevents the accidental release of lysosomal materials into the cytosol. LAMPs are believed to function in the maintenance of the structural integrity of the lysosomal membrane by protecting it from the hostile luminal environment. LAMP-1 and LAMP-2 exhibit considerable sequence homology and have similar domain structure and biochemical properties. It has been reported that absence of both LAMP-1 and LAMP-2 caused the embryonic lethality, double deficient LAMP-1/LAMP-2 mice proves that these two major lysosomal membrane

protein share common function *in vivo* (Eskelinen *et al*. 2004). Being a degradative compartment, lysosomes contain considerable number of proteases. Among them, CTSs family of proteases have been widely characterized and it is active in an acidic environment (Kirschke *et al*. 1998). These proteases are well known for intra/extracellular protein degradation for amino acid recruitment, and involved in various physiological processes (Turk *et al*. 2000). Bromme and Okamoto (1995) first reported that, CTSK a papain-like cysteine proteases, and that it has the ability of degradation of high matrix (collagen, elastin etc). Lysosomal CTSs which are enclosed in the lysosomes help to maintain the homeostasis of cell metabolisms by participating in the degradation of the materials.

In cattle, the study of lysosomal function and the expression of *LAMP-1, -2* and the presence of CTSK and LAMP-1 protein in blood leukocytes during early pregnancy remain unknown. Thus, current investigation was undertaken to elucidate the pregnancy specific response of lysosomal and lysosomal CTSs in PBLs, which is possibly induced by IFNT during maternal-fetal recognition period of pregnancy in dairy cows. The aim of this present study was to explore lysosomal function during maternal and fetal recognition period of pregnant cows by investigating (1) the lysosomal and lysosomal CTSs activity (2) the expression of *LAMP-1, -2* and (3) CTSK and LAMP-1 protein in PBLs.

2.2 Materials and Methods

2.2.1 Animals

Multiparous Holstein Friesian cows which belong to the Hokkaido University Dairy Farm were used for the experiment. All experimental procedures and protocols the care of

those cows was compiled in accordance with the guide lines of Hokkaido University, Sapporo, Japan.

2.2.2 Collection of blood sample and isolation of leukocytes

The cows were subjected to artificial insemination (AI) after estrus detection. Blood samples were collected from jugular vein on d18 after AI into tubes containing heparin (Terumo, Venoject II). Tubes were immediately placed on ice until processed. Blood samples were used for assessment of lysosomal CTSs activity, RT-qPCR, and immunostaining analysis. For separation of plasma, 5 ml of whole blood was taken into centrifugation tube and then tube was centrifuged at 1,200×*g* for 20 min at 4°C temperature. After centrifugation, most of the plasma was carefully removed. For isolation of the leukocytes, 8 ml of red blood cell (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 mixed by careful pipetting for rupturing the erythrocytes. Then the tubes were centrifuged at 1,200×*g* for 5 min at room temperature. After centrifugation, supernatant was removed carefully. This step was repeated until white precipitation of leukocytes became clear. The precipitate of leukocytes was washed with phosphate buffered saline (PBS) and cells were lysed in ISOGEN-II (Nippon Gene, Toyama, Japan) and stored at -80°C for further RNA extraction.

2.2.3 Assessment of lysosomal activity in leukocytes collected from pregnant and non-pregnant cows

Total leukocytes from d18 pregnant and non-pregnant cows were incubated in pre-warmed Roswell Park Memorial Institute (RPMI) 1640 medium (Wako) with 1 μM solution of DMSO-diluted LysoSensor Green DND-189 (L7535, Invitrogen by Thermo Fisher Scientific, Life Technologies Corporation, Eugen, Oregen, USA) for 15 min at 38.5°C, 5% CO_2 in air. Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) was added to stain the nuclei. After incubation, samples were washed two times with PBS. After washing, 20 μL cells were put onto glass slide covered with cover glass (18×18 mm) and observed under the fluorescence microscope (Leica Microsystems, Wetzlar, Germany) using 590 nm excitation filter at 630×. The fluorescence images were captured and measured the intensity by ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

2.2.4 Measurement of CTSs (B, K) activity in leukocytes collected from pregnant and non-pregnant cows

Detection of CTSs B and K activity was performed with fluorescent substrates. 10 μL of cell suspensions were taken into separate tubes. Magic Red CTSs (B and K) assay kits (MR-RR_2 and MR-LR_2) (Immunochemistry Technologies, MN, USA) were prepared by diluting the Magic Red stock solutions to 200× with RPMI 1640. To stain the nuclei, 1 μL Hoechst 33342 was added, and tubes were incubated for 40 min at 38.5°C, 5% CO_2 in air. After incubation, sample was washed two times with PBS. 20 μL of sample was taken onto glass slide with cover slip (18×18 mm) and immediately placed the slide to the fluorescence microscope (Leica Microsystems) and observed using a 550 nm excitation filter at 630×. All images were captured in the same exposure conditions. The fluorescence

intensity was measured by ImageJ analysis software (National Institutes of Health).

2.2.5 RNA extraction, cDNA synthesis and RT-qPCR

Total RNA of leukocytes was extracted and stored at -80°C until analysis. After standardizing the total RNA concentration using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA), cDNA was synthesized with ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO, Osaka, Japan). The synthesized cDNA was stored at -30°C. To assess the gene expression, quantitative PCR (qPCR) was performed using KAPA SYBR FAST PCR Kit optimized for Roche LightCycler nano (Roche Diagnostics, Mannheim, Germany). Specific primers for *ISG15*, *MX1* and *MX2* were used as a type 1 IFN induced positive marker gene, lysosomal associated membrane protein *LAMP-1*, *-2* and *CTSs* (*B*, *K*) were designed and commercially synthesized based on reported sequence.

The reaction was carried out in normal qPCR 8 strip tubes, in a total volume of 10 µL containing 1 µL (0.5 µL reverse and 0.5 µL forward) of each primer, 5 µL KAPA SYBR FAST qPCR Kit and 4 µL of cDNA and subjected to the following cycling condition: a denaturation step at 95°C for 30 sec, an amplification step of 45 cycles at 95°C for 10 sec, 55°C for 15 sec and 72°C for 30 sec. The expression level of each gene was normalized by *β-actin* as the internal standard and details of using for qPCR primers are presented in Table 1.

2.2.6 Immunodetection of CTSK and LAMP-1 protein in leukocytes from pregnant and non-pregnant cows

Total leukocytes collected from pregnant and non-pregnant cows on d18 after AI were fixed with 4% (w/v) paraformaldehyde (PFA) dissolved by PBS in 0.5 ml tube for 24h at 4ºC and processed using standard procedures. Fixed cells were washed with PBS and then permeabilized with PBS containing 0.2% TritonX-100 (PBST) for 10 min. Then cells were blocked with PBST containing 1% BSA for 1h at room temperature. Incubation with the primary CTSK rabbit polyclonal antibody (Pab) (anti-rabbit Ig, 1:1,000) (Wuxi App Tec, AB GENT, San Diego CA, USA) and LAMP-1 monoclonal anti-mouse antibody (Anti-mouse IgG1, 1:500) (Novus Biologicals, Littleton, CO, USA) was performed for 1h at room temperature. The cells were then incubated with the fluorescence in conjugated secondary antibody (donkey-antirabbit IgG, 1:1,000) (GE Healthcare Bio-Sciences, Uppasala, Sweden) for 1h at room temperature in dark condition. Negative control (NC) was set by substituting the primary antibody with PBST+1% BSA at the respective dilution. Between each step, cells were washed three times with PBS for 5 min. Slides were mounted with Vectashield with DAPI (Vector Laboratories Inc. CA, USA) and observed by fluorescence microscopy (Leica Microsystems). All images were captured by same exposure conditions using a 550 nm excitation filter at 630×. The fluorescence intensity of acquired images was measured by ImageJ analysis software (National Institutes of Health).

2.3 Statistical analysis

Data were analyzed using Graph Pad prism version 5 for Windows (Graph Pad

software, La Jolla, CA, USA Inc., www.graphpad.com) and were presented as the mean ± S.E.M., one way ANOVA and student t test. P values less than 0.05 ($p<0.05$) were considered to represent statistically significant differences.

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

Gene name		Primer sequence (5'-3')	Tm (ºC)	Product length (bp)	Gen Bank accession number
ISG15	FWD:	TGAGGGACTCCATGACGGTA	59.7	72	AC_000173.1
	REV:	GCTGGAAAGCAGGCACATTG	60.4		
MX1	FWD:	GCCAACTAGTCAGCACTACATTGTC	61.5	139	AC_000158.1
	REV:	GCTCTTGGACTCCATATCTTCAC	62.8		
MX2	FWD:	CAGAGACGCCTCAGTCGAAG	60.2	113	AC_000158.1
	REV:	GAGACGTTTGCTGGTTTCCATG	60.4		
LAMP-1	FWD:	GTGAAGAATGGCAACGGAC	66.6	250	AC_000169.1
	REV:	GCATCAGCTGGACCTCGTAA	65.2		
LAMP-2	FWD:	AAGAGCAGACCGTTTCCGTG	66.5	110	AC_000187.1
	REV:	CGAACACTCTTGGGCAGTAG	62.4		
CTSB	FWD:	CACTTGGAAGGCTGGACACA	66.3	141	AC_000180.1
	REV:	GCATCGAAGCTTTCAGGCAG	67.2		
CTSK	FWD:	TCCGGCCACATTATCCACAC	67.9	99	AC_000174.1
	REV:	ACCACAGGCAGCAGTAGAAC	61.9		
β-actin	FWD:	TGGACTTCGAGCAGGAGATG	65.5	221	AC_000182.1
	REV:	GTAGAGGTCCTTGCGGATGT	63.0		

FWD: Forward; REV: Reverse

2.4 Results

2.4.1 Assessment of lysosomal and CTSs (B, K) activity in leukocytes from pregnant and non-pregnant cows

Fluorescence intensity of lysosomes was increased significantly on d18 after AI in leukocytes from pregnant cows when compared with that of non-pregnant cows (Figure 1). Like as, lysosomal activity, and fluorescence of CTSB activity was also detected in leukocytes. The fluorescence intensity of CTSB activity was significantly higher in leukocytes from pregnant cows when compared with that from non-pregnant cows (Figure 2). Similar to CTSB, CTSK activity was detected in the leukocytes in pregnant cows. The fluorescence intensity of CTSK was significantly higher in leukocytes of pregnant cows than that of non-pregnant cells (Figure 3).

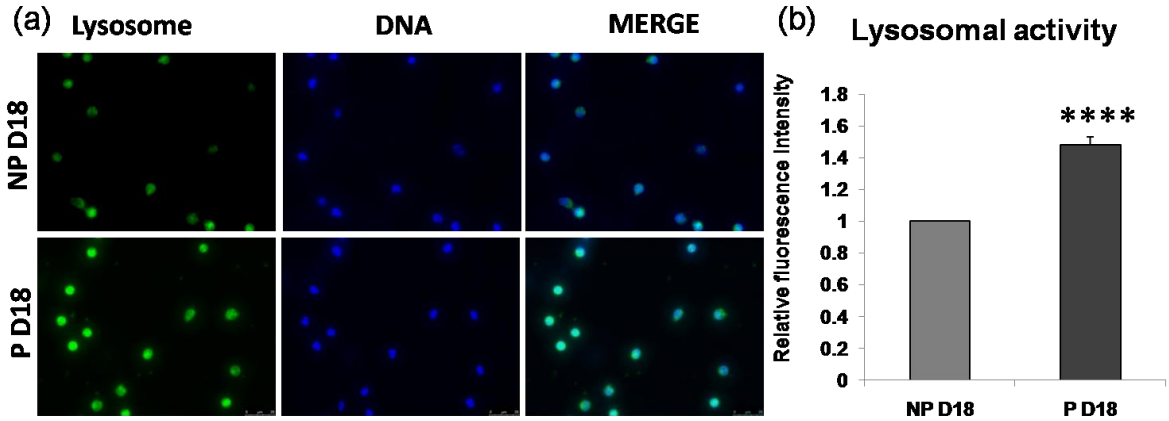


Figure 1. Lysosomal activity in leukocytes of pregnant and non-pregnant cows on d18 after AI. (a) Lysosomal activity fluorescence image in pregnant (P) and non-pregnant (NP) cows on d18 after AI. (b) The relative lysosomal activity in leukocytes on d18 pregnant (P) and non-pregnant (NP) cows after AI. All data are shown as the mean ± standard error of the mean (S.E.M.). Asterisks (****; $p < 0.0001$) indicate significant difference in between the pregnant (P) and non-pregnant (NP) cows on d18 after AI determined by student t test.

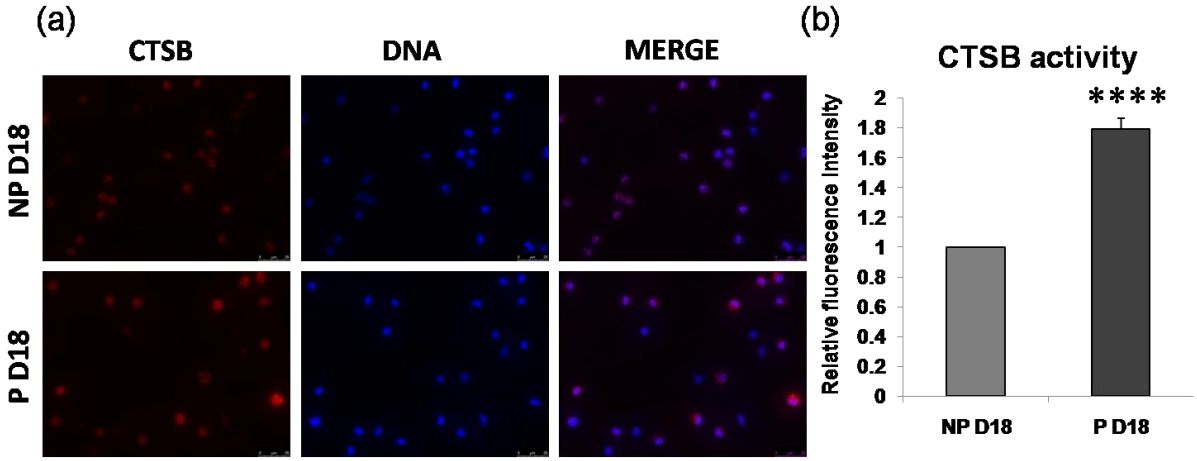


Figure 2. CTSB activity in leukocytes of pregnant and non-pregnant cows on d18 after AI. (a) CTSB activity fluorescence image in leukocytes on d18 in pregnant (P) and non-pregnant (NP) cows. (b) The relative CTSB activity of pregnant (P) and non-pregnant (NP) cows on d18 after AI. All data are shown as the mean ± standard error of the mean (S.E.M.). Asterisks (****; $p < 0.0001$) indicate significant difference in between the pregnant (P) and non-pregnant (NP) cows on d18 after AI determined by student t test.

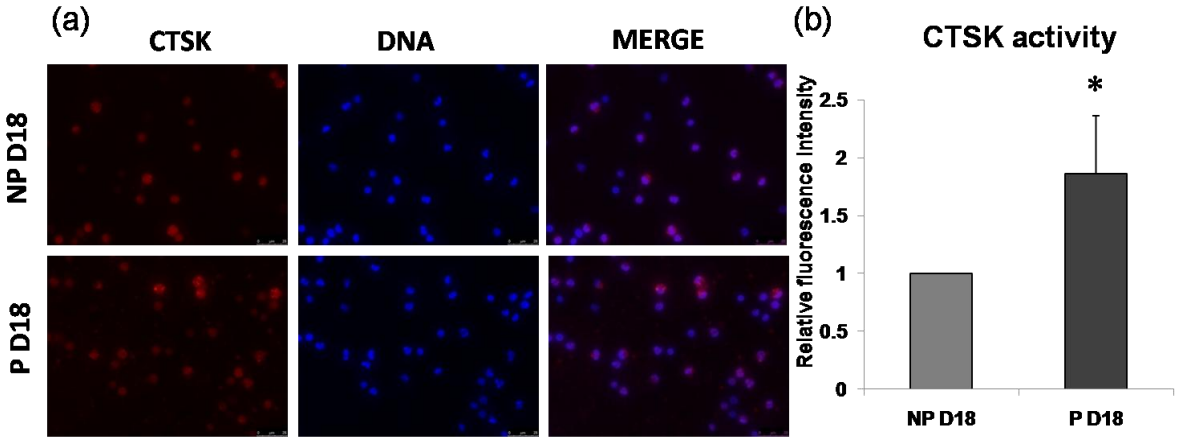


Figure 3. CTSK activity in leukocytes of pregnant and non-pregnant cows on d18 after AI. (a) CTSK activity fluorescence image in leukocytes on d18 in pregnant (P) (lower side) and non-pregnant (NP) cows (upper side) cows. (b) The relative CTSK activity of pregnant and non-pregnant cows on d18 after AI. All data are shown as the mean ± standard error of the mean (S.E.M.). All data are shown as the mean ± standard error of the mean (S.E.M.). Asterisks (*; $p < 0.05$) indicate significant difference in between the pregnant (P) and non-pregnant (NP) cows on d18 after AI determined by student t test.

2.4.2 Expression of ISGs (*ISG15*, *MX1* and *MX2*), *CTSs* (*B*, *K*) and *LAMP-1*, *-2* in leukocytes from pregnant and non-pregnant cows

To confirm that leukocytes of pregnant cows are affected by IFNT stimulation,

expression of *ISG15*, *MX1* and *MX2* as positive markers of IFNT induced genes of bovine leukocytes during early pregnancy was evaluated. The mRNA expression levels of *ISG15*, *MX1* and *MX2* genes were significantly higher in blood leukocytes collected from d18 pregnant cows than those collected from non-pregnant cows (Figure 4a, b, c). The expression of *CTSB* gene was significantly higher in leukocytes from d18 pregnant cows than that from non-pregnant cows, but not differed significantly (*p=0.1572*) (Figure 4d). Similarly, *CTSK* mRNA expression level was higher in leukocytes from d18 in pregnant cows when compared to leukocytes from non-pregnant cows (Figure 4e).

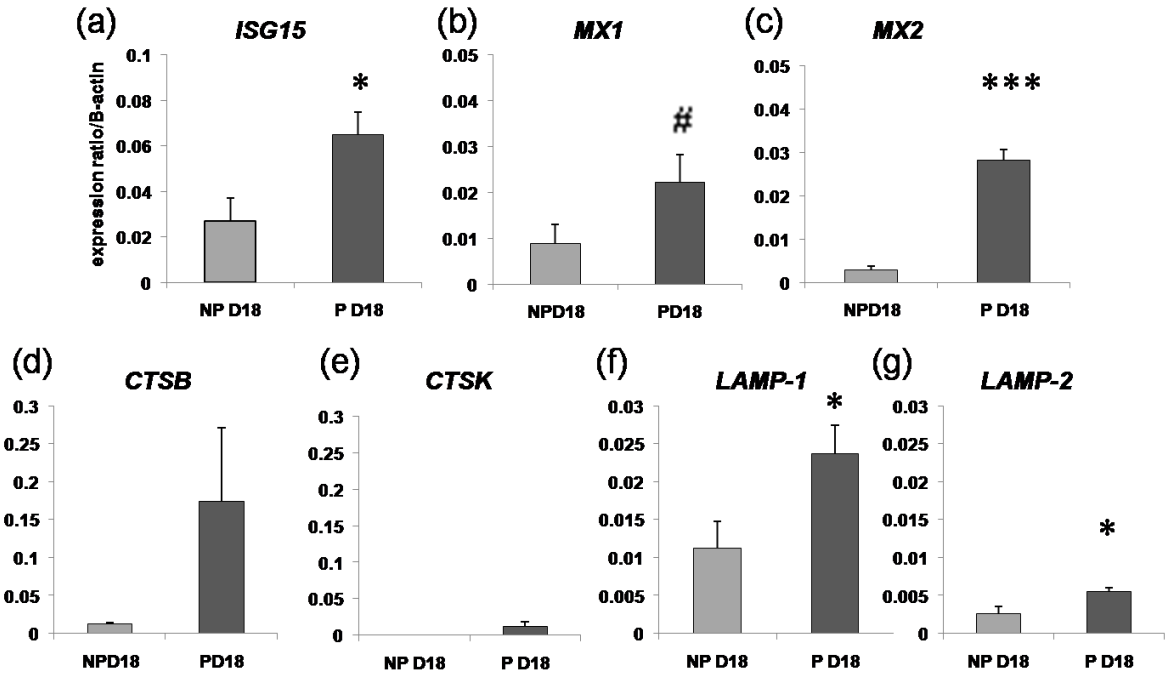


Figure 4. Expression of ISGs (*ISG15*, *MX1* and *MX2*), *CTSs* (*B*, *K*) and *LAMP-1*, *-2* mRNA leukocytes in pregnant and non-pregnant on d18 after AI. The vertical line shows the relative expression level of (a) *ISG15* (b) *MX1* (c) *MX2* (d) *CTSB* (e) CTSK (f) *LAMP-1* and (g) *LAMP-2* mRNA in leukocytes using RT-qPCR. All data are shown as the mean ± standard error of the mean (S.E.M.). Asterisks (***; *p<0.001*,*; *p<0.05*) indicate significant difference in between the pregnant (P) and non-pregnant (NP) cows on d18 after AI determined by students t test. Additionally, a sign (#) indicates a tendency for significant differences (*p<0.1*).

In consistence, *LAMP-1* and *LAMP-2* mRNA expression levels were significantly

higher in leukocytes obtained from pregnant cows compared to those obtained from non-pregnant cows (Figure 4f, g).

2.4.3 Immunodetection of CTSK and LAMP-1 proteins in leukocytes from pregnant and non-pregnant cows

CTSK protein was also detected in leukocytes collected from pregnant and non-pregnant cows. Fluorescent intensity of CTSK was significantly higher in d18 pregnant cows when compared with d18 non-pregnant cows (Figure 5a, b). LAMP-1 protein was also significantly increased in leukocytes from d18 pregnant cows in compared with that of d18 non-pregnant cows (Figure 5c, d).

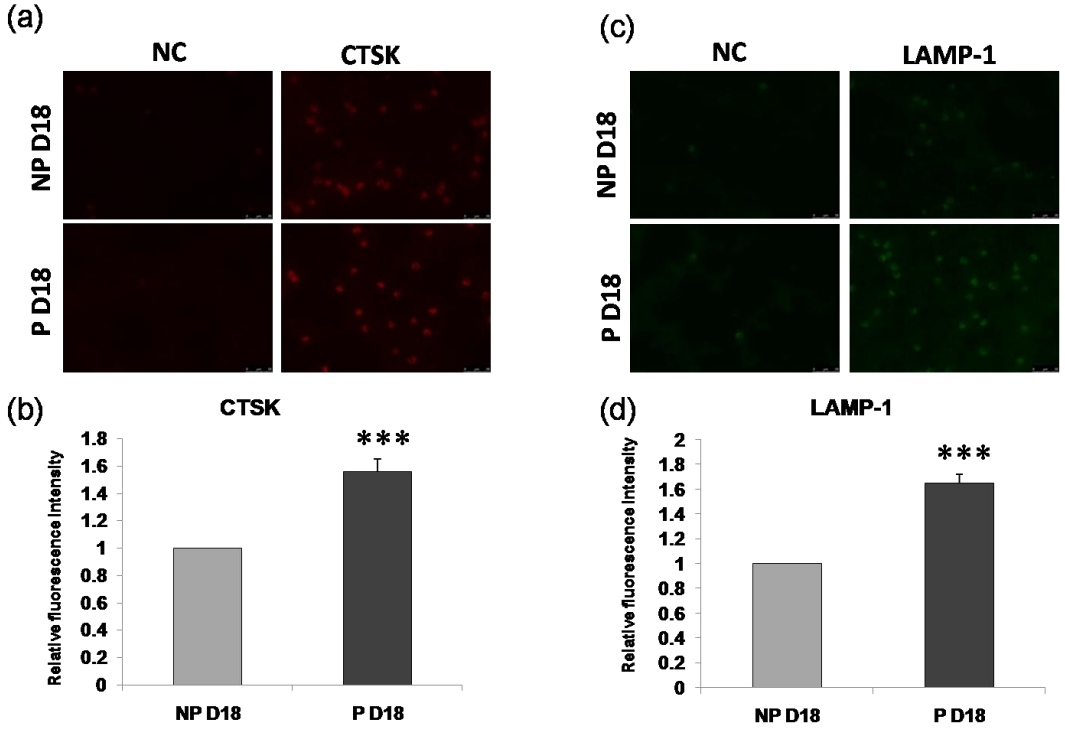


Figure 5. Immunodetection of CTSK and LAMP-1 protein in leukocytes from pregnant and non-pregnant cows after AI. (a) Immunostaining of CTSK in leukocytes from d18 in pregnant (lower part) and non-pregnant (upper part) cows. (b) Relative fluorescence intensity of CTSK protein. (c) Immunostaining for detection of LAMP-1 protein in pregnant (lower part) and non-pregnant (upper part) cows (d) Relative fluorescence intensity of LAMP-1 protein. NC: negative control. All data are shown as the mean ± standard error of the mean (S.E.M.). Asterisks (***; $p<0.0001$) indicate significant difference in between the pregnant (P) and non-pregnant (NP) cows after AI on d18 determined by students t test.

5. Discussion

In the present study, I clarified the pregnancy-specific activation of lysosomes and cathepsins, expression of *CTSs* (*B, K*) genes and lysosomal associated membrane proteins *LAMP-1, -2* as well as protein expression in bovine leukocytes for the first time. This research will contribute to the current era of finding more promising tool to understand the mechanisms of lysosomal activation in blood leukocytes. These results potentially used to find new candidates of genes which are induced by type 1 interferon (*i.e.*, IFNT) and also used as potential markers of pregnancy through immune cells for early pregnancy diagnosis in cattle.

Fluorescence intensity of lysosomal and CTSs (B, K) activities data in the present study clearly revealed that these activities are significantly increased in d18 pregnant cows compared to non-pregnant cows. I hypothesized that, this significant increase of lysosomal CTSs activity in leukocytes during implantation period is due to IFNT. CTSs are lysosomal cysteine proteases capable of digesting matrix proteins and activating other proteases involved in matrix degradation (Kirschke *et al*. 1998). It has been reported that lysosomal protease such as CTSs are regulated by certain cytokines. Fiebiger *et al*. (2001) demonstrated that CTSs S and B activities were increased in human dentritic cells (DCs) in response to cytokines such as TNF alpha and IL1 beta, leading to increased class 2 MHC (major histocompatability complex) 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).

Lysosomes are organelles that contain acidic environment as the result of

protonation. The proton pump activity of the V-ATPase generates a transmembrane voltage, which has to be compensated by either an efflux of cations or an influx of anions to allow efficient lysosomal acidification. This protonation also relieves the fluorescence quenching of the dye by its weak base side chain, resulting in an increase in fluorescence intensity. Thus, the lysosensor reagents exhibit a pH dependent increase in fluorescence intensity upon acidification. These probes usually used to investigate the acidification of lysosomes and alterations of lysosomal function or trafficking that occur in cells.

In the present study, *ISG15*, *MX1* and *MX2* were selected as pregnancy-positive markers in PBL which are induced by IFNT. Expressions of all genes were significantly increased in pregnant cows compared to non-pregnant cows, which have been reported in PBMCs (Puglisi *et al*. 2014, Shirasuna *et al*. 2012, Kizaki *et al*. 2013), and in leukocytes (Green *et al*. 2010, Gifford *et al*. 2007, Yankey *et al*. 2001). Therefore, the increase of lysosomal and lysosomal CTSs activities in PBL is highly pregnancy dependent.

Proteolytic enzyme families such as cysteine proteases degrade extracellular matrix (ECM) during implantation and placentation (Salamonsen LA, 1999 & Barrett AJ, 1994). No data were available for discussing about the expression pattern of CTSs mRNA in PBL, although their presence in unspecified ovary mRNA samples has been reported previously (Soderstrom *et al*. 1999, Kirschke *et al*. 1998, Rantakokko *et al*. 1996, Bromme and Okamoto, 1995, Petanceska and Devi, 1992).

Importantly, the pregnancy specific expression of *LAMP-1* and *LAMP-2* in leukocytes suggests that leukocytes are affected by pregnancy-specific factors in the blood. These proteins play important role to maintain the integrity of the organelle. An issue of

interest and controversy that has been taken the pathway by LAMPs after leaving the Golgi complex. It could be confirmed that in pregnancy, the respective genes were elevated compared to non-pregnant cows. These results strongly indicate that the pregnancy specific factor(s) derived from conceptus in the uterus reaches to peripheral circulation through immune cells. Although LAMPs are distributed within the cell primarily in the lysosome, they are also constitutively expressed at the cell surface of platelets, T and B lymphocytes and monocytes and human several lymphocytes cell lines. Indeed, an alternative pathway targeting for LAMP-1 and LAMP-2, aside from direct transport form the rough endoplasmic reticulum and Golgi to the lysosome, involves a cell surface intermediary which is shuttle to the lysosome through endocytosis (Carlsson *et al*. 1992, Mathews 1992, Harter and Mellman, 1992).

Immunostaining analysis also confirmed the up-regulation of CTSK protein in pregnant leukocytes. In the present study, one of the major goals was to detect CTSK and LAMP-1 signal in blood cells during early pregnancy in cows. Hou *et al*. (2001) reported that CTSK is expressed in synovial fibroblasts from joints of patients with rheumatoid arthritis. Other cysteine proteases CTSB expression has been observed in murine endometrial luminal and glandular epithelium and CTSL expression in decidualizing stroma (Afonso *et al*. 1997). Immunodetection of CTSK and LAMP-1 protein in blood cells has not been reported yet.

The present results revealed for the first time that lysosomal activation, expression as well as CTSs activities are pregnancy specific reaction in bovine leukocytes during maternal-fetal recognition period.

Chapter III

Activation of lysosomes and lysosomal cathepsins in bovine PBMCs and PMNs during early pregnancy

3.1 Introduction

The IFNT modify tissues and cells to activate the signals for recognition of pregnancy and implantation. The possible marker genes using whole blood leukocytes and peripheral blood mononuclear cells (PBMCs) in several studies were investigated previously. PBMCs contain T and B cells which are required for immune response (Shirasuna *et al*. 2012). Assessing of the cell specific difference on lysosomal CTSs activity and expression is a new approach to detect early pregnancy because whole blood leukocytes contain different type of cells such as T lymphocytes, monocytes and polymorphonuclear granulocytes (PMNs) and each type of cells have specific characteristics. Pregnancy specific response of lysosomal CTSs function in PBMCs and PMNs could be possible markers for pregnancy evaluation. Expression of interferon stimulated genes (ISGs) was clearly up-regulated in blood leukocytes in the earlier stages of pregnancy (Oliveira *et al*. 2008, Gifford *et al*. 2007, Han *et al*. 2006). These reports suggested that the increase of ISGs in circulated leukocytes is high possible by type 1 IFN in the blood at the time of pregnancy recognition. 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, new approaches are necessary to investigate pregnancy-specific responses in the PBMCs and PMNs.

In cattle, lysosomal CTSs activity during early pregnancy, including the gene expression patterns of lysosomal membranes and lysosomal CTSs (B, K), and the presence of CTSB proteins in separated blood cells remains unknown. Thus, the present research was undertaken to investigate the cell different responses the status of lysosomes and lysosomal CTSs in bovine PBMCs and PMNs during early pregnancy.

3.2 Materials and Methods

3.2.1 Animals

The animals used in this study were the multiparous Holstein Friesian cows which belong to the Hokkaido University Dairy Farm. All experimental procedures and protocols for the care of the cows were compiled in accordance with the guide lines of Hokkaido University, Sapporo, Japan.

3.2.2 Collection of blood samples and isolation of PBMCs and PMNs

After AI, blood samples were collected from the jugular vein from cows on day (d)0, d7, d14, and d18 after AI. Blood samples were collected with tubes containing heparin (Terumo). Pregnancy was confirmed by trans-rectal ultrasonography at 40-60 days after AI. For the isolation of PBMCs, whole blood was mixed with an equal volume of phosphate buffered saline (PBS). 3 ml 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 440×*g* 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 440×*g*. 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 carefully removed. This step was repeated until white precipitation, a layer of granulocytes (PMNs), was clearly observed. The PMNs precipitate was washed with PBS and the separated PBMC and PMN samples were lysed in ISOGEN-II (Nippon Gene) and stored at -80°C for further RNA extraction. The separated cells were then used for measuring the activities of CTSs B, K and L gene expression analysis, immunostaining, and lysosomal activity.

3.2.3 Activities of CTSs (B, K and L) in PBMCs and PMNs from pregnant and non-pregnant cows

Detection of CTS activity was performed with fluorescent substrates. Cell suspensions (10 µL) of both PBMCs and PMNs were used. Magic Red CTSs (B, K and L) assay kits (MR-RR_2 MR-LR_2 and MR-FR_2) (Immunochemistry Technologies) were prepared by diluting the Magic Red stock solutions to 200× with RPMI 1640 containing 5% FBS. To stain the nuclei, 1 µL of Hoechst 33342 (Sigma-Aldrich) 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. 10 µL of cell suspension was dropped onto a slide glass with a cover slip (18×18 mm) and immediately observed the fluorescent under a fluorescence microscope (Keyence, Osaka, Japan) using a 550 nm excitation filter at 400×. All images in both PBMCs and PMNs were captured with the same exposure conditions. The fluorescence intensity was measured by ImageJ analysis software (National Institutes of Health).

3.2.4 RNA extraction, cDNA synthesis and qPCR

Total RNA of PBMCs and PMNs from *in vivo* collected 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 with gDNA remover (TOYOBO). 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 LightCycler nano (Roche Diagnostics). Primers for *ISG15* as a pregnancy positive marker, *CTSs* (*B*, *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 µL containing 1 µL (0.5 µL reverse and 0.5 µL forward) of each primer, 5 µL KAPA SYBR FAST qPCR Kit and 4 µL of cDNA, and subjected to the following cycling condition: a denaturation step at 95°C for 30s, an amplification step of 45 cycles at 95°C for 10s, 55°C for 15s and 72°C for 30s. The expression level of each gene was normalized

using glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) and *β-actin* as the internal standard. The qPCR primer sequences used are presented in Table 2.

3.2.5 Immunodetection of CTSB protein in PBMCs and PMNs from pregnant and non-pregnant cows

Separated PBMCs and PMNs collected from d18 pregnant and non-pregnant cows were fixed in 4% paraformaldehyde (PFA) in 0.5 ml tubes for 24h at 4ºC and processed using standard procedures. The fixed 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 1h at room temperature. After blocking, incubation with the primary CTSB polyclonal antibody (anti-rabbit IgG, 1:1,000) (Calbiochem, EMD Millipore Corporation, USA) was performed for 2h at room temperature. The cells were also incubated with the fluorescence in conjugated secondary antibody (donkey-antirabbit IgG, 1:1,000) (GE Healthcare Bio Sciences AB, Uppsala, Sweden) for 1h at room temperature in the 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 respectively. After washing with PBST, the cell suspension was dropped onto a glass slide and coved with a cover glass (18×18 mm). All images were captured with a fluorescent microscope (Leica Microsystems) in 1.5s exposures time using a 550 nm excitation filter at 400×. The fluorescence intensity was measured by ImageJ analysis software (National Institutes of Health).

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

Separated PBMCs and PMNs from d18 pregnant and non-pregnant cows, were incubated in pre-warmed RPMI 1640 medium with 1µM solution of DMSO diluted with LysoSensor Green DND 189 (L7535, Invitrogen by Thermo Fisher Scientific) for 15 min at 38.5°C, 5% CO_2 in air. 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).

3.3 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 t test were performed. P values less than 0.05 ($p<0.05$) were considered to represent statistically significant differences.

Table 2. List of primer and primer sequence used for RT-qPCR

Gene name		Primer sequence (5'-3')	Tm (ºC)	Product length (bp)	GenBank accession number
ISG15	FWD:	TGAGGGACTCCATGACGGTA	59.7	72	AC_000173.1
	REV:	GCTGGAAAGCAGGCACATTG	60.4		
CTSB	FWD:	CACTTGGAAGGCTGGACACA	66.3	141	AC_000180.1
	REV:	GCATCGAAGCTTTCAGGCAG	67.2		
CTSK	FWD:	TCCGGCCACATTATCCACAC	67.9	99	AC_000174.1
	REV:	ACCACAGGCAGCAGTAGAAC	61.9		
LAMP-1	FWD:	GTGAAGAATGGCAACGGAC	66.6	250	AC_000169.1
	REV:	GCATCAGCTGGACCTCGTAA	65.2		
LAMP-2	FWD:	AAGAGCAGACCGTTTCCGTG	66.5	110	AC_000187.1
	REV:	CGAACACTCTTGGGCAGTAG	62.4		
GAPDH	FWD:	CACCCTCAAGATTGTCAGCA	63.9	103	AC_000162.1
	REV:	GGTCATAAGTCCCTCCACGA	63.8		
β-actin	FWD:	TGGACTTCGAGCAGGAGATG	65.5	221	AC_000182.1
	REV:	GTAGAGGTCCTTGCGGATGT	63.0		

FWD: Forward; REV: Reverse

3.4 Results

3.4.1 Activities of CTSs (B, K and L) in PBMCs and PMNs in pregnant and non-pregnant cows

Fluorescence indicating the activities of CTSs (B, K and L) was detected both in PBMCs and PMNs (Figure 6a, b, c). The relative fluorescence intensity of CTSB activity in PBMCs was significantly higher on d14, and PMNs was significantly higher on d14 and d18 respectively, when compared to that in early day of pregnancy (Figure 7a, b), but was not different in PBMCs and PMNs compared with non-pregnant cows. Similar to CTSB,

strong fluorescence from CTSK activity was detected in the PBMCs and PMNs of pregnant cows. The fluorescence intensity of CTSK in PBMCs was higher on d14 and d18 of pregnancy than on d0 and d7 (Figure 7c). The fluorescence intensity of CTSK was significantly higher in the pregnant d18 PMNs than non-pregnant d18 cells (Figure 7d). In addition, fluorescence intensity CTSL was detected in PBMCs and PMNs. The fluorescence intensity of CTSL was significantly higher in PMNs on d18 among the day of pregnancy (Figure 7f).

3.4.2 Gene expression of *CTSs* (*B, K*) and *ISG15* in PBMCs and PMNs from pregnant and non-pregnant cows

Expression of the *CTSB* gene in PBMCs was significantly higher in pregnant d14 and d18 cows than non-pregnant cows (Figure 8a). *CTSB* expression was also significantly higher in PMNs of d18 pregnant cows than non-pregnant cows (Figure 8b). *CTSK* mRNA expression levels were not affected in PBMCs or PMNs (Figure 8c, d). 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 PBMCs, *ISG15* mRNA expression levels were significantly higher on d18 in pregnant cows than in other stages (Figure 8e). In PMNs, expression of *ISG15* mRNA was significantly higher on d18 than at other stages (Figure 8f).

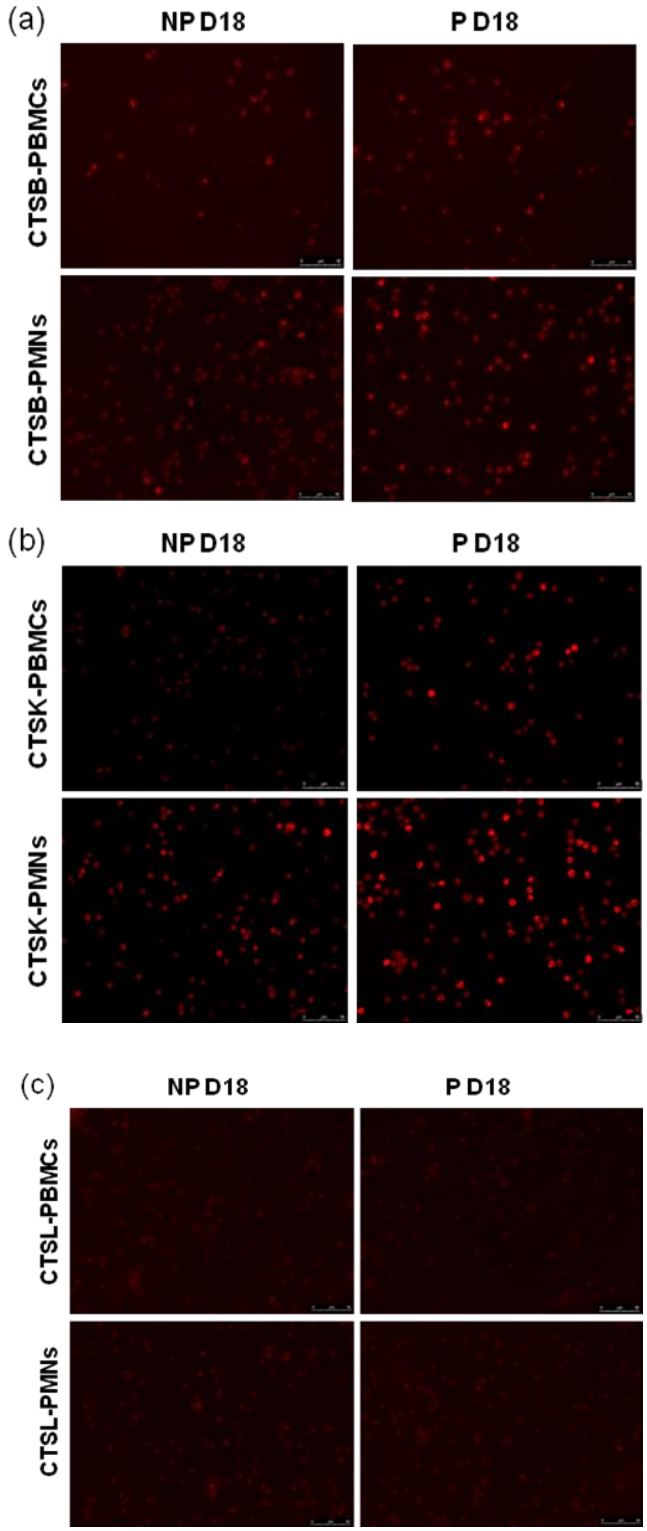


Figure 6. Activities of CTSs (B, K and L) in PBMCs and PMNs collected from d18 pregnant and non-pregnant cows. Photos show the fluorescence images of CTSB (a), CTSK (b), and CTSL (c) in PBMCs and PMNs on d18 in pregnant (P) and non-pregnant (NP) cows. The scale bar represents 50 µm.

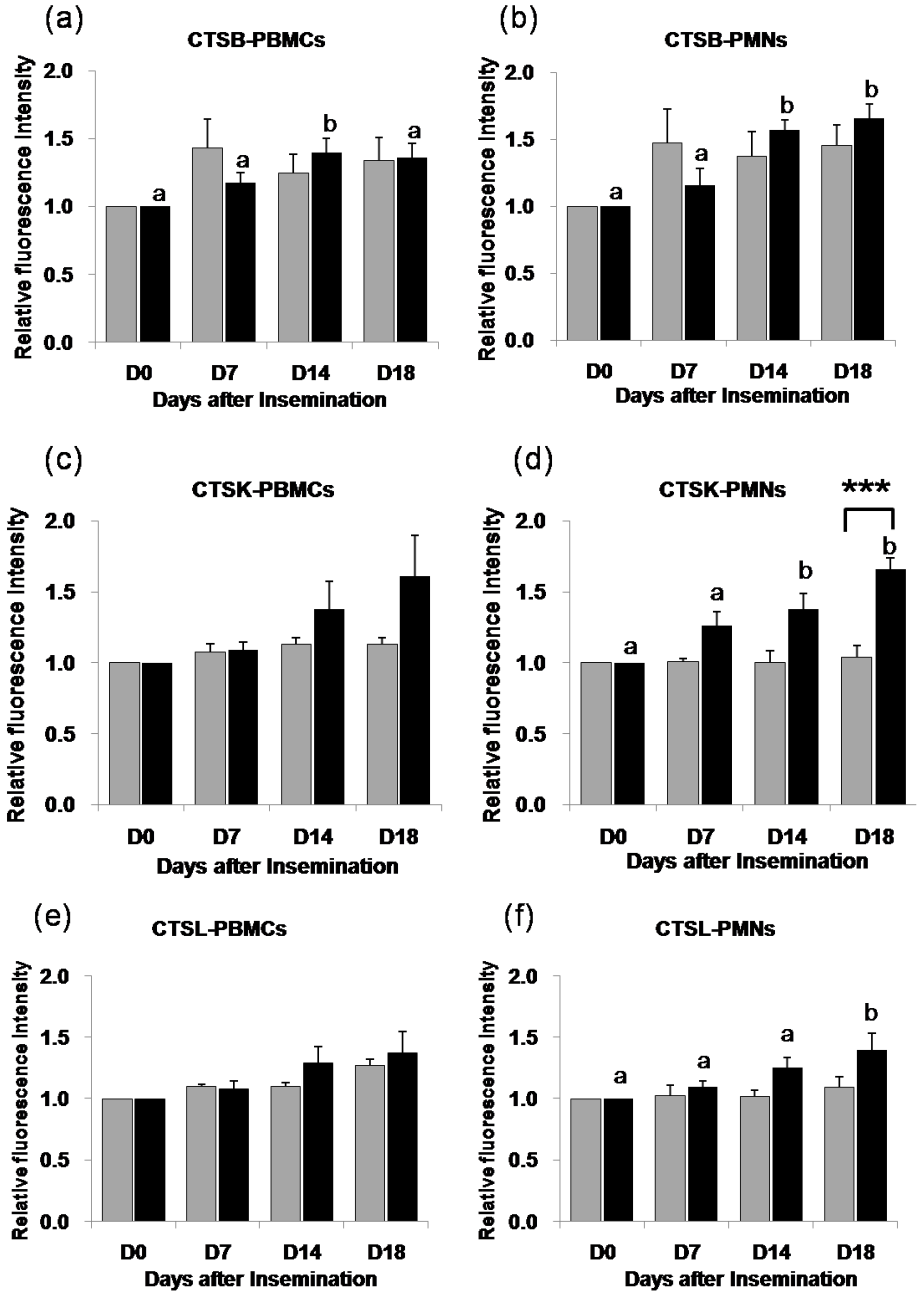


Figure 7. Changes in the relative activities of CTSs (B, K and L) in PBMCs and PMNs collected from pregnant and non-pregnant cows. Relative activities of CTSB in PBMCs (a) and PMNs (b), CTSK in PBMCs (c) and PMNs (d), CTSL in PBMCs (e) and PMNs (f) on d0-18 after insemination in dairy cows that were later diagnosed as pregnant (black bars) or non-pregnant (gray bars). All data are shown as the mean ± 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 significant differences (**$p<0.0001$, student t test) between pregnant (P) and non-pregnant (NP) cows on the same days after AI.

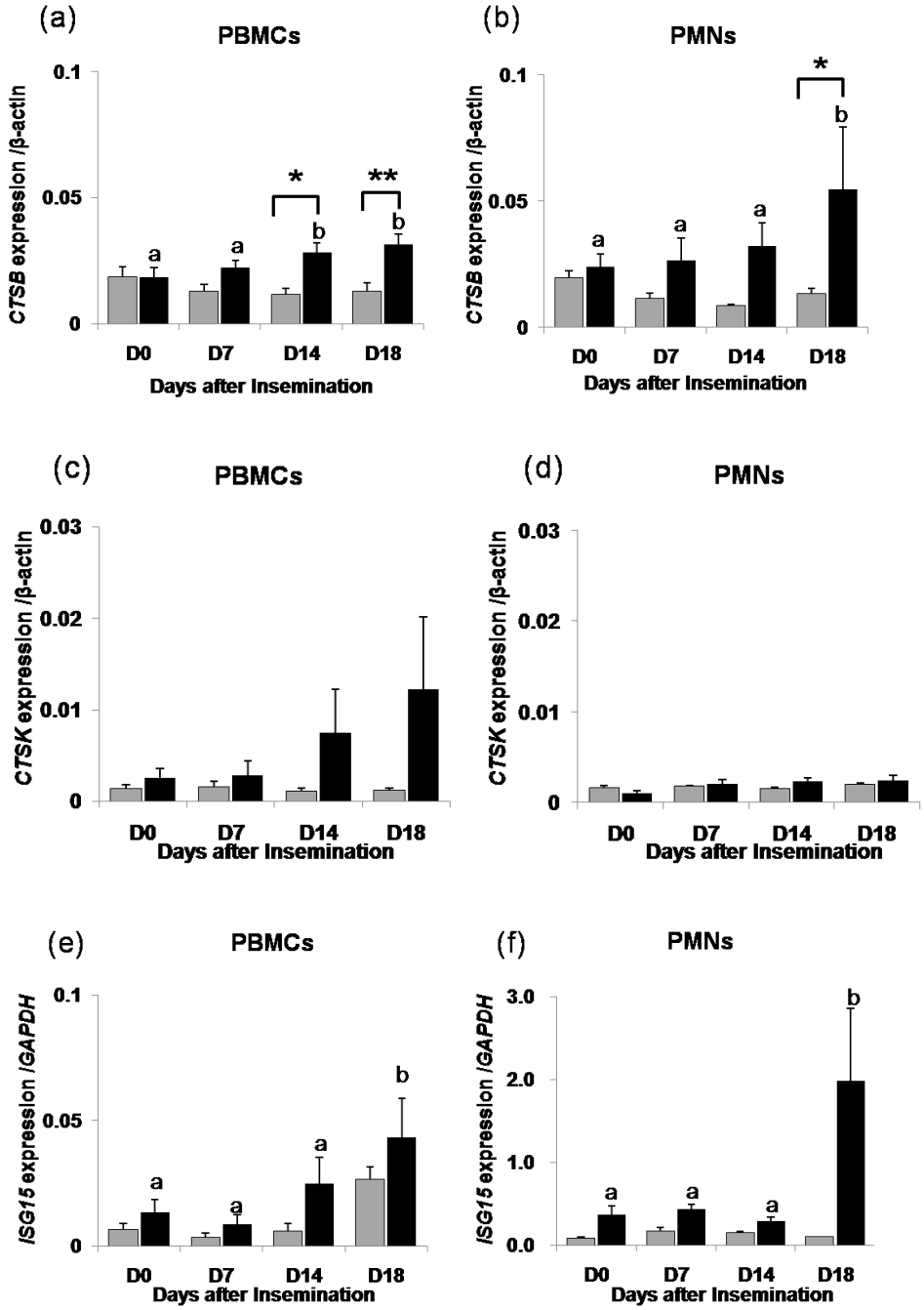


Figure 8. Expression of *CTSs* (*B*, *K*) and *ISG15* genes mRNA in PBMCs and PMNs of pregnant and non-pregnant cows after AI. The graphs show the expression levels of *CTSB* in PBMCs (a) and PMNs (b), and *CTSK* in PBMCs (c) and PMNs (d), and *ISG15* in (e) PBMCs and (f) PMNs standardized by *GAPDH* and *ß-actin*. All data are shown as the mean ± 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 significant differences (*$p<0.05$, **$p<0.01$, student t test) between pregnant (P) and non-pregnant (NP) cows on the same days after AI.

3.4.3 Immunodetection of CTSB protein in PBMCs and PMNs from pregnant and non-pregnant cows

In immunostaining experiment, CTSB was clearly detected in *in vivo* collected PBMCs and PMNs (Figure 9a). Image analysis showed that fluorescent intensities of CTSB in pregnant PBMCs and PMNs were significantly higher than those in non-pregnant cows (Figure 9b, c).

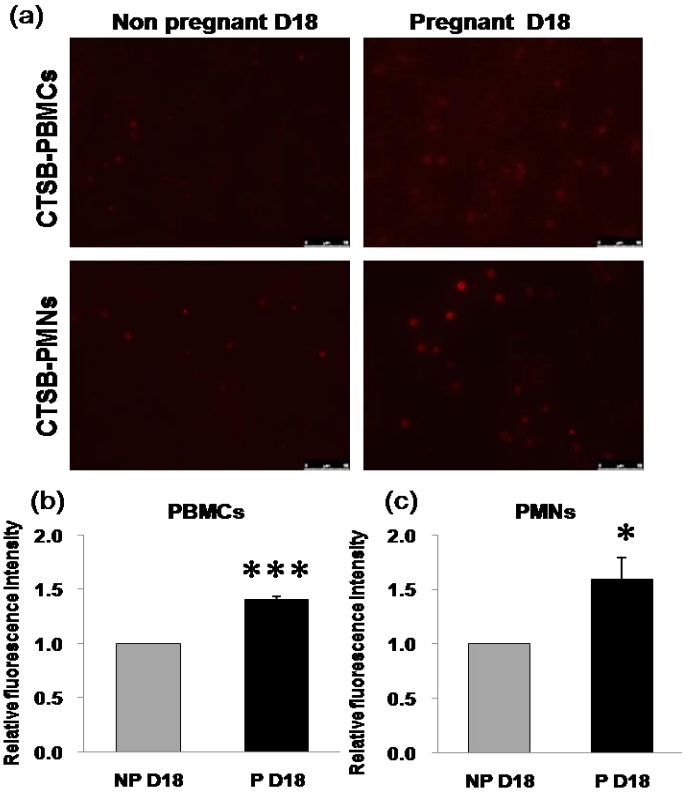


Figure 9. Immunostaining and detection of CTSB protein in PBMCs and PMNs after AI. (a) Immunohistochemical detection of the CTSB protein from in pregnant (P) and non-pregnant (NP) cows on d18 in PBMCs (upper part) and PMNs (lower part). The scale bar represents 50 µm. Asterisks (***$p < 0.0001$, *$p < 0.05$, student t test) indicate significant differences between the pregnant (P) and non-pregnant (NP) cows after AI.

3.4.4 Lysosomal activity in PBMCs and PMNs from pregnant and non-pregnant cows

Clear fluorescence indicating the acidification status of lysosomes was observed both in PBMCs and PMNs (Figure 10a). The fluorescence intensities of PBMCs and PMNs from pregnant cows were significantly higher than those from non-pregnant cows (Figure 10b, c).

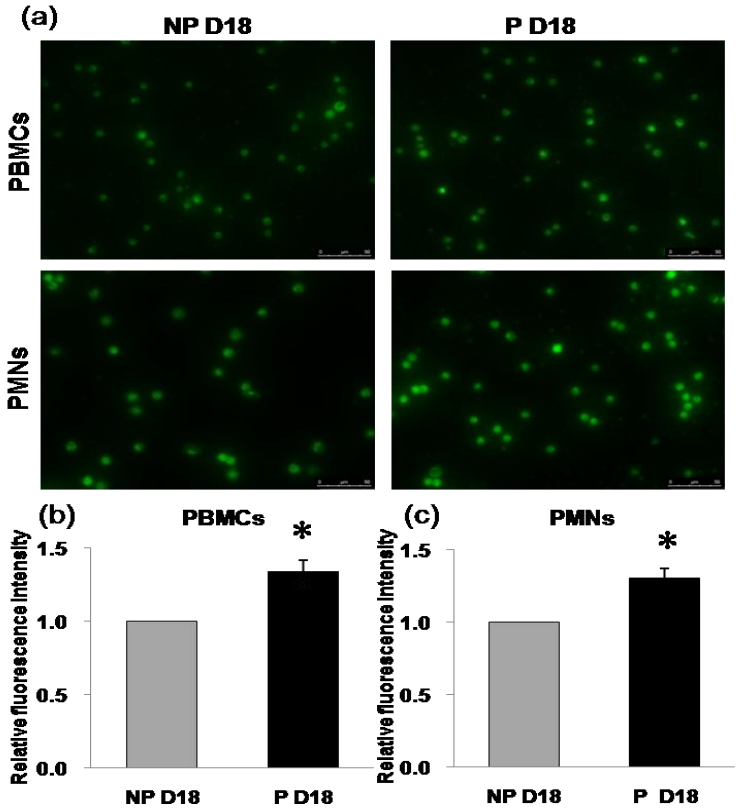


Figure 10. Lysosomal activities in PBMCs and PMNs after AI. (a) Fluorescence image of lysosomal acidification in pregnant (P) and non-pregnant (NP) cows in PBMCs (upper part) and in PMNs (lower part). Asterisks (**p<0.01, *p<0.05, student t test) indicate significant difference in between the pregnant (P) and non-pregnant (NP) cows on d18 after AI.

3.4.5 Gene expression of *LAMP-1* and *LAMP-2* in PBMCs and PMNs from pregnant and non-pregnant cows

Expression of *LAMP-1* was significantly higher in both PBMCs and PMNs on d18 in pregnant cows (Figure 11a, b). Similarly, expression of *LAMP-2* was significantly higher in both cells of d18 pregnant cows (Figure 11c, d).

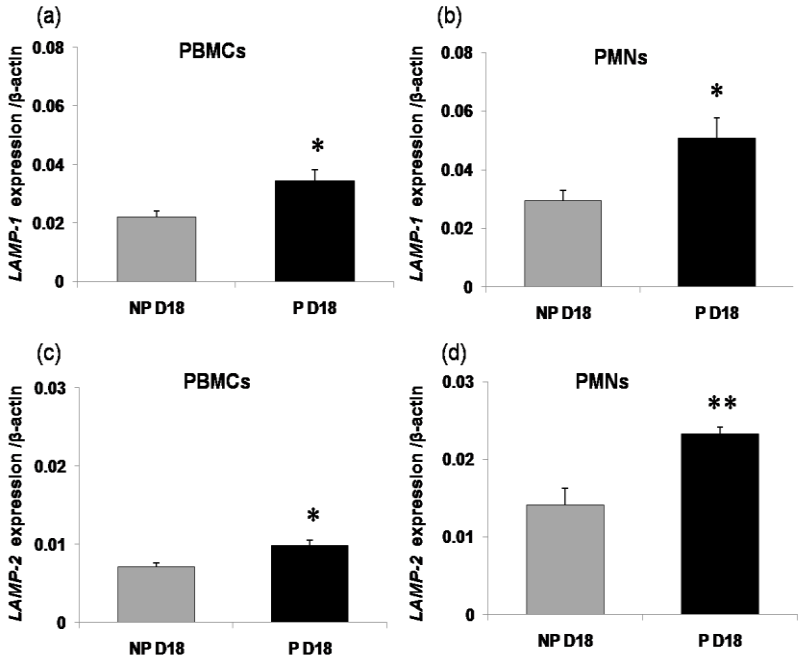


Figure 11. Expression of *LAMP-1* and *LAMP-2* mRNA in PBMCs and PMNs after AI. Asterisks indicate significant differences for *LAMP-1* (**p*<0.05) and for *LAMP-2* (**p*<0.05, ***p*<0.01) between pregnant (P) and non-pregnant (NP) cows after AI, determined by student t test.

3.5 Discussion

In the present study, I investigated the cell-specific lysosomal functions associated with CTSs in PBMCs and PMNs during early pregnancy using *in vivo* experiments.

Results in this study revealed a significant increase the activity of CTSs B, K and L in PMNs in pregnant cows on d18 compared with that of cells collected from d0 post-AI cows. To confirm the cell-specific response of CTSs activity in PBMCs and PMNs during pregnancy time is caused by IFNT. *ISG15* was also analyzed as a potential positive marker of pregnancy, which has been well established as a suitable early pregnancy detection marker in many studies (Puglisi *et al*. 2014, Shirasuna *et al*. 2012, Green *et al*. 2010). These results showed high *ISG15* expression levels in pregnant cows, indicating that the PBMCs and PMNs were affected by IFNT *in vivo*. Therefore, the increase of CTSs activities on d18

was possibly due to IFNT, as its secretion is maximal at this stage.

The expression differences in *CTSs* (*B*, *K*) genes in PBMCs and PMNs in pregnant cows suggest that, these cells have diversified function which is responsible for the expression of *CTSs* genes that support the implantation of the conceptus. The results of the present study support the hypothesis that CTSs are dynamically expressed in pregnant bovine PBMCs and PMNs and they play important roles in the establishment and maintenance of pregnancy. In the present study, CTSB activity and gene expression were increased in pregnant cows on d18 in PBMCs and PMNs. However, despite high activity of CTSK in PBMCs and PMNs in pregnant cows on d18, gene expression was not clearly affected by pregnancy. The change in the expression of these genes might be related to changes in the concentration of IFNT during pregnancy. The different expression patterns of CTSs B, K, L, and S are reported in osteoclasts (Ishibashi *et al*. 2001) even though CTSB and CTSK belong to same cysteine protease.

Cysteine CTSs are optimally active in a slightly acidic pH, becoming mostly unstable at neutral pH, and different CTSs 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 CTSB and CTSK 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.

Interestingly, in the present study, clear activation of lysosomes by increasing acidification was detected, as well as the increase of gene expression of *LAMP-1* and *LAMP-2* in PBMCs and PMNs from pregnant cows. There is no direct pathway to explain how IFNT respond to increase these membrane proteins. Results in this study indicates that cell specific response PMNs is more sensitive than PBMCs and PMNs could be used as suitable marker in early pregnancy. However, the cell specific high response mechanism in PMNs by IFNT is still unclear. PMNs have short life (less than one week) in leukocytes is one of the possible reason to higher response (Shirasuna *et al*. 2012). Although, lysosomes are membrane bound dynamic organelles that are indispensable for endocytosis, phagocytosis, and autophagy (Eskelinen *et al*. 2003). It contains enzymes that breakdown unused intracellular materials, such as acid hydrolases 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). LAMP-1 and LAMP-2 are major lysosomal transmembrane proteins which regulate lysosomal functions including acidification (Saftig and Klumperman 2009). Therefore, the

increase of lysosomal acidification as well as CTSs activity caused by low pH can be explained by an increase of LAMP-1 and LAMP-2, 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).

The present study first showed the cell type specific activation of lysosomes and CTS enzymes in PBMCs and PMNs at the time of maternal and fetal recognition of pregnancy. In chapter IV, I have discussed about *in vitro* IFNT stimulation of PBMCs and PMNs.

Chapter IV

Effect of IFNT on stimulation of the activity of lysosomes and lysosomal cathepsins in bovine PBMCs and PMNs

4.1 Introduction

As described detail in chapter II and III, in cattle, pregnancy related lysosomal CTSs activity, mRNA expression and the presence of CTSB proteins in PBMCs and PMNs were clarified. However, it remains unknown about the direct involvement of IFNT. So, the present study was aimed to investigate the effect of IFNT on lysosomal activation. Therefore, the current investigation was undertaken to study the effect of IFNT on lysosomal CTSs activity, mRNA expression and the presence of CTSB proteins in PBMCs and PMNs from non-AI cows.

4.2 Materials and Methods

4.2.1 Animals

Multiparous Holstein Friesian cows which belong to the Hokkaido University Dairy Farm. All experimental procedures and protocols for the care of the cows were compiled in accordance with the guide lines of Hokkaido University, Sapporo, Japan.

4.2.2 *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 estrus stage. The cells were cultured in the

RPMI 1640 (Wako) containing 5% FBS with or without recombinant IFNT (rbIFNT, 1,000 IU/ml) for 3, 6, and 12h at 38.5°C, 5% CO_2 in air. 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 1,000 IU/ml IFNT was designed based on the antiviral activity of plasma collected from d15 of pregnant ovine uterine vein (500-1,000 U/ml) that might be a possible stimulating concentration to leukocytes locally (Oliveira *et al*. 2008). At the end of each treatment, cells were collected and centrifuged at 1,200×*g* for 10 min, followed by washing twice with PBS. For measuring CTSs activity, 10 µL cell suspensions were removed from whole cell suspension and 200× diluted CTSs substrates were added and gently mixed. Cells were cultured for 40 min at 38.5°C, 5% CO_2 in air. After incubation, the samples were washed twice with PBS solution. The cells were then used for measuring CTSs (B, K and L) activity, gene expression analysis, and immunostaining for detection of CTSB protein.

4.2.3 Activities of CTSs (B, K and L) in PBMCs and PMNs from non-AI cows

Detection of CTSs activity was performed with fluorescent substrates. Cell suspensions (10 µL) of both PBMCs and PMNs were used. Magic Red CTSs (B, K and L) assay kits (MR-RR_2, MR-LR_2 and MR-FR_2) (Immunochemistry Technologies) were prepared by diluting the Magic Red stock solutions to 200× with RPMI 1640 containing 5% FBS. To stain the nuclei, 1 µL of Hoechst 33342 (Sigma-Aldrich) was added, and the tubes were incubated for 40 min at 38.5°C, 5% CO_2 in air. After incubation, the samples

were washed twice with PBS solution. 10 µL sample was dropped onto a slide glass with a cover slip (18×18 mm) and immediately placed under a fluorescence microscope (Keyence) using a 550 nm excitation filter at 400×. All images in both PBMCs and PMNs were captured in the same exposure conditions. The fluorescence intensity was measured by ImageJ analysis software (National Institutes of Health).

4.2.4 RNA extraction, cDNA synthesis and qPCR

Total RNA of PBMCs and PMNs from *in vitro* cultured samples was separately extracted and stored at -80°C until analysis. After standardizing the total RNA concentration using a NanoDrop spectrophotometer (Thermo Fisher Scientific), cDNA was synthesized with a ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO). 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 LightCycler nano (Roche Diagnostics). Primers for *ISG15* as a pregnancy positive marker, CTSs (*B, 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 µL containing 1 µL (0.5 µL reverse and 0.5 µL forward) of each primer, 5 µL KAPA SYBR FAST qPCR Kit and 4 µL of cDNA, and subjected to the following cycling condition: a denaturation step at 95°C for 30s, an amplification step of 45 cycles at 95°C for 10s, 55°C for 15s and 72°C for 30s. The expression level of each gene was normalized using *β-actin* as the internal standard. The qPCR primer sequences used are presented in

Table 2.

4.2.5 Immunodetection of CTSB in *in vitro* cultured PBMCs and PMNs from non-AI cows

Separated PBMCs and PMNs collected from samples after 6h of *in vitro* stimulation by IFNT, were fixed in 4% PFA in 0.5 ml tubes for 24h at 4ºC and processed using standard procedures. Cells were washed with PBS 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 1h at room temperature. After blocking, incubation with the primary CTSB polyclonal antibody (anti-rabbit IgG, 1:1,000) (Calbiochem, EMD Millipore Corporation, USA) was performed for 2h at room temperature. The cells were also incubated with the fluorescence in conjugated secondary antibody (donkey-antirabbit IgG, 1:1,000) (GE Healthcare Bio Sciences) for 1h 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 with PBS for 5 min. After washing with PBST, the cell suspension was dropped onto a glass slide and coved with a cover glass (18×18 mm). All images were captured with a fluorescent microscope (Leica Microsystems) in 1.5s exposures time using a 550 nm excitation filter at 400×. The fluorescence intensity was measured by ImageJ analysis software (National Institutes of Health).

4.2.6 Assessment of lysosomal activity in PBMCs and PMNs from non-AI cows

Separated PBMCs and PMNs from samples after 6h of *in vitro* stimulation by IFNT, were incubated in pre-warmed RPMI 1640 medium (Wako) with 1 µM solution of DMSO diluted with LysoSensor Green DND 189 (L7535, Invitrogen by Thermo Fisher Scientific) for 15 min at 38.5°C, 5% CO_2 in air. Then Hoechst 33342 (Sigma-Aldrich) was added to stain the nuclei. After incubation, samples were washed twice with PBS. 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).

4.3 Statistical analysis

Data were analyzed using GraphPad prism version 5 for Windows (GraphPad software) and were presented as the mean ± S.E.M., one-way ANOVAs and student t test were performed. P values less than 0.05 ($p<0.05$) were considered to represent statistically significant differences.

4.4 Results

4.4.1 Effect of IFNT stimulation on activity of CTSs (B, K and L) in PBMCs and PMNs from non-AI cows

An increase in CTSB activity was observed both in PBMCs and PMNs after the addition of IFNT (Figure 12a). Significant increases of fluorescence intensity were

observed in PBMCs after 12h and in PMNs 6h after stimulation with IFNT (Figure 12b, c). In addition, IFNT significantly increased CTSK activity in both PBMCs and PMNs (Figure 13a). Fluorescence intensity was significantly increased in PBMCs after 6h and in PMNs after 3h of IFNT treatment (Figure 13b, c). Moreover, fluorescence activity showed CTSL activity (Figure 14a). CTSL activity increased significantly after IFNT stimulation at 3, 6h in PBMCs and 6h period in PMNs respectively (Figure 14b, c).

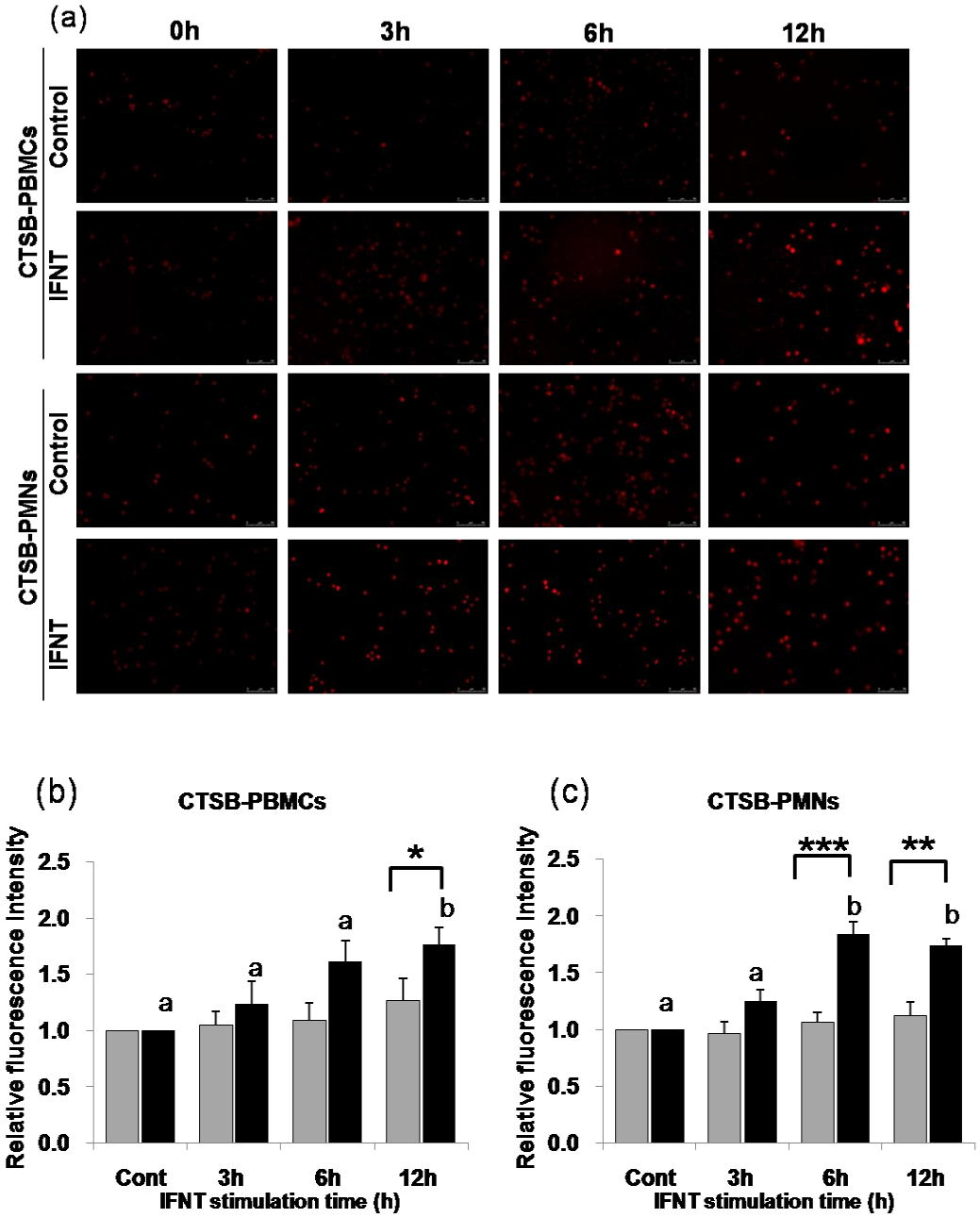


Figure 12. CTSB activities in PBMCs and PMNs after *in vitro* stimulation by IFNT.
Photos show the fluorescence images of CTSB in PBMCs and PMNs after 3, 6, and 12 h of IFNT stimulation (a). Relative fluorescence intensity of CTSB activity of PBMCs (b) and PMNs (c). The scale bar represents 50 µm. Gray bars show the fluorescence intensity of the non-treated group and black bars the IFNT-treated group. All data are shown as the mean ± S.E.M. Different letters indicate significant difference ($p<0.05$) among IFNT treatment times according to ANOVA followed by Fisher's as a multiple-comparison test. Asterisks indicate significant differences (*$p<0.05$, **$p<0.01$, ***$p<0.0001$, student t test) between groups with the same incubation time.

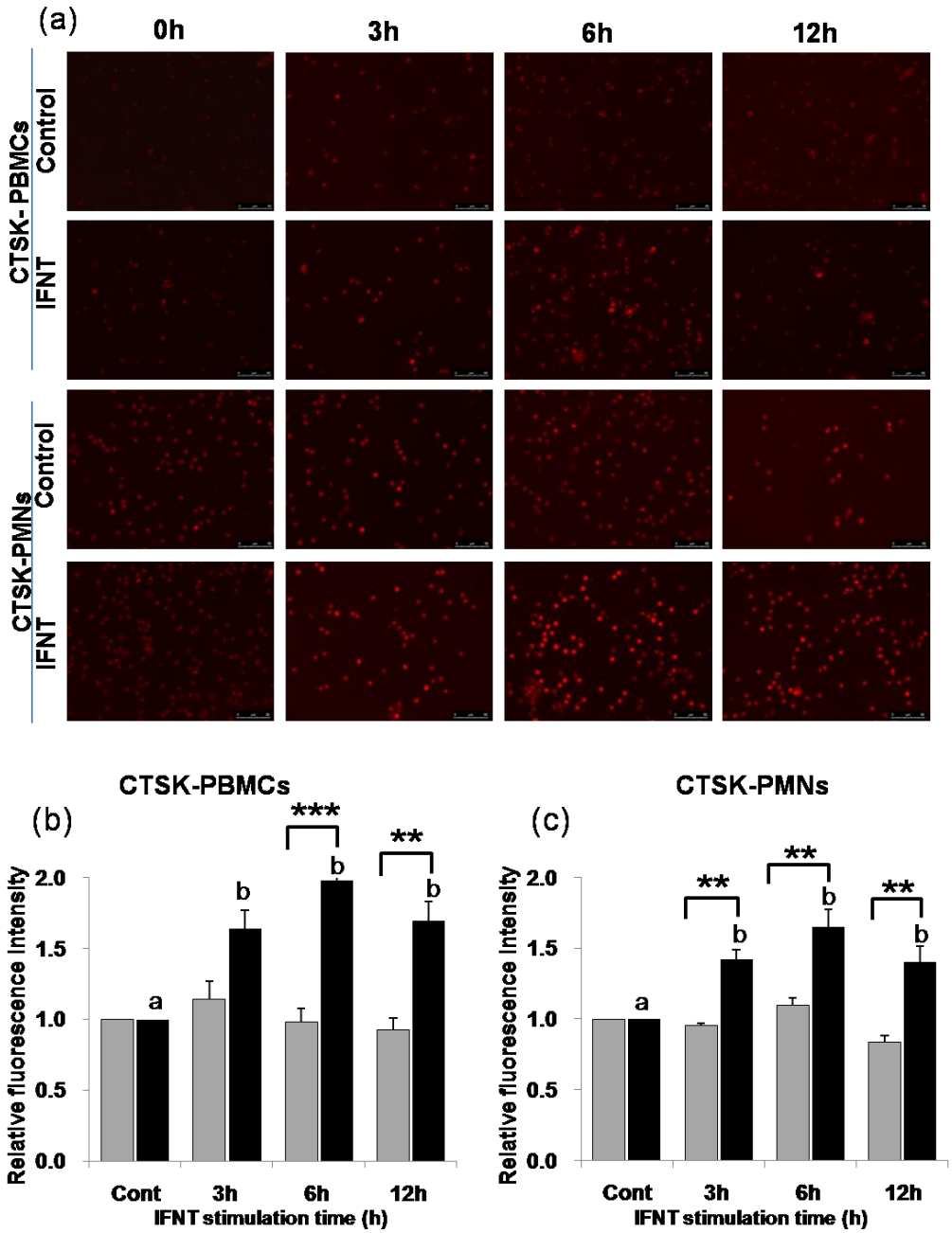


Figure 13. CTSK activities in PBMCs and PMNs after *in vitro* stimulation of IFNT.
Photos show the fluorescence images of CTSK in PBMCs and PMNs after 3, 6, and 12 h of IFNT stimulation (a). Relative fluorescence intensity of CTSK activity of PBMCs (b) and PMNs (c). The scale bar represents 50 µm. Gray bars show the fluorescence intensity of the non-treated group and black bars the IFNT-treated group. All data are shown as the mean ± S.E.M. Different letters indicate significant difference ($p<0.05$) among the IFNT treatment times according to ANOVA followed by Fisher's as a multiple-comparison test. Asterisks indicate significant differences (**$p<0.01$, ***$p<0.0001$, student t test.) between groups with the same incubation time.

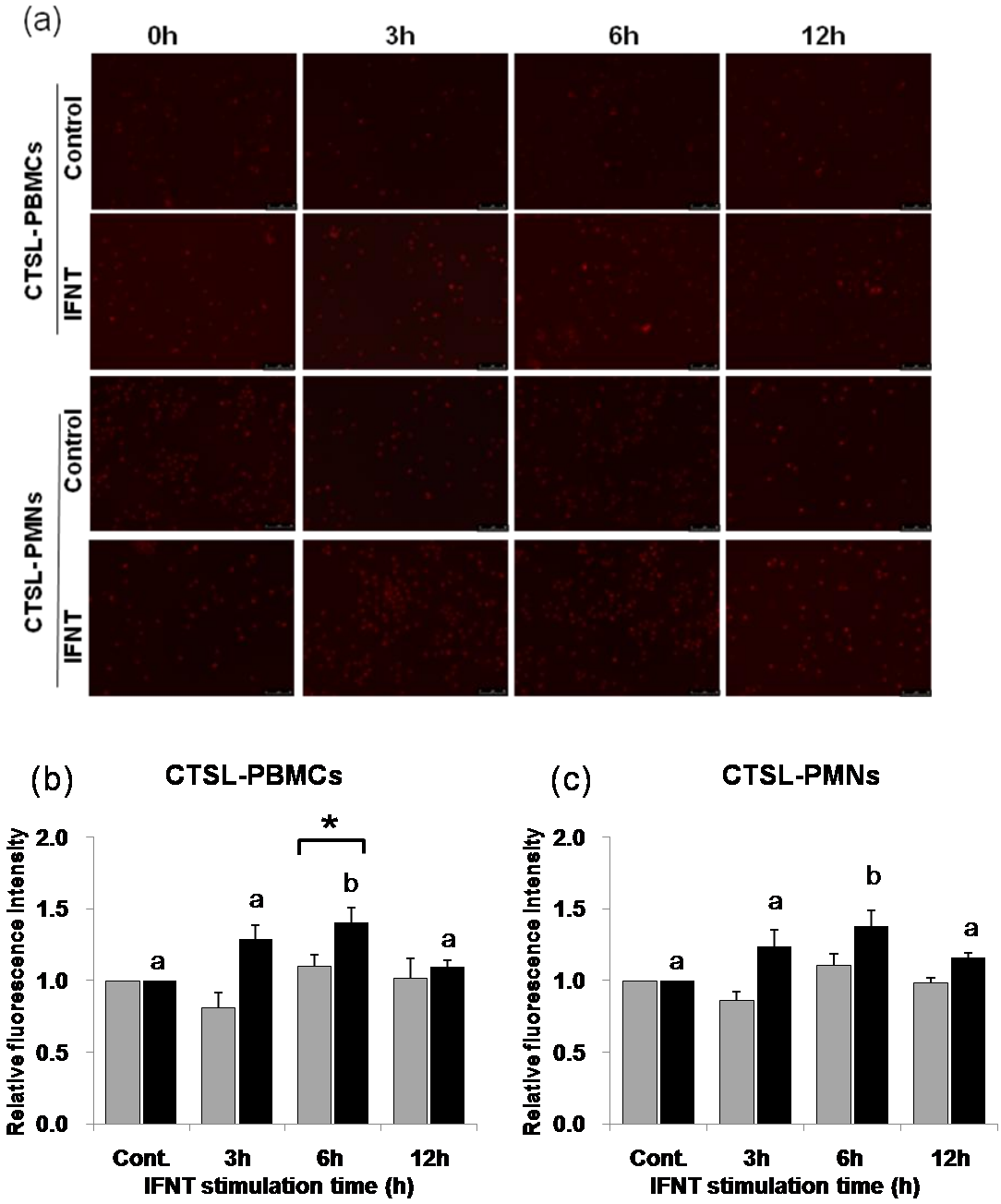


Figure 14. CTSL activities in PBMCs and PMNs after *in vitro* stimulation of IFNT.
Photos show the fluorescence images of CTSL in PBMCs and PMNs after 3, 6, and 12 h of IFNT stimulation (a). Relative fluorescence intensity of CTSL activity of PBMCs (b) and PMNs (c). The scale bar represents 50 μm. Gray bars show the fluorescence intensity of the non-treated group and black bars the IFNT-treated group. All data are shown as the mean ± S.E.M. Different letters indicate significant difference ($p<0.05$) among the IFNT treatment times according to ANOVA followed by Fisher's as a multiple-comparison test. Asterisks indicate significant differences (*$p<0.05$ by student t test) between groups with the same incubation time.

4.4.2 Effect of IFNT stimulation on the expression of *CTSs* (*B, K*) and *ISG15* in PBMCs and PMNs from non-AI cows

Expression of *CTSB* mRNA in PBMCs and PMNs was significantly higher after 6h of IFNT stimulation (Figure 15a, b). *CTSK* mRNA level was not affected by IFNT treatment (Figure 15c, d). *ISG15* mRNA expression was significantly increased in both PBMCs and PMNs by IFNT treatment (Figure 15e, f).

4.4.3 Immunodetection of CTSB protein in PBMCs and PMNs from non-AI cows

In immunostaining experiment, CTSB was clearly detected in *in vitro* stimulation of both cells by IFNT and showed a significant increase of fluorescence compared with non-stimulated cells (Figure 16a, b, c).

4.4.4 Lysosomal activity after *in vitro* IFNT stimulation in PBMCs and PMNs from non-AI cows

Clear fluorescence indicating the acidification status of lysosomes was observed both in PBMCs and PMNs (Figure 17a). *In vitro* stimulation of both cells by IFNT showed significant increases in fluorescence intensity compared with non-stimulated control cells (Figure 17b, c).

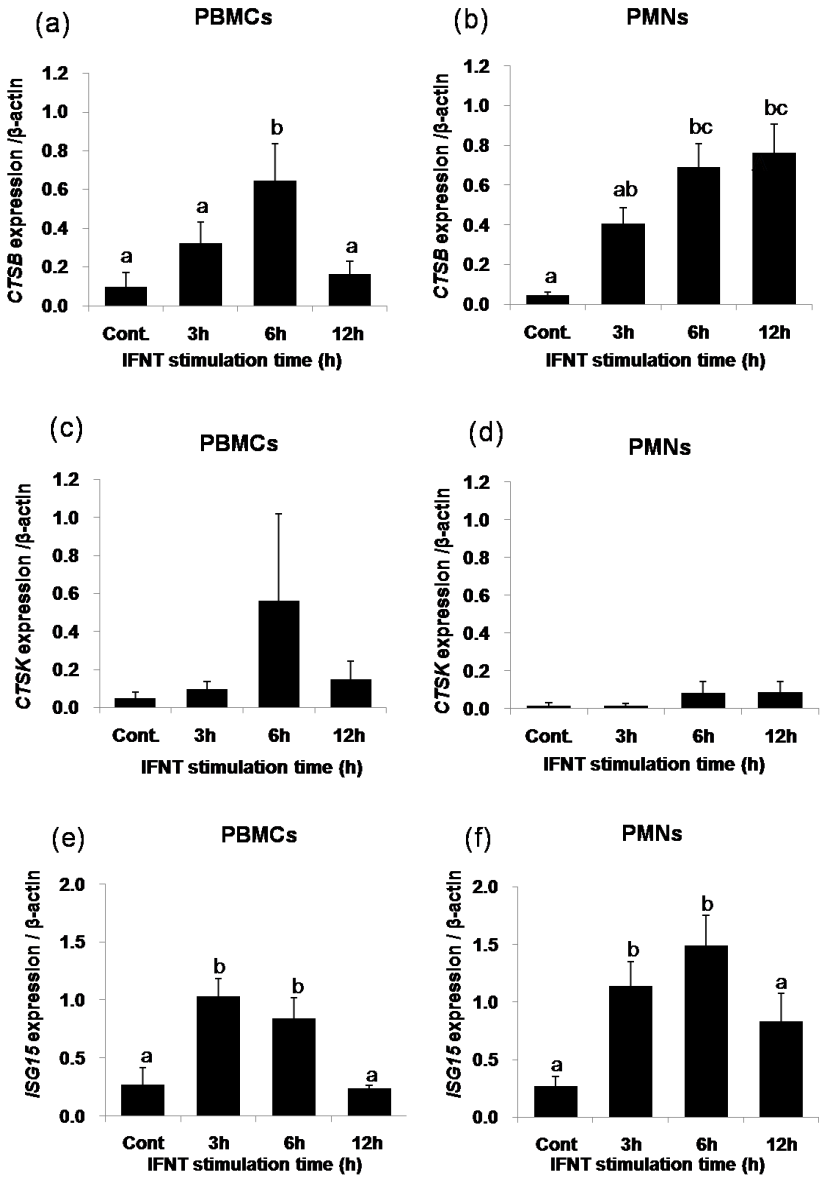


Figure 15. Effect of IFNT stimulation on the expression of *CTSs* (*B*, *K*) and *ISG15* mRNA in PBMCs and PMNs from non-AI cows. Expression levels of *CTSB* in PBMCs (a) and PMNs (b), and *CTSK* in PBMCs (c) and PMNs (d), and *ISG15* in PBMCs (e) and PMNs (f) using RT-qPCR standardized with the reference gene *ß-actin*. IFNT stimulation was performed for 3, 6, and 12 h. Different letters indicate significant differences ($p<0.05$) among the IFNT stimulation times by ANOVA followed by Fisher's PLSD procedure as a multiple-comparison test.

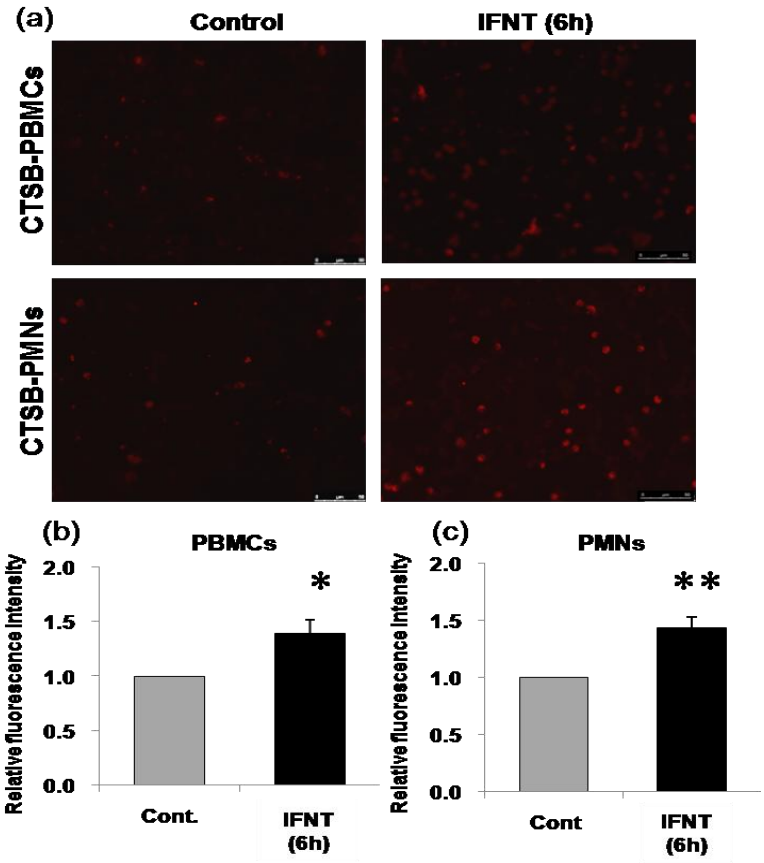


Figure 16. Immunostaining and detection of CTSB protein in PBMCs and PMNs after IFNT stimulation. (a) Immunohistochemical detection of the CTSB protein in control and IFNT treatment groups in PBMCs (upper part) and PMNs (lower part). The scale bar represents 50 µm. The vertical line shows the relative fluorescence intensity of CTSB protein after IFNT stimulation in (b) PBMCs and (c) PMNs. All data are shown as the mean ± standard error of the mean (S.E.M.). Asterisks (*p<0.05, **p<0.01, student t test) indicate significant difference between control and IFNT treatment group.

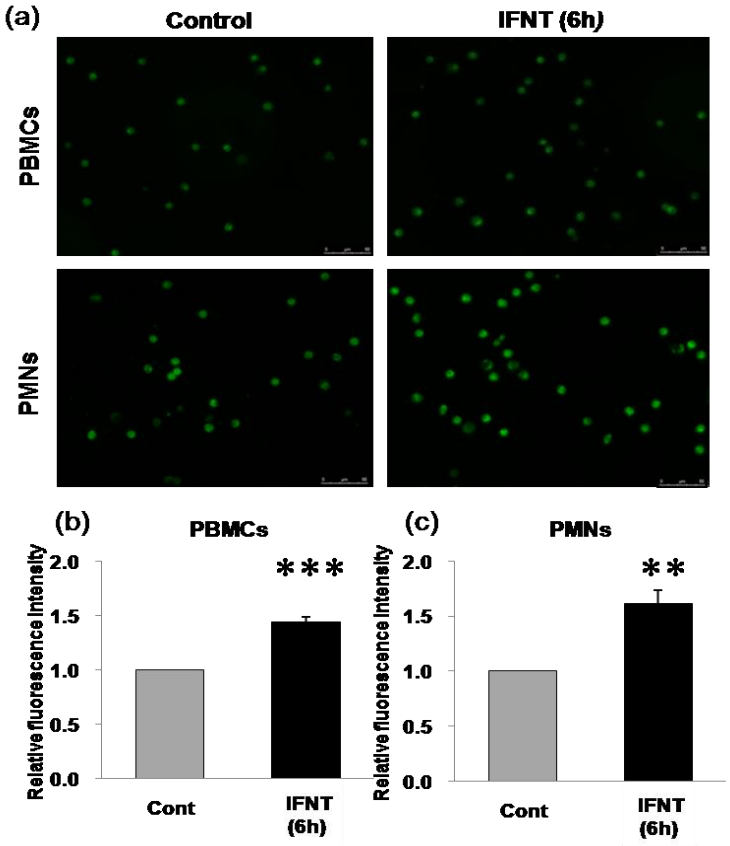


Figure 17. Lysosomal activities in PBMCs and PMNs after IFNT stimulation. (a) Lysosomal activity fluorescence image in control and IFNT treatment group in PBMCs (upper part) and in PMNs (lower part). The scale bar represents 50 µm. The vertical line shows the relative fluorescence intensity of lysosomal activity after IFNT induction in (b) PBMCs and (c) PMNs. All data are shown as the mean ± standard error of the mean (S.E.M.). Asterisks (****p<0.0001*, ***p<0.05*, student t test) indicate significant difference between the control and IFNT treatment groups.

4.5 Discussion

In the present study, I investigated the effect of IFNT on function of lysosomal CTSs in PBMCs and PMNs. In chapter III, *in vivo* analysis revealed a significant increase in the activity of CTSs B, K and L in PBMCs and PMNs in pregnant cows on d18 compared with that of cells collected from d0 post-AI cows. To confirm whether the

pregnancy-related cell specific CTSs activation is caused by IFNT, an *in vitro* study was conducted. As expected, the fluorescence intensity of these CTSs enzyme activity was increased by IFNT stimulation both in PBMCs and PMNs. These results strongly suggest that the activation of the CTSs in immune cells could be stimulated by IFNT.

To confirm that the increase of CTSs activity is pregnancy-specific in PBMCs and PMNs, potential and widely expressed interferon stimulated genes *ISG15* was also analyzed. Its response to *in vitro* stimulation using IFNT, 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). Our results showed high *ISG15* expression levels, indicating that the PBMCs and PMNs were affected by IFNT stimulation in *in vitro*. Therefore, the increase of CTS activities was possibly due to IFNT-mediated stimulation.

CTSs B, K and L 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 (Turk *et al.* 2012, Reiser *et al.* 2010). The present study indicated that type 1 interferon (*i.e.*, 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.

In the present study, CTSB activity and gene expressions were clearly increased by

IFNT both in PBMCs and PMNs. This indicates the IFNT-induced CTSB activity, transcription of gene followed by an increase of the protein. However, despite high activity of CTSK in PBMCs and PMNs IFN stimulated cells, gene expression was not clearly affected. *In vitro*, *CTSB* and *CTSK* mRNA levels in PBMCs increased significantly after 3 and 6h of IFNT stimulation, compared to the control group. Also, in PMNs, these mRNA levels significantly increased at 3, 6, and 12h 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 CTSs are optimally active in a slightly acidic pH, becoming mostly unstable at neutral pH, and different CTSs 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 CTSB and CTSK may not correlate with enzymatic activities. In addition, detection of fluorescent activity was achieved with different fluorescent substrates for CTSs B, K and L. 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 after IFN stimulation *in vitro*. CTSB could be a potential marker for pregnancy detection in the blood cells.

The increase of lysosomal acidification as well as CTSs activity is caused by low pH can be explained by an increase of LAMP-1 and LAMP-2, 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 roles of lysosomal membrane proteins. MxA, a type 1 IFN stimulated gene (protein), co-localizes with LAMP-1 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 blood cells. It is still necessary to clarify the mechanisms.

The present study first showed the IFNT-mediated activation of lysosomes and CTS enzymes in PBMCs and PMNs. These findings suggest a possible marker for potential early pregnancy detection.

Chapter V

Involvement of type 1 IFN signal transduction pathway on lysosomal functions in bovine leukocytes

5.1 Introduction

IFNT binds to the type 1 interferon receptor (IFNAR1 and IFNAR2), whose function is essential for the generation of the biological activities of all the different type 1 IFNs. IFNAR1 and IFNAR2 induce cell signaling via the Janus activated kinases (JAKs) and Tyrosine kinase 2 (TYK2) pathways, respectively (Platanias 2005, Der *et al*. 1998, Darnell *et al*. 1994). Activation of the JAKs that are associated with the type 1 IFNs receptors results in tyrosine phosphorylation of STAT (signal transducer and activator of transcription) 1 and STAT2, that leads to the formation of STAT1-STAT2-IFR (IFN regulatory factor) 9 complexes, which is known as ISGF (IFN stimulated gene factor) 3 complexes. These complexes translocate to the nucleus which binds and activates interferon stimulated response elements (ISREs) in DNA to initiate amplify effects of type 1 IFNs (Choi *et al*. 2001, Spencer *et al*. 1998). During pregnancy recognition period, IFNT affects uterine tissues with activating pregnancy-specific signal pathway such as increase of interferon stimulated genes *ISG15*, *MX1*, and *MX2* (Oliveira *et al*. 2008, Hicks *et al*. 2003, Ott *et al*. 1998). Besides, ISGs expression is clearly up-regulated in blood leukocytes in earlier stage of pregnancy (Gifford *et al*. 2007, Han *et al*. 2006).

AZD1480 is a potent small ATP (adenosine tri phosphate) competitive JAK (Janus activated kinase) 2 inhibitor and exert its biological effects by preventing the

phosphorylation and activation of STAT3 both *in vivo* and *in vitro* (Shuang Yan *et al*. 2013). Suppressing STAT3 activation using AZD1480 blocks downstream targets of STAT3 genes, such as cell cycle regulator Cyclin D1 and the anti-apoptotic genes (Bcl-2 and survivin). As a result, AZD1480 inhibits cell proliferation and induces cell apoptosis (Shuang Yan *et al*. 2013 and Derenzini E *et al*. 2011). Hong Xin *et al*. (2011) also reported that AZD1480 blocks STAT3 dependent VEGF (vascular endothelial growth factor) expression, leading to the inhibition of tumor angiogenesis and metastasis.

In cattle, determining the pathway of IFNT-mediated lysosomal function and the expression of *LAMP-1, -2* as well as the expression of CTSK and LAMP-1 proteins in PBLs remain unknown. The aim of this present study was to unveil the mechanism by which IFNT-mediates lysosomal function by investigating (1) lysosomal and CTSs activities (2) expression of *LAMP-1, -2* and (3) CTSK and LAMP-1 protein in leukocytes after *in vitro* stimulation of IFNT and AZD1480 JAK inhibitor.

5.2 Materials and Methods

5.2.1 Animals

Multiparous Holstein Friesian cows which belong to the Hokkaido University Dairy Farm. All experimental procedures and protocols the care of those cows was compiled in accordance with the guide lines of Hokkaido University, Sapporo, Japan.

5.2.2 *In vitro* induction of IFNT with AZD1480 JAK inhibitor for assessment of lysosomal and CTSs (B, K) activity in leukocytes from non-AI cows

Total leukocytes collected from healthy lactating non-AI cows at mid-late estrus stage of non-AI cows were cultured in the RPMI 1640 medium (Wako). Experiment was achieved using three experimental groups: control (no addition of IFNT), IFNT (500 IU/ml), and IFNT (500 IU/ml) with inhibitor (10 nM AZD1480 JAK inhibitor). After culture for 12h at 38.5°C, 5% CO_2 in air. At the end of the treatment, cells were collected and centrifuged at 1,200×*g* for 5 min. For measuring of lysosomal activity, 10 µL of cell suspensions were taken into small tube and incubated in pre-warmed RPMI 1640 medium (Wako) with 1µM solution of DMSO-diluted LysoSensor Green DND-189 (L7535, Invitrogen by Thermo Fisher Scientific) for 15 min at 38.5°C, 5% CO_2 in air. Measuring of CTSs activity, 10 µL of cell suspensions were taken into small tube from whole cell suspension then 200× diluted CTSs substrates were added to the tubes and gently mixed. Cells were cultured for 40 min at 38.5°C, 5% CO_2 in air. Hoechst 33342 (Sigma-Aldrich) was added to stain the nuclei. After incubation, samples were washed two times with PBS. The images and measured the fluorescence intensity followed the same procedures described details in chapter II *in vivo* analysis.

5.2.3 RNA extraction, cDNA synthesis and RT-qPCR

Total RNA of leukocytes from non-AI cows was extracted and stored at -80°C until analysis. After standardizing the total RNA concentration using a NanoDrop spectrophotometer (Thermo Fisher Scientific), cDNA was synthesized with ReverTra Ace qPCR RT Master Mix with gDNA remover (TOYOBO). The synthesized cDNA was stored at -30°C. To assess the gene expression, quantitative PCR (qPCR) was performed using

KAPA SYBR FAST PCR Kit optimized for Roche LightCycler nano (Roche Diagnostics). Primers for *ISG15*, *MX1* and *MX2* used as a type 1 IFN induced positive marker gene, lysosomal associated membrane protein *LAMP-1*, *-2* and *CTSs* (*B*, *K*) were designed and commercially synthesized based on reported sequence (Table 1).

The reactions were carried out in normal qPCR 8 strip tube, in a total volume of 10 µL containing 1 µL (0.5 µL reverse and 0.5 µL forward) of each primer, 5 µL KAPA SYBR FAST qPCR Kit and 4 µL of cDNA and subjected to the following cycling condition: a denaturation step at 95°C for 30s, an amplification step of 45 cycles at 95°C for 10s, 55°C for 15s and 72°C for 30s. The expression level of each gene was *β-actin* as the internal standard and using for qPCR primers sequences are presented in Table 1 (chapter II).

5.2.4 Immunodetection of CTSK and LAMP-1 proteins in leukocytes after *in vitro* induction of IFNT and AZD1480 JAK inhibitor

After *in vitro* culture total leukocytes were fixed with 4% paraformaldehyde (PFA) in 0.5 ml tube for 24h at 4ºC and processed using standard procedures. Cells were washed with PBS and then permeabilized PBS containing 0.2% TritonX-100 (PBST) for 10 min. Then cells were blocked with PBST containing 1% BSA solution for 1h at room temperature. Incubation with the primary CTSK rabbit polyclonal antibody (Pab) (anti-rabbit Ig, 1:1,000) (Wuxi App Tec, AB GENT, CA, USA) and LAMP-1 monoclonal anti-mouse antibody (Anti-mouse IgG1, 1:5,00) was performed for 1h at room temperature. The cells were also incubated with the fluorescence in conjugated secondary antibody (donkey-antirabbit IgG, 1:1,000) (GE Healthcare Bio-Sciences) for 1h at room temperature in dark

condition. 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 with PBS for 5 min. Slides were mounted with DAPI and viewed with fluorescence microscopy (Leica Microsystems). All images captured in same exposures condition using a 550 nm excitation filter at 630×. The fluorescence intensity was measured by ImageJ analysis software (National Institutes of Health).

5.2.5 Statistical analysis

Data were analyzed using GraphPad prism version 5 for Windows (GraphPad software) were presented as the mean ± S.E.M., one way ANOVAs and student t test. P values less than 0.05 ($p<0.05$) were considered to represent statistically significant differences.

5.3 Results

5.3.1 *In vitro* induction of IFNT and AZD1480 JAK inhibitor and measurement of lysosomal and CTSs (B, K) activity in leukocytes from non-AI cows

The lysosomal activity was significantly increased after IFNT induction. Such increase in lysosomal activity was decreased significantly by AZD1480 in the presence of IFNT (Figure 18a, b). An increase in CTSB activity was observed in leukocytes after the induction of IFNT alone (Figure 19a). However, CTSB activity was decreased by AZD1480 when combined with IFNT induction, but not differed significantly (Figure 19b). In consistence, IFNT alone significantly increased CTSK activity. Using AZD1480 to inhibit

JAK significantly decreased CTSK activity in the presence of IFNT (Figure 20a, b).

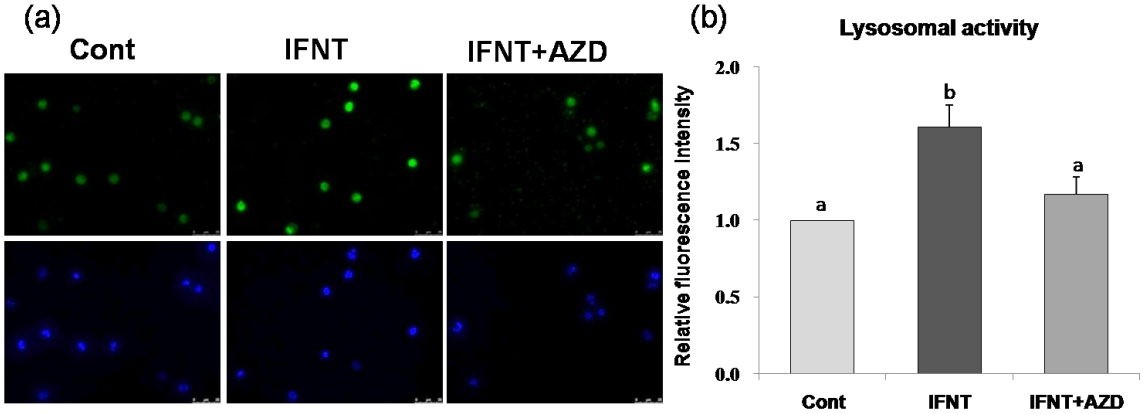


Figure 18. Lysosomal activity of leukocytes after stimulation of IFNT and AZD1480. (a) lysosomal fluorescence image in control, IFNT alone, and combination with IFNT and AZD1480 in leukocytes. The scale bar represents 25 µm. (b) the relative lysosomal activity on leukocytes after stimulation of IFNT and AZD1480. All data are shown as the mean ± standard error of the mean (S.E.M.). Letters (a *vs* b; $P<0.05$) indicate significant difference among the treatment group after incubation time according to ANOVA followed by Fishers as a multiple-comparison test.

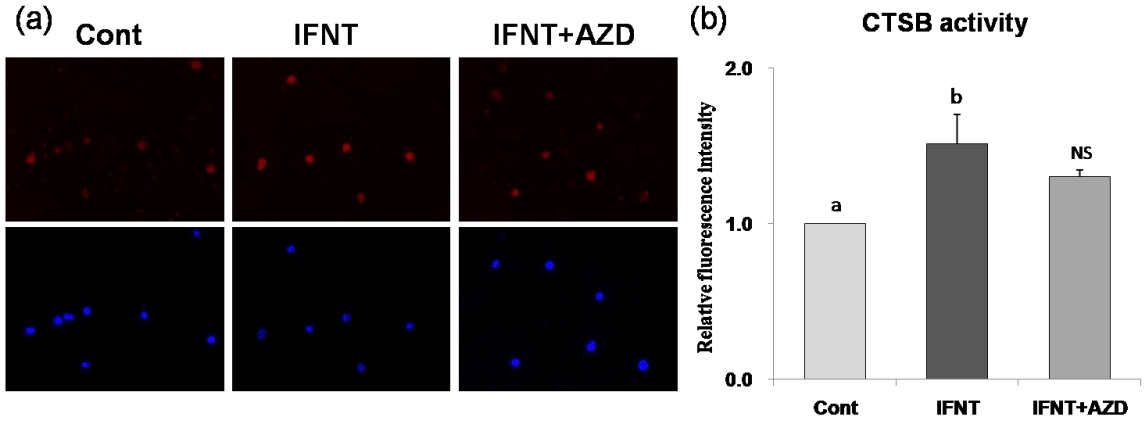


Figure 19. CTSB activity on leukocytes after IFNT stimulation and AZD1480. (a) CTSB fluorescence image in control, IFNT alone, and combination with IFNT and AZD1480 in leukocytes. The scale bar represents 25 µm. (b) the relative CTSB activity on leukocytes after IFNT stimulation and AZD1480. All data are shown as the mean ± standard error of the mean (S.E.M.). Letters (a *vs* b; $P<0.05$) indicate significant difference among the treatment group after incubation time according to ANOVA followed by Fishers as a multiple-comparison test.

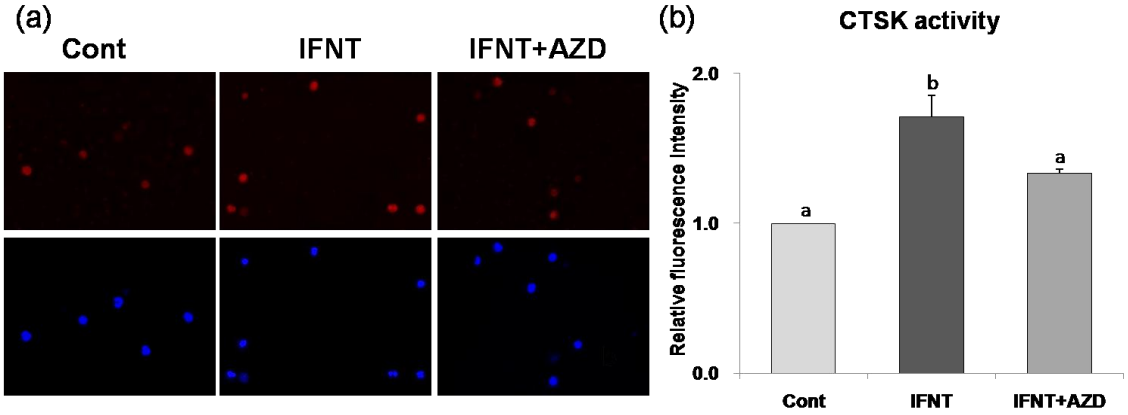
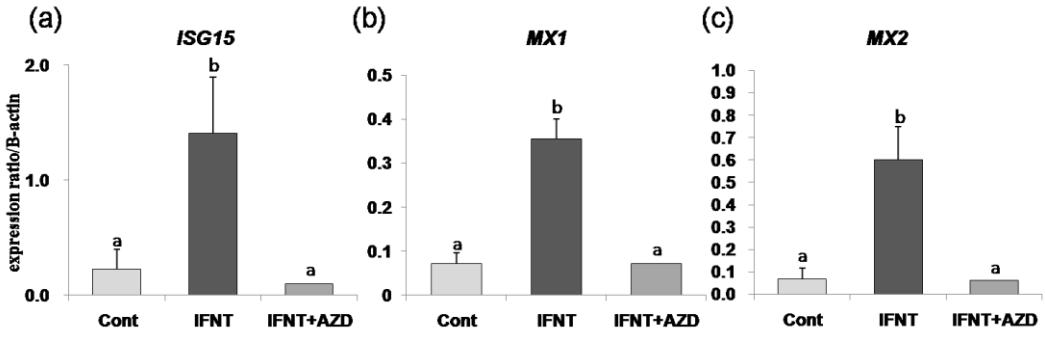


Figure 20. CTSK activity on leukocytes after IFNT stimulation and AZD1480. (a) CTSK fluorescence image in control, IFNT, and combination with IFNT and AZD1480 in leukocytes. The scale bar represents 25 µm. (b) the relative CTSK activity on leukocytes after IFNT stimulation and AZD1480. All data are shown as the mean ± standard error of the mean (S.E.M.). Letters (a *vs* b; *P<0.01*) indicate significant difference among the treatment group after 12h incubation time according to ANOVA followed by Fishers as a multiple-comparison test.

5.3.2 Expression of ISGs (*ISG15*, *MX1* and *MX2*), *CTSs* (*B*, *K*) and *LAMP-1*, *-2* mRNA after *in vitro* induction of IFNT and AZD1480 in leukocytes obtained from non-AI cows

Gene expression levels of *ISG15*, *MX1* and *MX2* were significantly increased by IFNT treatment when compared to controls. However, these increased transcript abundances in IFNT-treated leukocytes were significantly decreased when JAK was also inhibited using AZD1480 (Figure 21a, b, c).



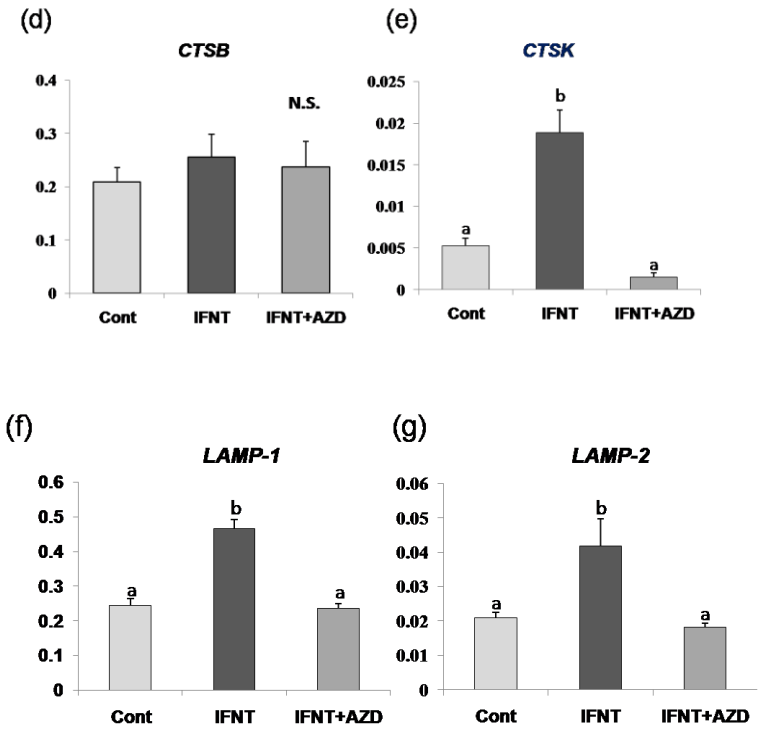


Figure 21. Expression of ISGs (*ISG15, MX1* and *MX2*), *CTSs (B, K*) and *LAMP-1, -2* mRNA in leukocytes after IFNT stimulation and AZD1480. The vertical line shows the mRNA expression level of (a) *ISG15* (b) *MX1* (c) *MX2* (d) *CTSB* (e) CTSK (f) *LAMP-1* and (g) *LAMP-2*. All data are shown as the mean ± standard error of the mean (S.E.M.). Letters (a *vs* b; *P<0.01*, *P<0.05*) indicate significant difference among the treatment group after 12h incubation time according to ANOVA followed by Fishers as a multiple-comparison test.

CTSB expression was not affected by IFNT stimulation. Besides, *CTSB* expression was not decreased by AZD1480 (Figure 21d). The expression of *CTSK* mRNA was significantly higher after IFNT induction and decreased significantly by the addition of AZD1480 in the presence of IFNT (Figure 21e). Similarly, the expression of *LAMP-1* and *LAMP-2* was significantly higher in IFNT treatment alone when compared to control, and decreased significantly by AZD1480 (Figure 21f, g).

5.3.3 Immunodetection of CTSK and LAMP-1 proteins after *in vitro* induction of IFNT and AZD1480 in leukocytes from non-AI cows

CTSK protein signal showed strong fluorescence (Figure 22a) and significantly increased after 12h of *in vitro* induction of IFNT. Addition of AZD1480 significantly decreased the CTSK activity in the presence of IFNT (Figure 22c). Regarding LAMP-1 protein, increase of fluorescence by IFNT stimulation and was significantly decreased by AZD1480 (Figure 22b, d).

5.4 Discussion

The effect of IFNT on lysosomal function and CTSs gene expression via type 1 IFN pathway in bovine leukocyte has not been studied until the present study. One of the major goals of the present study was to elucidate the involvement of the type 1 interferon signaling pathway in bovine blood leukocytes by investigating the lysosomal CTSs activity and mRNA expression as well. I also assure that IFNT stimulation significantly increased the lysosomal enzymatic activity and mRNA expression and that addition of AZD1480 a JAK inhibitor significantly decreased the lysosomal enzymatic activity and mRNA expression in the presence of IFNT. Given the results of my previous *in vivo* study (chapter II), I hypothesized that, the significant increase of lysosomal and lysosomal CTSs activity in pregnant cows is due to IFNT. So, the activation mechanism was actually triggered by IFNT or involvement of any other type 1 IFN pathway in immune cells. I have conducted this *in vitro* study with IFNT and a AZD1480. In this study I observed that, IFNT stimulation alone significantly increased lysosomal CTSs (B, K) as well as lysosomal

activity. I also found that, AZD1480 significantly decreased the activities these lysosomal and CTSs in the presence of IFNT. These results, suggest that lysosomal CTSs

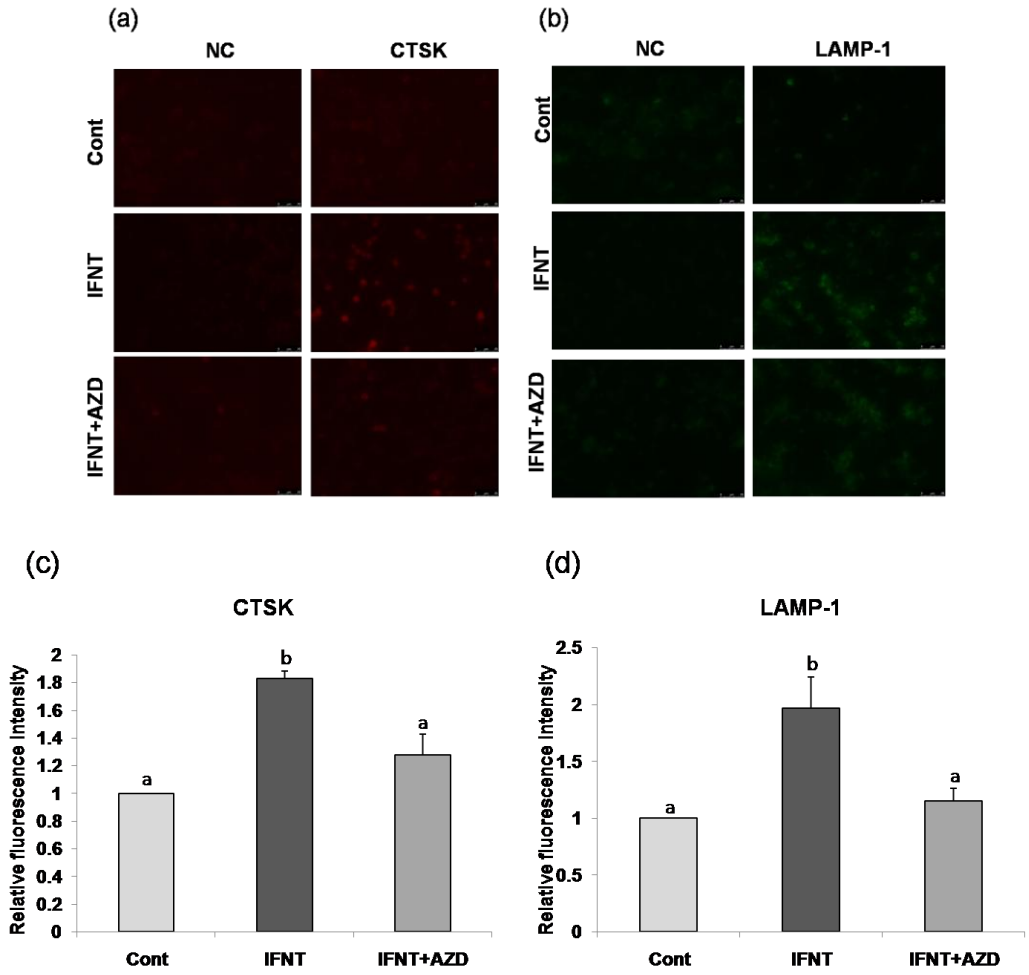


Figure 22. Immunodetection of CTSK and LAMP-1 protein in leukocytes after stimulation of IFNT and AZD1480. (a) Immunostaining for detection of CTSK and (b) LAMP-1 protein in leukocytes cells in control, IFNT, and combined with IFNT and AZD 1480. The images were captured with fluorescence microscope using 590 nm excitation filter at 630×. The scale bar represents 25 µm. The vertical line shows the relative fluorescence intensity of (c) CTSK and (d) LAMP-1 protein. NC-negative control. All data are shown as the mean ± standard error of the mean (S.E.M.). Letters (a *vs* b, $p<0.0001$ in case of CTSK and $P<0.05$ in case of LAMP-1) indicate significant difference among the treatment group after 12h incubation time according to ANOVA followed by Fishers as a multiple-comparison test.

activity is regulated by IFNT through type 1 IFN pathway mechanism in the leukocytes by suppressing the CTSs activity with the inhibitor. CTSs are lysosomal cysteine proteases

capable of digesting matrix proteins and activating other proteases involved in matrix degradation (Kirschke *et al*. 1998). It has reported that lysosomal protease such as CTSs activity is regulated by certain cytokines. Fiebiger *et al*. (2001) has been demonstrated that CTSs S and B activity increased in human DCs in response to cytokines such as TNF alpha and IL1 beta, leading to increased class 2 MHC (major histocompatability complex) dimer formations and T cell recognition. Altering endosomal pH and lysosomal protease activity can be modulated by cytokines such as IL6 and IL10 (Fiebiger *et al*. 2001, Drakesmith *et al*. 1998).

In the present study, *ISG15*, *MX1* and *MX2* were selected as potential positive markers of interferon stimulated genes (ISGs) and the difference in mRNA expression levels in leukocytes in pregnant cows were investigated. The expression of ISGs in leukocytes was significantly increased in IFNT treatment, and as significantly decreased after the addition of AZD1480 in the presence of IFNT. It might be suggested that, ISGs expression in leukocytes is regulated by the IFNT through type 1 IFN pathway. Expression of *ISG15* has been reported in PBMC's (Puglisi *et al*. 2014, Kizaki *et al*. 2013, Shirasuna *et al*. 2012), and in leukocytes (Green *et al*. 2010, Gifford *et al*. 2007, Yankey *et al*. 2001). Similar results have been reported for dairy cows in which *MX1* and *MX2* gene expression increases in PBLs of pregnant cows (Gifford *et al.* 2007).

CTSK, but not CTSB significantly increased after IFNT stimulation and significantly decreased after JAK inhibition. These expression results suggest that, CTSK activity and mRNA expression is regulated by type 1 IFN. In general, JAK inhibitor binds to the kinase to prevent its activation, thereby preventing the phosphorylation of type 1

IFNT receptor, STAT phosphorylation and the translocation of the STAT dimerized complex into the nucleus with subsequent inhibition of genes transcription.

Results of the present study established that, cysteine proteases were expressed in leukocytes and that the expression of *CTSs* (*B*, *K*) mRNA increased by IFNT stimulation. Expression of *CTSK* significantly decreased by the JAK inhibitor in the presence of IFNT. Interestingly, *CTSB* mRNA expression level did not affected by JAK inhibition, though CTSB and CTSK location is same within lysosomes. The inhibitory effect of *CTSB* genes transcription is unclear. Author thinks that, enzymatic activity and *CTSB* gene transcription follows the other independent pathway.

Importantly, the pregnancy specific expression of *LAMP-1*, *-2* in leukocytes suggests broad and diversified function in immune mechanisms responsible for expression of these genes that supports the conceptus implantation during establishment and maintenance of pregnancy. In this study, expression level of *LAMP-1*, *-2* was clearly up-regulated by IFNT stimulation, and decreased by AZD1480 in the presence of IFNT. It is suggested that, the expression of these membrane proteins are regulated by IFNT through type 1 IFN pathway. These results may indicate that the conceptus signal IFNT reaches peripheral circulation through the immune cells to up-regulate *IFNT* genes expression leading to apparent expression of these genes. Although LAMPs are distributed within the cell primarily in the lysosome, they are also constitutively expressed at the cell surface of platelets, T and B lymphocytes, monocytes and human several lymphocytes cell lines (Carlsson *et al*. 1992, Mathews 1992, Harter and Mellman, 1992).

Immunostaining analysis confirmed the up-regulation of CTSK and LAMP-1

protein after IFNT stimulation in leukocytes and decreased significantly by the AZD1480 in the presence of IFNT. Hou *et al*. (2001) reported that CTSK is expressed in synovial fibroblasts from joints of patients with rheumatoid arthritis, where it is thought to contribute to cartilage and sub-cortical bone degradation. Immunodetection of CTSK and LAMP-1 protein in blood cells has not been reported yet.

In conclusion, this study revealed for the first time that IFNT-induced lysosomal activation as well as expression, and lysosomal function are regulated by IFNT through type 1 IFN pathway in bovine leukocytes.

Chapter VI

General discussion

Study of lysosomes and lysosomal CTSs in white blood cells from early pregnant cows has not been studied until the present research. It is necessary to investigate the lysosomal proteases function during early pregnancy. Generally, ISGs function is known as a part of the innate immune response to viral infection. Furthermore, assaying for expression of ISGs in blood during early pregnancy provides a less window on conceptus-uterine cross-talk during early pregnancy (Gifford *et al*. 2008). The present study described new marker, which would be helpful to detect pregnancy within three weeks, especially 14-18 days after AI. The technique might be more specific and practical than the traditional methods of pregnancy diagnosis mentioned previously. Recent understanding of basic immune activity and conceptus biology associated with pregnancy recognition allowed the development of novel tools for early pregnancy diagnosis.

Currently, the most commonly used methods to diagnose pregnancy in dairy cows are relatively late following the maternal recognition of pregnancy after day 30. Consequently, early pregnancy detection is important for identifying non-pregnant cows and for promoting the efficient management of reproduction (Fiedrich *et al*. 2010). In dairy cattle, Lucy *et al*. (2004) proposed a re-insemination system that requires a method of pregnancy detection that is accurate on or before three weeks following the first insemination.

Accordingly, this research aimed to address the lysosomal CTSs activation by conceptus secretory protein IFNT and to specify the role of implantation of the conceptus.

It is clear that there is no precise pathway of IFNT mediated lysosomal CTSs activation.

Generally, functions of lysosomes is to complete degradation of internal proteins and produce amino acids and energy. Various factors that may contribute to the modulation of lysosomal proteolysis and increased the intralysosomal acidification, which has been seen in mature DCs is probably the most direct because it could favor proteolysis in several ways. The increased acidification would provide a pH environment closer to the optimal pH of most lysosomal enzymes (pH 4.5). A similar increase in proteolytic capacity in concert with lysosomal acidification was described for the regulation of yolk degradation (from pH 5.5 down to pH 4.5) (Fagotto and Maxfield 1994). Lysosomal acidification could also extend the proteolysis and formation of pro major histocompatability complex (pMHC) by favoring the activation and function of the proenzyme forms of gamma interferon inducible lysosomal thiol reductase (GILT) (Arunachalam *et al*. 2000). The role of lysosomes and lysosomal CTSs has long been considered to be housekeeping macromolecule turnover, but recent studies have revealed specific functions. Immunostaining experiments in this study and the detection of CTSs B, K and LAMP-1 in PBLs support this idea. Almost all cells express some level of papain-type lysosomal proteases. This appears to be required for the house keeping function of lysosomes in protein turnover by cells.

It is believed those cysteines CTSs are responsible for intracellular protein degradation. These proteins are monomeric and have homology of amino acid sequences and folds (Turk *et al*. 2001). It exhibits optimal activity at acidic pH and are unstable at neutral pH. Cysteine CTSs bind substrate in an extended conformation along the active site cleft (Turk *et al*. 2003, Turk *et al*. 1998). It can be summarized that, cysteine CTSs are non-

specific enzymes with no clear substrate recognition site. The specificity does not involve a single binding site, but is the result of contributions of all interactions.

However, the effects of increased ISGs expression on immune cell function are not known. Peripheral activation of ISGs suggests that immune cells respond to IFNT, although the details of these responses remain to be determined. Numerous T cells (CD4, CD8 and γδ), natural killer (NK) cells, DCs, B cells and macrophages (MΦ) are present at the conceptus-maternal interface during pregnancy recognition. However, surprisingly little is known about how these cells respond to the conceptus. We assumed that conceptus signals stimulate processes critical for establishment of pregnancy and induction of some immune regulators, stimulation of tissue remodeling and angiogenesis and induction of ISGs to increase innate immunity.

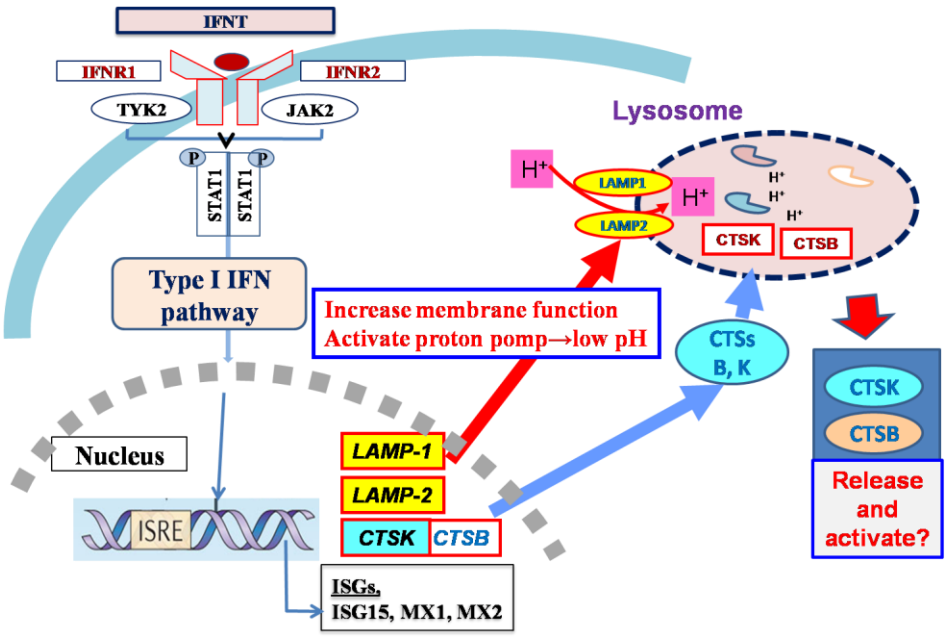


Figure 23. Schematic model of IFNT-mediated lysosomal activation

Chapter VII

Conclusion

Overall results obtained in the present study and clearly showed that, the pregnancy specific lysosomal and lysosomal CTSs (B, K) activities were up-regulated in leukocytes obtained from pregnant cows. Besides these activities, *LAMP-1*, *-2* and *CTSs* (*B*, *K*) mRNA expression levels were also up-regulated in leukocytes obtained from pregnant cows. The consideration of the expression of ISGs (*ISG15*, *MX1* and *MX2*) and the elevation of these lysosomal *CTSs* genes in pregnant cows compared to non-pregnant cows suggest the involvement of IFNT. As noted in the chapter III, cell specific response of lysosomal CTSs and mRNA expression in PBMCs and PMNs is affected during the maternal and fetal interface time. So, this evidence clearly indicates that the cell specific response in PBMCs and PMNs is highly possible by IFNT. Stimulation of IFNT showed time dependent activity within short period of incubation, and IFNT was able to induce CTSs activation described in chapter IV. As noted in the chapter V, it is clear that the up-regulation and inhibition mechanisms of lysosomal associated membrane proteins and *CTSs* genes in PBLs confirmed by *in vitro*. IFNT regulated many genes examined in this study, indicate that comparing differential gene expression on specific day of estrus cycle of pregnant cows and or control *vs* treatment by IFNT. It will be useful to select candidate genes for early identification of pregnancy.

Finally, it could be suggested that, in early pregnancy PBLs gene expression may be altered through the influence of pregnancy signal of IFNT. An increased knowledge of the interaction between conceptus and maternal immune system during early pregnancy in

ruminants is necessary to understand and elucidate the true cause for successful establishment of pregnancy. This study also provided the basis for new strategies for improving pregnancy outcomes and reproductive efficiency. In this study, we defined for the first time that IFNT induces lysosomal activation as well as the expressions of lysosomal proteins (CTSB, CTSK and LAMP-1). The PBL reaction to the IFNT was also verified *in vitro* by adding rbIFNT to leukocytes either in the presence or absence of JAK inhibitor. It is still remaining to address whether lysosomal function is regulated by the IFNT via type 1 IFN pathway in bovine leukocytes during maternal-fetal recognition period. Future study will explore the route of IFNT transport to lysosomes in leukocytes during the implantation period and expression of lysosomal CTSs in leukocytes and discover new functions of lysosomal CTSs by IFNT.

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Chemicals and reagents list

PBS (-):

KCl (Wako)	0.1 g
KH_2PO_4 (Wako)	0.1 g
$Na_2HPO_4.12H_2O$ (Wako)	1.45 g
NaCl (Wako)	4 g
Distilled water	500 ml

Gently stirring and stored at room temperature.

0.5 M EDTA (pH 8.0):

2 NA (EDTA. 2NA)	186.1 g
Distilled water	1 L

Gently stirring for proper mixing and keep it room temperature.

4% PFA:

PFA (Wako)	0.2 g
PBS (-)	5 ml

Sufficient boiling and sometime shaking for proper dissolve, and stored at 4°C.

PBS-T:

TritonX-100(Wako)	20 µl
PBS (-)	10 ml

TritonX put into warm water before use for proper dissolve, finally keep into 4°C.

1% BSA + PBS-T:

PBS-T	10 ml
BSA (Sigma)	0.1 g

TritonX keep warm water before use for proper dissolve the and finally store at 4°C.

TBS:

KCL (Wako)	0.2 g
Tris	3 g
NaCl (Wako)	8 g
Distilled water	1 L

Gently stirring for proper mixing and stored at room temperature.

TBST:

Tween 20 (Wako)	500 µl
TBS	500 ml

Gently stirring for proper mixing after adding Tween20 and stored at room temperature.

10 % SDS:

SDS (Wako)	10 g
Distilled water	100 ml

Stored at room temperature.

30% acrylamide/bis mixed solution

Acrylamide	14.6 g
Bis acrylamide	0.4 g
Distilled water	100 ml

Maximum storage time 1 month and stored at 4°C. Before use keep it room temperature.

1.5 M Tris HCl (pH 8.8):

Tris (Wako)	18.17 g
Distilled water	100 ml

About 80 ml full and measure pH 8.8 then fill up to 100 ml. Stored at 4°C.

0.5 M Tris HCl (pH 6.8):

Tris (Wako)	6.05 g
Distilled water	100 ml

About 80 ml full and measure pH 6.8 then fill up to 100 ml. Stored at 4°C.

10% APS (ammonium persulphate):

APS (Wako)	0.1 g
Distilled water	1.0 ml

Prepare every time or keep it -20^{0}C

Protein lysis buffer:

Distilled water	3.55 ml
D-solution	1.25 ml
B-solution	2.0 ml
Glycerol	2.5 ml
2-ME	0.5 ml
BPB	0.2 ml
Total	10 ml

Stored at 4°C

D-solution

Tris HCl (pH 6.8)	6 g
Distilled water	3.55 ml

Stored at 4°C

B-solution (10% SDS)

Sodium dodecyl sulphate	10 g
Distilled water	100 ml

Slowly mix, no bubble and stored at room temperature.

Red blood cells lysis buffer (10×)

NH_4Cl	8.26 g
$NaHCO_3$	1.19 g
EDTA2Na	0.037 g
Distilled water	3.55 ml

Use sterilized distilled water and maintain pH 7.3