The role of interferon-tau (IFNT) secreted from Day-7 embryo in uterine immune modulation in cattle

2018

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ウシの 7 日目の初期胚から分泌されるインターフェロン・タウの子宮内免疫調節の役割

平成 30 年

（2018）

帯広畜産大学大学院畜産学研究科

博士後期課程　畜産衛生学専攻

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Dedication

My beloved father and mother

In the memory of my dear father…

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Abbreviations

BSA	Bovine serum albumin
BUECs	Bovine uterine epithelial cells
cDNA	Complementary deoxyribonucleic acid
CIDR	Controlled internal drug releaser
CL	Corpus luteum
CM	Conditioned media
DCs	Dendritic cells
DMEM/F12	Dulbeccos's Modified Eagle Medium: Nutrient Mixture F-12
E2	Estrogen
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbant assay
FCS	Fetal calf serum
FRT	Female reproductive tract
FSH	Follicle stimulating hormone
HBSS	Hank's balanced salt solution
IFN	Interferon
IFNT	Interferon-tau
IL	Interleukin
IL10	Interleukin 10
IL1B	Interleukin 1B
ISG15	Interferon-stimulated gene-15
ISGs	Interferon-stimulated genes
MRP	Maternal recognition of pregnancy
MHC	Major hiscompatibility complex
Mo	Monocyte
MX2	Myxovirus resistance 2
МΦ	Macrophage
NFkB	Nuclear factor kappa B
NK	Natural killer cell

OAS1	2′-5′ oligoadenylatesynthetase 1
P4	Progesterone
PAG	Pregnancy associated glycoprotein
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PGE2	Prostaglandin
PGF2α	Prostaglandin F-2alpha
PL	Placental lactogens
PMN	Polymorpho nuclear neutrophil
PTGES	Prostaglandin E synthase
PTGES	Prostaglandin E synthase
rbIFNT	Recombinant interferon-tau
Real-time PCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
RPMI-1640	Roswell Park Memorial Institute-1640
SOV	Superovulation
TGFB	Tissue growth factor beta
Th	helper T cells
TNFA	Tumor necrosis factor apha
Treg	T regulatory cells
UF	Uterine flush

General Introduction

Successful pregnancy establishment is the prerequisite for high reproductive performance in dairy cattle. It requires good quality of viable embryo, maternal recognition signal for its existence and an immunologically receptive uterus. The developing bovine embryo after successful fertilization needs to start a bio-molecular dialogue with the maternal immune system to set up a modified immune response to the semi-allogenic conceptus. The maternal immune system is very important for embryo-maternal crosstalk during pregnancy and indeed disrupt immune response may lead to the early embryonic loss. Since bovine implantation is non-invasive and the embryo is to be suspended on uterine fluid for a long time until implantation start, bioactive molecules secreted either from embryo or uterus play a crucial role for embryo survival and acceptance in the uterus. Interferon-tau (IFNT), a trophectoderm derived embryonic signal molecule, is to be considered as one of the main factors in embryo-uterus crosstalk resulting rescue of corpus luteum (CL) from lysis and modulation of the uterine immune environment to accept a semi-allogenic embryo.

1. Early embryo development and implantation in cattle

The early bovine embryo develops in the female reproductive tract (FRT) through a series of biological events and establishes pregnancy by initiating bio-molecular dialogue with the mother. After fertilization, one cell embryo undergoes mitotic division and form developing morulae that pass from oviduct to uterus around Day-4 to 5 and developed to spherical shaped blastocyst by Day-7. The blastocyst then hatched from protective zona pellucida on Day-9 to 10 and starts to elongate and turn into a filamentous conceptus that occupies the entire length of the ipsilateral uterine horn up to Day-19 [1, 2] (Fig. 1-1). During this time frame embryo has to depend solely on the maternal secretions (i.e. histotroph) for its survival [3]. Unlike human and mice species, bovine implantation is non-invasive and is

furnished by a superficial adhesion of the trophectoderm to caruncular and intercaruncular space, commencing on Day-19 [4].

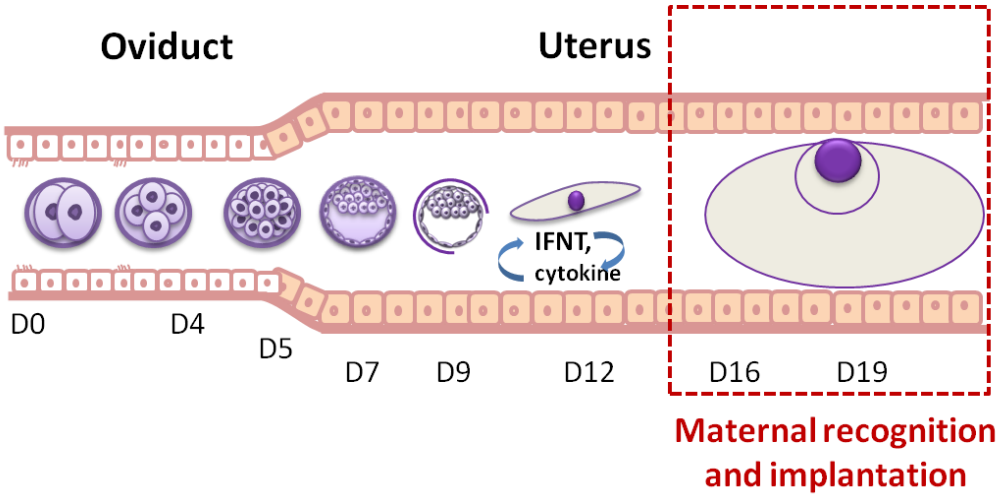

Fig. 1-1. Illustration showing the embryo development and implantation in the female reproductive tract during early pregnancy in cattle.

2. Immunogenic aspect of embryo

Well-furnished mucosal immune system in the female reproductive tract (FRT) regulates the local immunity through the release of several molecules which influence the functions of underlying resident immune cells and thereby provide immunological protection against foreign particles. Though embryo develops in the FRT as an independent living entity it is not immunologically analogical to the mother. It is a semi-allogenic entity as half of the genetic material is coming from allogenic father's sperm. Moreover, on Day-7 of pregnancy bovine blastocyst expresses the paternal antigen of MHC molecule I [5, 6]. Thus embryo may suffer from maternal immune attack and could be one of the main reasons for the early embryonic loss. In bovine, although fertilization rate is very high at around 90-95% calving rate is almost half (40-55%) and about 35-50% embryo has been lost of which major embryonic loss occur between 8-16 days after insemination [7] (Fig. 1-2).

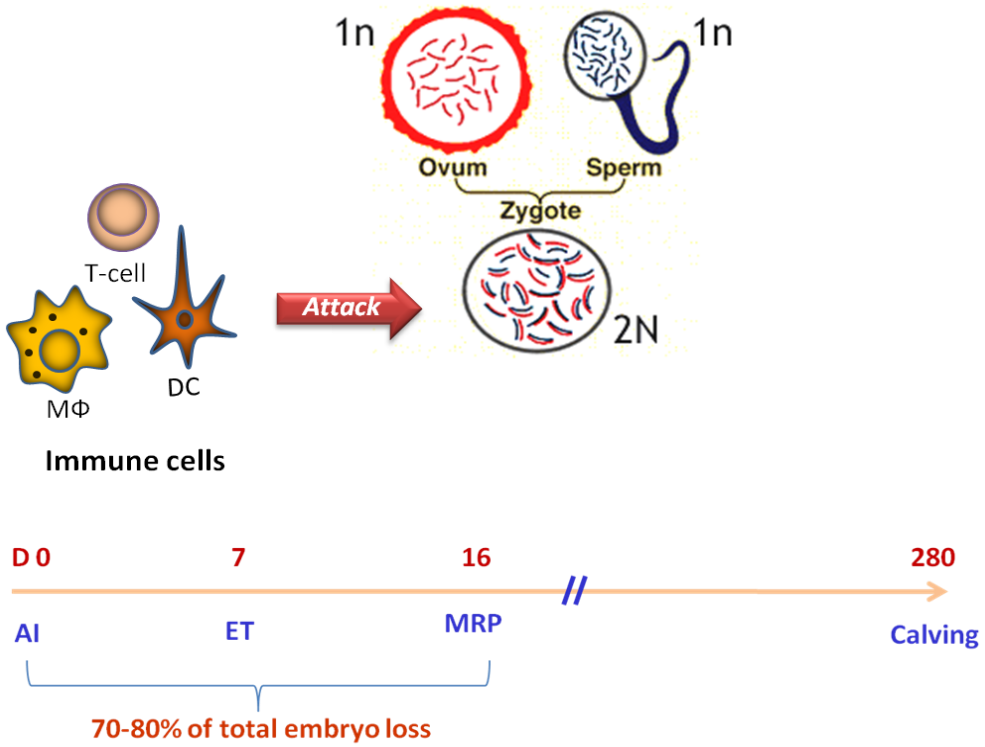

Fig. 1-2. Illustration showing the semi-allogenocity of embryo and rate of early embryonic loss during pregnancy in cattle.

3. Embryonic signals and maternal recognition in cattle

During the journey after fertilization to until before implantation, bovine early embryo has to face several major hurdles to survive i) giving the signal of it's existence to the mother, ii) avoiding attack by maternal immune systems and iii) inducing endometrial remodeling and angiogenesis for further development. Embryo releases a number of bioactive signaling molecules including interferon-tau (IFNT), pregnancy-associated glycoprotein (PAG), placental lactogens (PL) and prostaglandins E2 and $F_2\alpha$ (PGE2 & $PGF_2\alpha$) that start to communicate with the maternal part. Until now, IFNT previously known as trophoblastin, is a trophectoderm-derived unique cytokine that regarded as a molecule responsible for the process of maternal recognition of pregnancy (MRP) in ruminant ungulates [8, 9, 10). IFNT start to secrets from the functional trophoblast cells of the embryo around Day-8 of pregnancy and goes up thereafter along with the elongation of the conceptus. IFNT

production level touches the peak on Day-16 of pregnancy just before implantation start and then goes down rapidly as implantation process proceeds (Fig. 1-3). IFNT is no longer appears after complete attachment of the trophoblast with the maternal endometrium around by Day-22 of pregnancy [11, 12]. IFNT indirectly inhibits the pulsatile release of prostaglandin F_2 alpha from uterine endometrium into local and systemic circulation, thereby prevents corpus luteum (CL) from regression and maintains progesterone secretion necessary for pregnancy [9, 10]. Evidence suggests that the function of IFNT is not only limited to prevent luteolysis but to generate a suitable uterine immune condition for conceptus development and acceptance. It is also known to induce the expression of interferon-stimulated genes (*ISGs*) in the endometrium [13], CL [14] and peripheral blood mononuclear cells (PBMCs) [15, 16] during early pregnancy in cows. Recently, it was observed that a single Day-7 bovine blastocyst induces *ISGs* very locally in the anterior one-third of uterine horn [17]. At the same time, our laboratory demonstrated *in vitro* that Day-7 bovine embryos communicate with the uterine epithelial cells and also with immune cells, mediated in part by IFNT [18]. The findings from these two studies strongly suggest that Day-7 embryo starts to crosstalk locally with the uterus and possibly with immune cells *in vivo*.

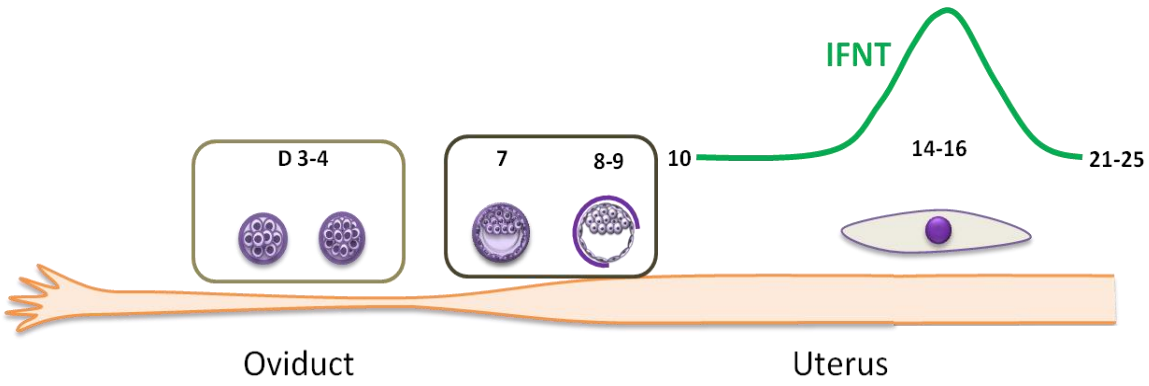

Fig. 1-3. Illustration showing the pattern of interferon-tau (IFNT) release during early pregnancy in cattle.

4. Immune cells in the bovine uterus

PMNs appear transiently in the uterine lumen during the follicular phase of the estrus cycle as predominant phagocytic cells. Other than estrus period, PMNs availability in the uterine lumen is treated as pathological [19].

Lymphocyte especially helper T lymphocytes (CD4+), B lymphocyte (CD21+) and macrophage (CD14+) are present in the sub-epithelial stroma of bovine endometrium [20]. The cytotoxic T lymphocytes (CD8+) are located exclusively in the luminal and glandular epithelium, as well as in the stroma immediately adjacent to the epithelium [21]. Macrophages (CD14+) and dendritic cells (CD11c+) has been identified in the endometrial stroma during early pregnancy [22]. Bovine natural killer (NK) cell has also recently discovered in the uterus but their role is still unclear [23]. There is no evidence regarding the presence of antigen presenting cells other than macrophages (Fig. 1-4).

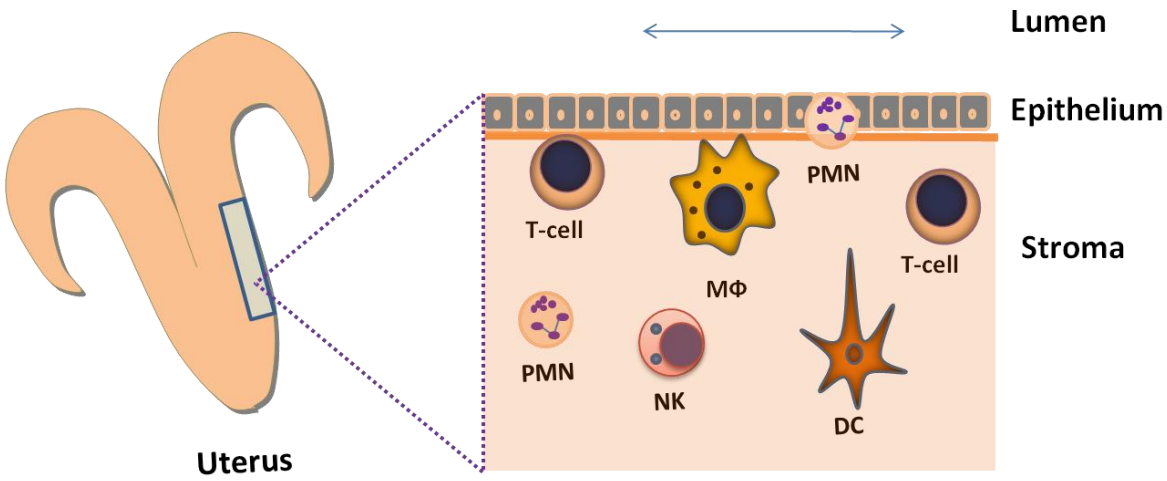

Fig. 1-4. Illustration showing the different types of immune cells in the uterus. T-cell= T-lymphocyte, DC= dendritic cell, MΦ = macrophage, NK= natural killer cell, PMN=polymorpho nuclear neutrophil; PMNs migrate into the lumen of the FRT during pre-ovulatory/estrus period in cows.

5. Response of maternal immune cells to early pregnancy in cattle

The maternal immune system needs to be strictly-controlled during pregnancy to support the embryo as well as at the same time provide protection against the pathogen. The immune cells located at the feto-maternal interface may provide tolerance to the embryo as well act as surveillance cells to protect infection. Till now, the mechanism on how the maternal immune cells respond to the semi-allogenic embryo is completely unknown. Evidence suggests that early pregnancy modulates the local uterine immune cells response as well as extra-uterine tissue including peripheral blood cells in cattle. It is observed that pregnancy initiate expansion of monocyte (Mo), CD14+-cells (MΦ), and CD172a–CD11c+ n(DC) populations in the endometrium on Day-13 of pregnancy in cattle [24], which might play an essential role for pregnancy establishment. A recent study in dairy heifer demonstrated that conceptus increases myeloid lineage cells (macrophages and DCs) population with a tolerogenic phenotype in the endometrium on Day-17 of pregnancy which may play a pivotal role for tolerance of a semi-allogenic embryo [22]. A reduced number of CD45+ cells with up-regulated IDO expression in the endometrium has also reported for rescuing the semi-allogenic conceptus from maternal rejection on Day-18 of pregnancy in the bovine [25]. Moreover, transcript of the endometrial genes revealed that Th1 cytokine (*IL1B*) was suppressed, while Th2 cytokine (*IL10*) was induced on Day-13 to16 of pregnancy in cattle [26].

In addition to the local uterine immune response, conceptus secretion also reported to induce sensing about embryo in extra-uterine tissue including peripheral blood cells (PBL) and the corpus luteum [27]. It has been shown that classical interferon-stimulated gene (*ISGs*) significantly expressed in peripheral immune cells [28, 29] and CL [14] in response to conceptus IFNT during early pregnancy (Day-18) in cattle. Although *ISGs* expression

activated in peripheral blood immune cells, the role of IFNT in terms of immune-modulation is still remaining unanswered. Even though it was observed that Th2 cytokines (*IL10*) were up-regulated with activation *ISGs* expression in PBMC on Day-8 of pregnancy in cow [15], suggesting that circulatory immune cell can recognize the early embryo existence in the uterus and thus generates a Th2 immune response.

6. Interferon-tau (IFNT) and immune regulation

Interferon-tau, an embryo derived unique cytokine is reported to as an immune suppressive molecule that inhibits lymphocytes proliferation in culture and thus may play a pivotal role for the protection of semi-allogenic embryo from maternal immune attack [30]. In ovine endometrium, it regulates recruitment and distribution of immune cells through activation of the IP-10 protein [31]. A recent study showed that IFNT plays an anti-inflammatory role in *Staphylococcus aureus*-induced endometritis in mice, through suppression of the nuclear factor kappa B (NFkB) pathway and inhibition of TNFA and IL1B production [32]. It was reported that IFNT induced IL10 secretion in a dose-dependent manner that thereby suppressed the secretion of IL1B in human THP-1 macrophages [33]. Since uterine immune cells [20], as well as circulatory immune cells [28], responded to conceptus-signal, IFNT during early pregnancy in cow, there might be IFNT mediated immune regulation exists in the uterus during early pregnancy. Till now, there is little or no information regarding direct evidence of IFNT involvement in the immune modulation during early pregnancy in the cow. Thus, It could be hypothesized that IFNT released from the early embryo in the uterine fluid may involve in the modulation of the local immune environment in the uterus.

7. Objectives of the study

Being immunologically foreign, the semi-allogenic embryo escapes from attack by the maternal immune systems and establishes pregnancy in the mother uterus. The bio-molecular mechanism by which embryo avoids the maternal immune attack is nearly unknown. Recent studies from ours and others suggest that Day-7 bovine embryo starts to communicate with the uterine epithelium through interferon-tau (IFNT) signaling. However, the immune-modulatory role of IFNT in the uterus just after the embryo moves from the oviduct is unclear. I hypothesized that Day-7 embryo secretes very small amount of IFNT in the uterus which then communicates locally with the uterine epithelium for generation of an anti-inflammatory response in immune cells (Fig. 1-5). It is, however, difficult to collect a number of local immune cells from the uterus for molecular analysis, so that an interaction between the embryo, IFNT, and immune cells has not yet been investigated.

Therefore, I used both *in vivo* and *in vitro* models;

(i). To investigate the effect of uterine flush (UF) from Day-7 pregnant cow with multiple embryos (*in vivo*) on interferon-stimulated genes (*ISGs)* as well as immune-related genes in peripheral blood mononuclear cells (PBMCs) and to further examined on whether or not IFNT directly regulated this immune-related gene expression in PBMCs through the use of bovine uterine epithelial cells (*in vitro*).

(ii). To observe the impact of IFNT (100 pg/ml) infusion in the uterus of Day-8 cyclic cow for 24 h (*in vivo*) on the immune-related gene expressions in PBMCs.

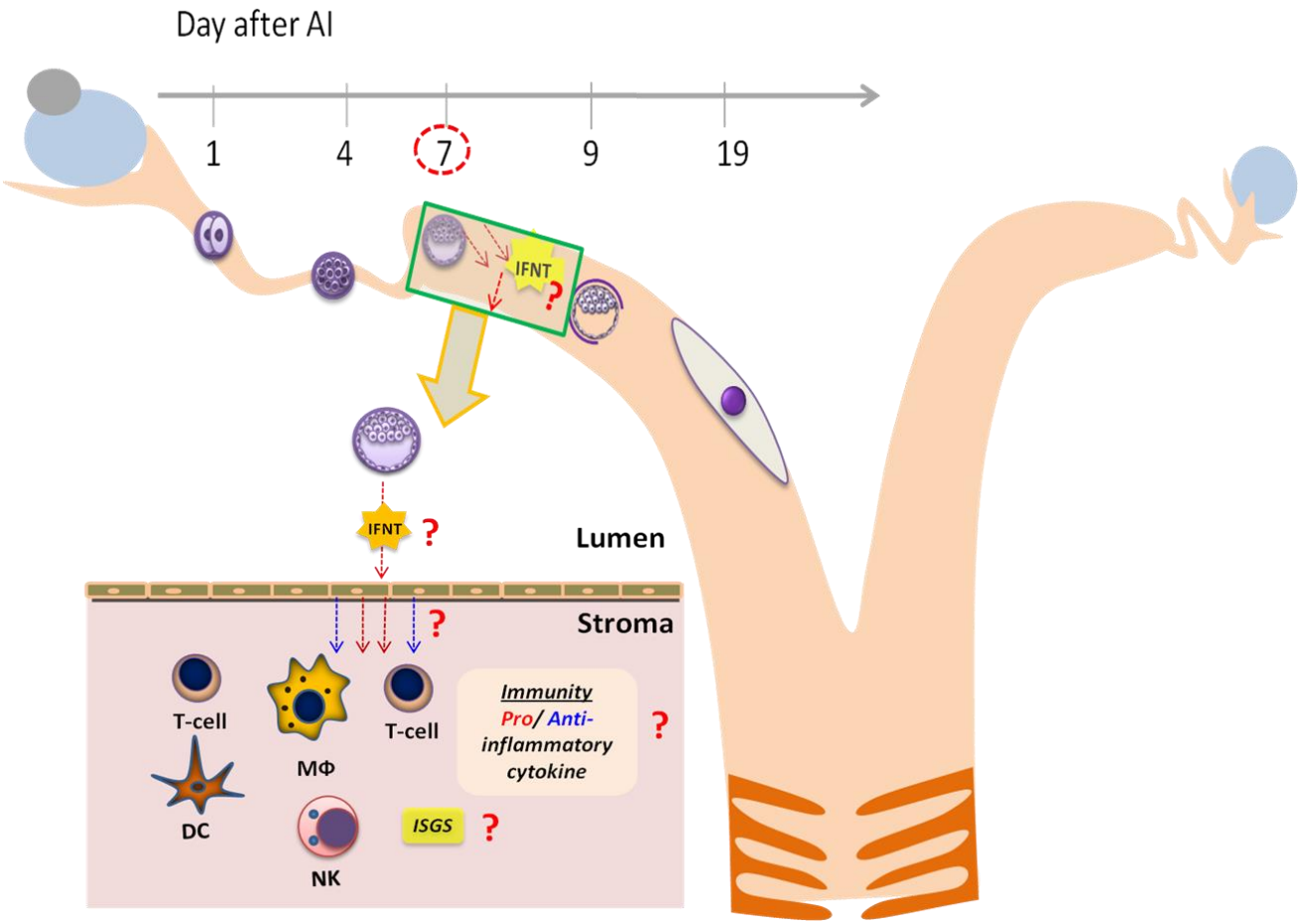

Fig. 1-5. Illustration showing the possible local immune regulation by IFNT secreted from Day-7 bovine embryo in the uterus.

Chapter I

Evidence that interferon-tau secreted from Day-7 embryo *in vivo* generates anti-inflammatory immune response in the bovine uterus

1. Introduction

Interferon-tau (IFNT), a trophectoderm-derived cytokine, is regarded as a molecule responsible for the process of maternal recognition of pregnancy (MRP) in ruminant ungulates. During MRP process, IFNT indirectly inhibits the pulsatile release of prostaglandin F2 alpha from uterine endometrium into local and systemic circulation, thereby prevents corpus luteum (CL) from regression and maintains progesterone secretion necessary for pregnancy [9, 10]. IFNT is also known to induce the expression of interferon-stimulated genes (*ISGs*) in the endometrium [13], CL [14] and peripheral blood mononuclear cells (PBMCs) [15] during early pregnancy in cows. Recently, it was observed that a single Day-7 bovine blastocyst induces *ISGs* very locally in the anterior one-third of uterine horn [17]. At the same time, our laboratory demonstrated *in vitro* that Day-7 bovine embryos communicate with the uterine epithelial cells and also with immune cells, mediated in part by IFNT [18]. The findings from these two studies strongly suggest that Day-7 embryo starts to crosstalk locally with the uterus and possibly with immune cells *in vivo*.

Immune cells especially T lymphocytes, B lymphocytes, macrophage and dendritic cells have been identified in the bovine uterus [20]. Several studies investigated the immunological interaction between embryo and endometrium during peri-implantation period particularly on Day-16 and 17 [13, 34], but not in the earlier stage (Day-7) of pregnancy in cows. It is, however, difficult to collect a number of local immune cells from the uterus for molecular analysis, so that an interaction between embryo, IFNT, and immune cells has not yet been investigated.

It is reported that Day-7 bovine embryo expresses the paternal antigen of MHC molecule I [5, 6], thus there is a possibility of immunological rejection of the Day-7

blastocyst by the uterine immune system in cows. Importantly, IFNT is also regarded as an immunosuppressive molecule that inhibits lymphocytes proliferation and thus may play a pivotal role to protect the embryo from maternal immune attack [30]. I hypothesized that Day-7 embryo secretes very small amount of IFNT in the uterus which then communicates locally with the uterine epithelium for generation of an anti-inflammatory response in immune cells.

Therefore, I aimed to investigate the effect of uterine flush (UF) from superovulated cows at Day-7 after artificial insemination (donor cows of embryo transfer program) on the expression of interferon-stimulated genes (*ISGs*) as well as immune-related genes in PBMCs. Because bovine embryos secrete many factors other than IFNT, which affect uterine gene expression and contents of UF, and because IFNT or other embryo secreted factors directly act on PBMCs and also on uterine epithelial cells, I further examined on whether or not IFNT directly regulated various gene expression in PBMCs through the use of bovine uterine epithelial cells (BUECs).

2. Materials and Methods

2.1. Ethics statement

All animal experiments were conducted under the approval of Animal Experiments Ethics Committee, Obihiro University of Agriculture and Veterinary Medicine, Japan (Permit number 25–101) and Animal Ethics Committee, Animal Research Center, Hokkaido Research Organization, Japan.

2.2. Experimental model

I used both *in vivo* and *in vitro* studies (Fig. 2-1) to investigate the role of IFNT on gene expression in immune cells. In *in vivo* study (Fig. 2-1a), cows were superovulated by

using routine hormonal treatment for superovulation regimen of embryo transfer program. After estrus (Day-0), cows were either inseminated (n=12; pregnant group) or remained non-inseminated (n=5; control group). On Day-7, the first UF was collected (20-25 ml) and pregnancy was confirmed with presence of multiple embryos in UF. In *in vitro* study **(**Fig. 2-1b**)**, BUECs after first passage were stimulated with IFNT (100 pg/ml) for 24 h and conditioned media (CM) was collected (IFNT-stimulated CM). CM from BUECs without IFNT stimulation served as controls and CM supplemented with IFNT (100 pg/ml) served as recombinant IFNT-CM. Subsequently, PBMCs cultured in UF (*in vivo*), IFNT-stimulated CM (*in vitro*, indirect) or CM supplemented with IFNT (*in vitro*, direct) was analyzed for their gene expression.

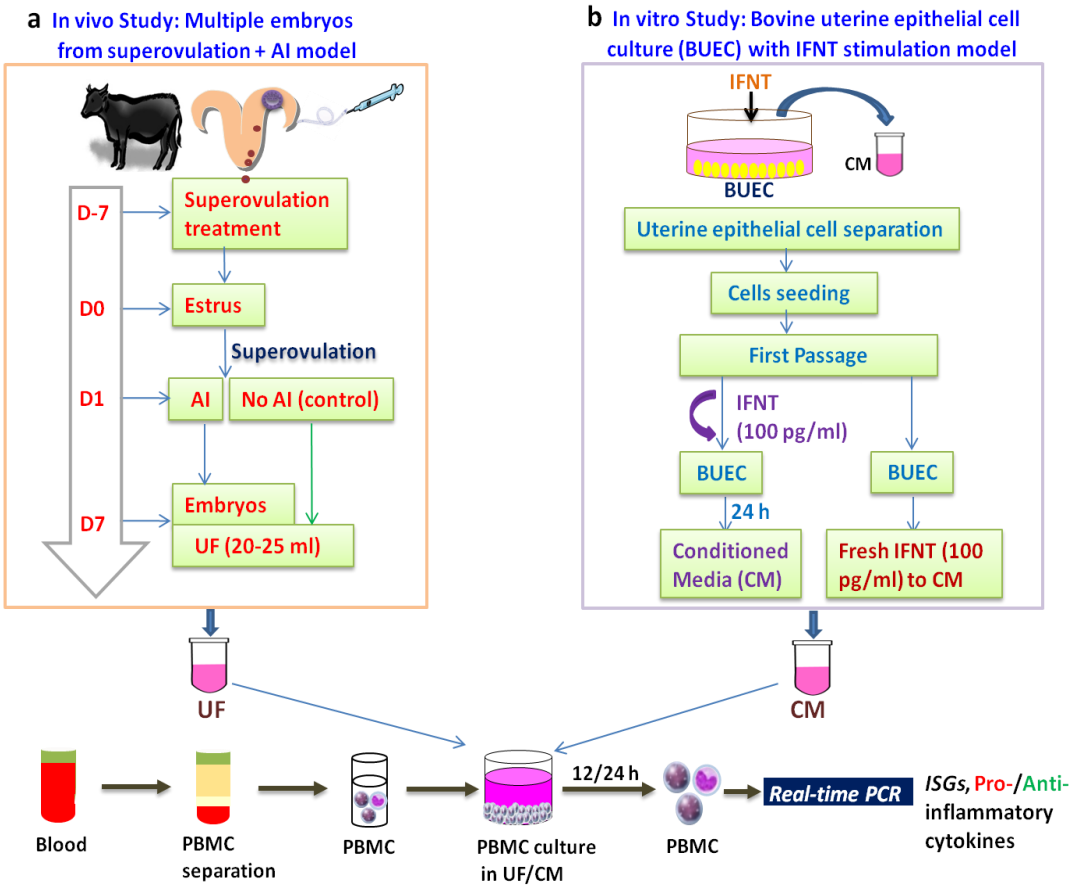

Fig. 2- 1. Schematic representation of the experimental model; (**a**) Cows was superovulated by using routine hormonal treatment for superovulation regimen. After estrus (Day-0), cows were either inseminated (n=12; pregnant group) or remained non-inseminated (n=5; control group). On Day-7, embryos were flushed out and

first UF was collected (20-25 ml). (**b**) BUECs after first passage were stimulated with IFNT (100 pg/ml) for 24 h and conditioned media (CM) was collected (IFNT-stimulated CM). CM from BUECs without IFNT stimulation served as control and CM supplemented with IFNT (100 pg/ml) served as recombinant IFNT-CM. Subsequently, PBMCs were cultured in UF or CM with or without IFNT from all groups and gene expressions were analyzed.

2.3. In vivo study: [Multiple embryos from superovulation + AI model]

2.3.1. Induction of superovulation (SOV) in cow

Reproductively normal multiparous Japanese Black Cows (Wagyu, n=23; average body weight 400±15 kg) were randomly selected in a commercial dairy herd (Nobel's Co. Ltd, Obihiro, Japan) and Animal research center (Shintoku, Hokkaido, Japan) for carrying out the experiment. All the cows were provided with balanced ration and ad-libidum water supply throughout the whole experimental period. Induction of superovulation (SOV) was done as previously described [35] with minor modifications. Superovulation protocol was started at 7 days before of standing estrus. Controlled internal drug release devices (CIDR 1900, Pfizer Japan, Tokyo, Japan) were inserted into the vagina along with single intramuscular injection of 0.5 mg of estradiol benzoate (EB, Kyoritsu seiyaku, Tokyo, Japan). Three days after, in total 20 IU of follicle-stimulating hormone (FSH) (AntrinR-10, Kyoritsu Seiyaku, Tokyo, Japan) was intramuscularly administered to each cow twice daily in decreasing doses over 3 days. The CIDR was removed 48 h after initiation of FSH treatment, and prostaglandin F2α (cloprostenol 0.5 mg/cow, Estrumate [SP] 20, Intervet, Tokyo, Japan) was intramuscularly injected to induce luteolysis. After onset of estrus, a single GnRH (100 µg fertirelin acetate/cow, Consulean, Aska Animal Health, Tokyo, Japan) injection was given intramuscularly with or after AI to ensure ovulation.

2.3.2. Collection of uterine flush (UF) on Day-7

All normal multiparous Japanese Black cows were brought into superovulated condition by widely using hormonal induced super-ovulation protocol as describe above. Cows were monitored for onset of estrus (standing heat) three times per day 30 minute each time. After onset of estrus (Day-0) cows were either inseminated with frozen thawed semen from the same batch of bull of proven fertility (n=18 for Day-7 of pregnancy) or remained non-inseminated and served as controls (n=5 for Day-7 superovulated cyclic). Ovulation was confirmed using a trans-rectal ultrasonic device equipped with a 5.0-MHz linear transducer (HS-101V, Honda Electronics, Toyohashi, Japan). On Day-7 embryos were flushed out non-surgically by using lactic acid containing Ringer solution (SOLULACT 1000 ml, Terumo Corp., Tokyo, JAPAN) supplemented with 3% modified PBS (Embryotech, Zenoaq, Koriyama, Japan). An epidural injection (5 ml procaine hydrochloride) was given to the donor cow to relax the bowel for easy palpation. After washing the perineum with lactacyd soap (Sanofi-aventis, Paris, France) and drying with paper towels soaked with alcohol, a balloon catheters multieyes type (Fujihira Industries, Tokyo, Japan) was inserted into the vagina, through the cervix until the horn of the uterus. An inflatable cuff on the catheter was filled with air to hold the catheter in place and flushing fluid (30-50 ml) was run into the uterine horn. Uterine flush was then run back through the catheter along with very fine filter (filter used to catch the embryos). First part of aspirating uterine flush (20-25 ml) with embryos was collected. UF was then centrifuged at 1000 g for 15 minutes and stored at -80 °C for further experimental use. Embryos were examined under a stereo-microscope (Fig. 2-2). Number of recovered embryos per cow was 9.5 ± 1.4 (Mean ± SEM). UF was similarly collected from control cows on Day-7. Cows were re-grouped on Day-7 after evaluation of embryo recovered into the following study group: pregnant (n=12) and control (n=5). As

expected, some inseminated cow were excluded (lacked embryo or inappropriate size and number of embryo) from further analysis.

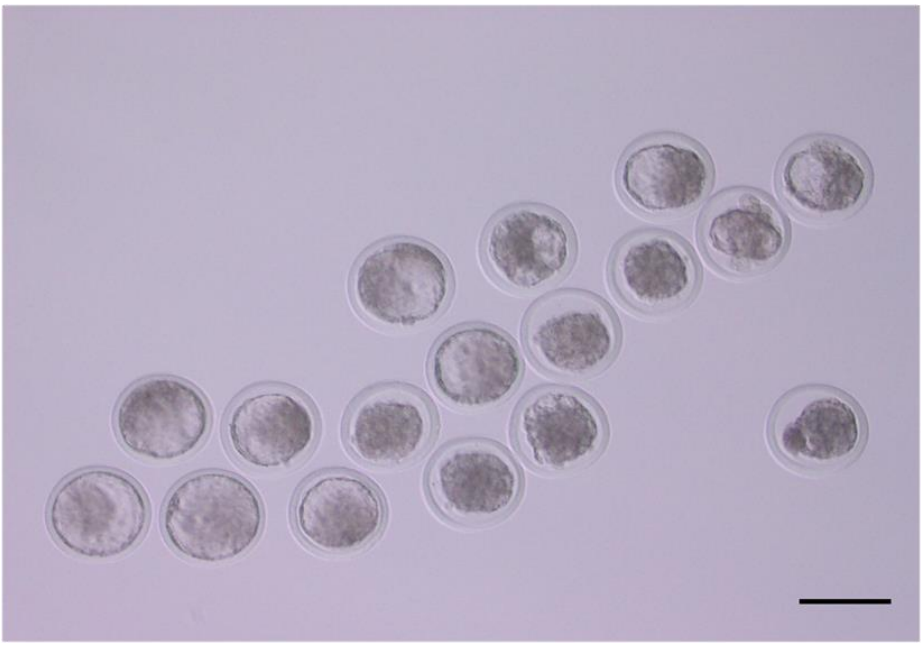

Fig. 2-2. Microphotograph of bovine morulae and blastocysts collected by uterine flushing on Day-7 of pregnancy. Scale bar represents 200 µm.

2.3.3. Isolation of PBMCs

PBMCs were isolated as previously described [15] with minor modifications. Blood was collected during early luteal phase in heparinized blood tube (20 ml) and mixed with an equal volume of PBS-/-, slowly layered over Ficoll-paque solution (Lymphoprep, Axis Shield, Norway), and centrifuged at 1000 x *g* for 35 min at 10 °C. The white buffy coat was collected as PBMCs, mixed with twice volume of hemolysis buffer (NH_4Cl 155 mM, $KHCO_3$ 9.9 mM, EDTA 96.7µM) for 3 minutes, and centrifuged at 500 x *g* for 5 min at 10 °C to remove red blood cells. After centrifugation, the cell pellet was washed twice with PBS-/-. The purity of PBMCs as evaluated by flow cytometry was >98% and the viability as assessed by Trypan blue staining was 98%.

2.3.4. Culture of PBMCs in UF

PBMCs (5×10^6 cells) were cultured in a 12-well plate (Nalge Nunc International) in 1ml UF from Day-7 pregnant cows or in UF from non-pregnant cows (control) for 12 h in a humidified atmosphere at 38.5 ℃ in 5% CO_2. UF of all cases was supplemented with 0.1% FCS (Bio Whittaker, Walkersville, MD). After 12 h of incubation, the cells were collected, lysed using Trizol (Thermo Fisher Scientific, Waltham, MA), and stored at -80 °C until RNA extraction.

2.3.5. Neutralization of IFNT in UF from pregnant cows using anti-bovine IFNT antibody (anti-bIFNT Ab)

First, UF from pregnant cow was incubated in 48-well plate (Nalge Nunc International) in the presence of anti-bIFNT Ab (1:300, Eurofins Genomics, Tokyo, Japan) which was confirmed the specificity to IFNT but not interferon-alpha (IFNA) for 1 h in a humidified atmosphere at 38.5 ℃ in 5% CO_2. PBMCs (5×10^6 cells) were then added to that media and cultured for 12 h. PBMCs cultured in UF (control) from non-pregnant cows and in UF from pregnant cow without anti-bIFNT Ab served as negative and positive controls, respectively. At the end of experiments, PBMCs were collected, lysed using Trizol (Thermo Fisher Scientific), and stored at -80 °C until RNA extraction. Anti-bIFNT Ab (1:300) dose was chosen on the basis of preliminary experiments, where different dilutions of anti-bIFNT Ab (1:300, 500, 1000 and 2000) were used to block the effect of IFNT (known dose 50 pg/ml) on gene expressions in PBMCs (data not shown).

2.4. In vitro study: [Bovine uterine epithelial cell culture with IFNT stimulation model]

2.4.1. Culture of BUECs

Bovine uterine epithelia cells (BUECs) were collected and cultured as previously described [18] with minor modifications. Briefly, uterus at luteal phase (Day7-9) were collected from a local slaughterhouse (Hokkaido Livestock Co., Doto Plant Tokachi Factory; Obihiro, Hokkaido, Japan) and transported to the laboratory in physiological saline containing 1% penicillin-streptomycin (Gibco, Grand Island, NY, USA) and 1% amphotericin B (Gibco). The uterine horn, ipsilateral to the corpus luteum, was used for isolation and culture of epithelial cells. Epithelial cells were separated from the endometrium of the uterine horn using enzymatic cell separation protocol. Thereafter, cells were cultured in DMEM/F12 (Gibco) supplemented with 2.2% $NaHCO_3$ (Sigma-Aldrich, Steinheim, Germany), 1% penicillin-streptomycin, 1% amphotericin B and 10% FCS (Bio Whittaker, Walkersville, MD). The cells were seeded in 25 cm^2 culture flasks (Nalge Nunc International, Roskilde, Denmark) and cultured at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 48 h until growing BUECs reached to 70-80% confluence, at which point cells were given a first passage. The cells were trypsinized (0.05% trypsin EDTA; Amresco, Solon, OH, USA), re-plated in 12-well plates and cultured until sub-confluence (80-90%). The BUECs from the first passage were supplemented with 5 ng/ml progesterone (P4) (Sigma-Aldrich) and 3 pg/ml estradiol 17β (E2) (Sigma-Aldrich). The purity of the epithelial cells was evaluated by immune-fluorescence staining using a monoclonal antibody against cytokeratin (anti-cytokeratin 8+18; ab53280, Abcam, Tokyo, Japan). The purity of the cultured uterine epithelial cells was > 98%.

2.4.2. Stimulation of BUECs with recombinant bovine IFNT (rbIFNT)

The sub-confluent BUECs monolayer from first passage were washed twice and cultured in medium supplemented with 0.1% FCS (Bio Whittaker) in combination with 100 pg/ml of rbIFNT (bIFNT 2B, specific activity = 4.15×10^7 U/mg; Zenoaq, Koriyama, Japan) for 24 h in a humidified atmosphere at 38.5 °C in 5% CO_2. BUECs without IFNT treatment served as a control. The dose of IFNT (100 pg/ml) was chosen from our previous *in vitro* study [18] where a similar magnitude of *ISG15* mRNA stimulation was observed in BUECs with same dose as with the embryo. At the end of BUECs culture, the supernatant was collected and stored as CM at -80 °C for further use.

2.4.3. Culture of PBMCs in conditioned media (CM)

PBMCs (5×10^6 cells) were cultured in a 12-well plate in 1ml CM from IFNT-stimulated or not stimulated (control) BUECs culture or in CM from BUEC culture plus recombinant IFNT for 24 h in a humidified atmosphere at 38.5 °C in 5% CO_2. After 24 h of incubation, cells were collected, lysed using Trizol (Thermo Fisher Scientific) and stored at -80 °C until RNA extraction. The experiment in triplicates was performed three times independently.

2.5. RNA extraction and cDNA synthesis

RNA extraction from PBMCs using the Trizol reagent (Thermo Fisher Scientific) was performed according to the previously described protocol [36]. Extracted RNA was detected by ultraviolet (UV) spectroscopy (optical density, 260 nm) and the concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at 260 and 280 nm absorbance values. The total extracted RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at –80 °C until cDNA production. The cDNA synthesis was carried out as previously described protocol [37]. Briefly, DNase treatment step was performed using RQ1

RNase-Free DNase kit (Promega, Madison, WI, USA) to remove residual genomic DNA and other contaminations. The extracted RNA (1 μg in 7 μl) was incubated for 30 min at 37 °C with 1 unit of the 10× RQ1 RNase-free DNase reaction buffer and 2 μl of the 1 μg/μl RNase-free DNase. To terminate the reaction, 1 μl of the RQ1 DNase Stop solution (20 mM EDTA) was added to the sample, and the mixture was incubated for 10 min at 65 °C. First-strand cDNA synthesis was conducted according to the commercial protocol described in the Super Script II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The mixture was prepared using 1.5 μl of 50 ng/μl random primer (Invitrogen, Carlsbad, CA, USA), 1.5 μl of 10 mM PCR Nucleotide Mix (dNTP; Roche Diagnostics, Indianapolis, IN, USA), and 4 μl of H_2O to obtain a total volume of 18 μl per sample. This mixture was then incubated at 65 °C for 5 min in a thermal cycler (Bio-Rad, Munich, Germany). The samples were kept on ice while the second mixture, which consisted of 3 μl of 0.1M dithiothreitol (DTT, Invitrogen, Carlsbad, CA, USA), 1.5 μl of 40 units/μlRNasin Ribonuclease Inhibitor (Promega, Madison, WI, USA), and 6 μl of 5× First-Strand Buffer (Invitrogen, Carlsbad, CA, USA), was added to each tube. The samples were incubated for 2 min at 42 °C, and 0.2 μl of 200 units/μl of Super Script II Reverse Transcriptase was added to each tube. The thermal cycler was programmed at 25 °C for 10 min, 42 °C for 50 min, and then 70 °C for 15 min. The synthesized cDNA was stored at -30 °C.

2.6. Real-time PCR

Quantitative real-time PCR of specific target genes (Table 2-1) was performed using QuantiTect SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) by an iCycler iQ (Bio-Rad Laboratories, Tokyo, Japan). The amplification program was set up according to a previously described protocol [37]. Briefly, this program was run with first activation step (15 min at 9 °C), followed by 40 cycles of PCR (15 sec denaturation at 95 °C,

30 sec annealing at 55–58 °C, and 20 sec extension at 72 °C). At the end of the program, the melting curve was evaluated to check the specificity of the amplification. The cycle threshold values that are calculated after ending of this program were normalized using *B-actin* as the internal standard by applying the Delta-Delta comparative threshold method [38] to quantify the fold change between samples.

Table 2-1. List of the primers used in real-time PCR

Gene		**Sequence of nucleotide (5'→3')**	**Accession no.**	**Fragment size (bp)**
B-actin	Forward	TCACCAACTGGGACGACATG	NM_173979.3	51
	Reverse	CGTTGTAGAAGGTGTGGTGCC		
ISG15	Forward	TCTGAGGGACTCCATGACGG	NM_174366	51
	Reverse	TTCTGGGCGATGAACTGCTT		
OAS1	Forward	TAGGCCTGGAACATCAGGTC	NM_001040606.1	105
	Reverse	TTTGGTCTGGCTGGATTACC		
PTGES	Forward	AAAATGTACGTGGTGGCCGT	NM_174443.2	51
	Reverse	CTTCTTCCGCAGCCTCACTT		
TNFA	Forward	CAAAAGCATGATCCGGGATG	NM_173966.3	51
	Reverse	TTCTCGGAGAGCACCTCCTC		
IL1B	Forward	AATCGAAGAAAGGCCCGTCT	NM_174093.1	51
	Reverse	ATATCCTGGCCACCTCGAAA		
TGFB1	Forward	CTGCTGAGGCTCAAGTTAAAAGTG	NM_001166068.1	90
	Reverse	CAGCCGGTTGCTGAGGTAG		
IL10	Forward	GAGATGCGAGCACCCTGTCT	NM_174088.1	51
	Reverse	GGCTGGTTGGCAAGTGGATA		

2.7. Statistical analysis

Data are presented as Mean ± SEM. Statistical analysis was performed using SPSS software version 14.0 (SPSS Inc., Chicago, USA). Student's t- test was applied for two groups and one-way ANOVA for more than two groups. *P* values <0.05 were considered to be statistically significant.

3. Results

3.1. Uterine flush (UF) from Day-7 of pregnant cows stimulated interferon-stimulated genes (ISGs) and influenced immune gene expression towards anti-inflammatory condition in PBMCs

I investigated whether UF from pregnant cows induced *ISGs* as well as immune gene expression in PBMCs. As compared with UF from non-pregnant cows, UF from pregnant cows induced *ISGs (ISG15, OAS1*), down-regulated pro-inflammatory cytokines (*TNFA, IL1B)* and up-regulated anti-inflammatory cytokine (*IL10)* expression in PBMCs (Fig. 2-3). These results indicated that activation of *ISGs* in PBMCs could have been induced by the embryo-derived IFNT present in UF from pregnant cows. In addition, changes of other cytokines expressions suggest that Day-7 embryo generates an anti-inflammatory response in immune cells of the uterus in cows.

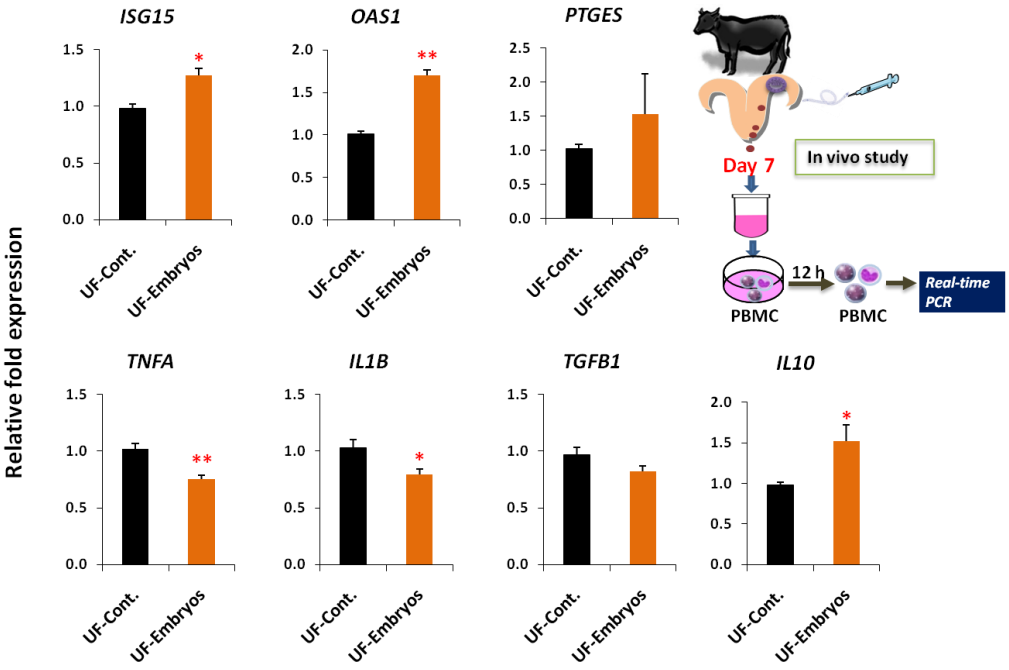

Fig. 2-3. Uterine flush (UF) from pregnant cows stimulated interferon-stimulated genes (*ISGs*) and influenced immune gene expression towards anti-inflammatory condition in PBMCs. UF from non-pregnant cows served as the control. Relative mRNA expression of interferon-stimulated genes (*ISGs*; *ISG15*, *OAS1*), an enzyme

involved in prostaglandin E synthesis (*PTGES*), pro-inflammatory cytokines (*TNFA*, *IL1B*) and anti-inflammatory cytokines (*TGFB1*, *IL10*). Data are presented as Mean ± SEM. Asterisks denote significant difference: * $P<0.05$, ** $P<0.01$ when compared to the control.

3.2. Neutralization of UF from pregnant cows using anti-bovine IFNT antibody blocked changes in gene expression in PBMCs

Antibody neutralization of the UF from pregnant cows using an anti-bovine IFNT antibody blocked the increase in interferon-stimulated gene (*ISG15*, *OAS1)* in PBMCs. After neutralization of IFNT, UF did not suppress pro-inflammatory cytokines (*TNFA*, *IL1B*), nor did it stimulate *IL10* (Fig. 2-4). These results indicated the IFNT from Day-7 embryos was involved in the regulation of PBMCs gene expression.

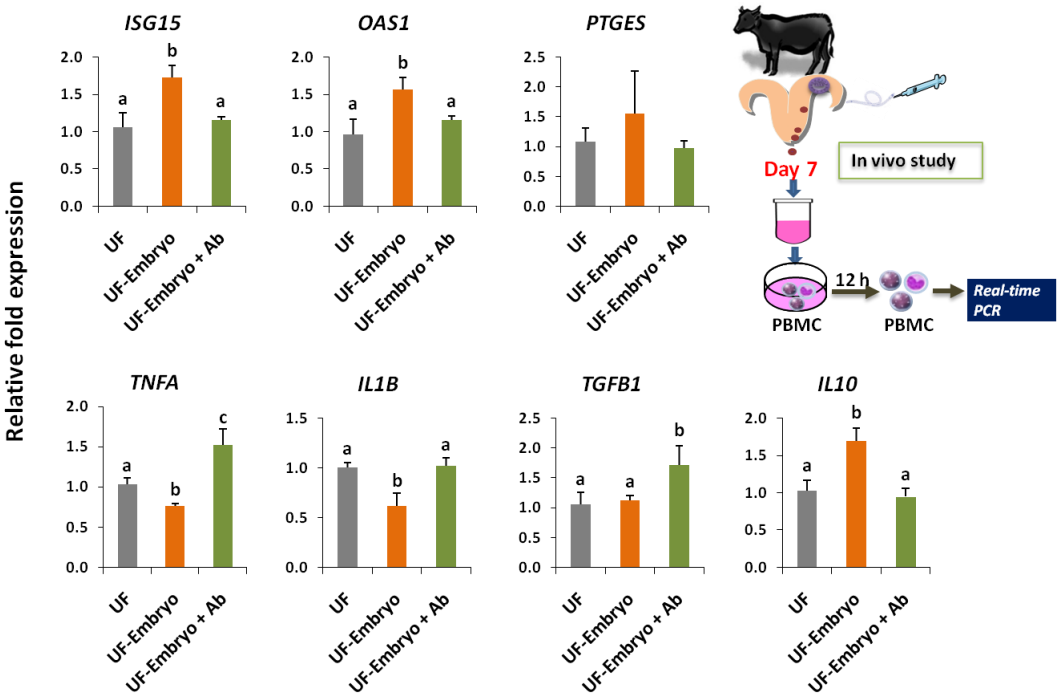

Fig. 2-4. Antibody neutralization of UF from pregnant cows blocked changes in gene expression in PBMCs. UF from non-pregnant cows served as the control and UF from pregnant cows without antibody neutralization served as positive control. Relative mRNA expression of interferon-stimulated genes (*ISGs*; *ISG15*, *OAS1*), an enzyme involved in prostaglandin E synthesis (*PTGES*), pro-inflammatory cytokines (*TNFA*, *IL1B*) and anti-inflammatory cytokines (*TGFB1*, *IL10*). Data are presented as Mean ± SEM. Different letters denote significant difference at $P<0.05$ level, when compared to the control.

3.3. Conditioned media (CM) from IFNT-stimulated BUEC culture (in vitro) regulated immune gene expression towards anti-inflammatory condition in PBMCs

To confirm the direct action of IFNT in the effect on immune cells gene expression, I incubated PBMCs in CM from IFNT-stimulated BUECs culture. CM from IFNT-stimulated BUECs culture down-regulated pro-inflammatory cytokines (*TNFA*, *IL1B*) and up-regulated anti-inflammatory cytokine (*TGFB1*, *IL10*) expression along with stimulation of *ISGs* in PBMCs as compared with controls CM (Fig. 2-5). These *in vitro* results revealed the similar response of PBMCs to those by UF from Day-7 pregnant cows.

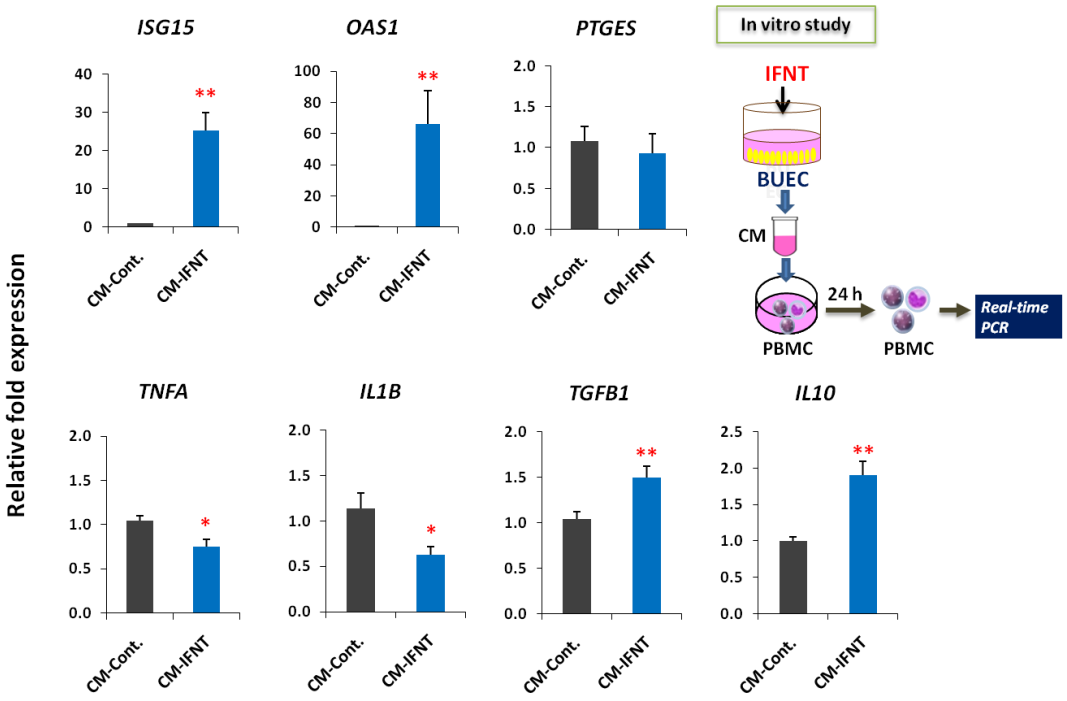

Fig. 2-5. Conditioned media (CM) from IFNT stimulated BUECs culture (*in vitro*) modulated immune gene expression towards anti-inflammatory condition in PBMCs. CM from BUEC culture without IFNT stimulation served as the control. Relative mRNA expression of interferon-stimulated genes, *PTGES*, pro-inflammatory cytokines and anti-inflammatory cytokines. Data are presented as Mean ± SEM of three independent experiments performed in triplicates. Asterisks denote significant difference: * $P<0.05$, ** $P<0.01$ when compared to the controls.

3.4. Addition of recombinant IFNT to CM (control) induced similar immune gene expression in PBMCs as did by IFNT-stimulated BUEC-CM

I investigated whether IFNT alone given in CM (control) directly regulates PBMCs gene expressions compared with those by IFNT-treated BUEC-CM. Unlike IFNT-treated BUEC-CM, a recombinant IFNT alone suppressed *TNFA* and stimulated *IL10*, however, it did not suppress *IL1B* and stimulate *TGFB1* (Fig. 2-6). These results indicated that IFNT acts as one of the major factors for regulation of PBMCs gene expressions, but some factor(s) from BUECs stimulated by IFNT might also be involved in this immunological crosstalk between the embryos and immune cells in the uterus.

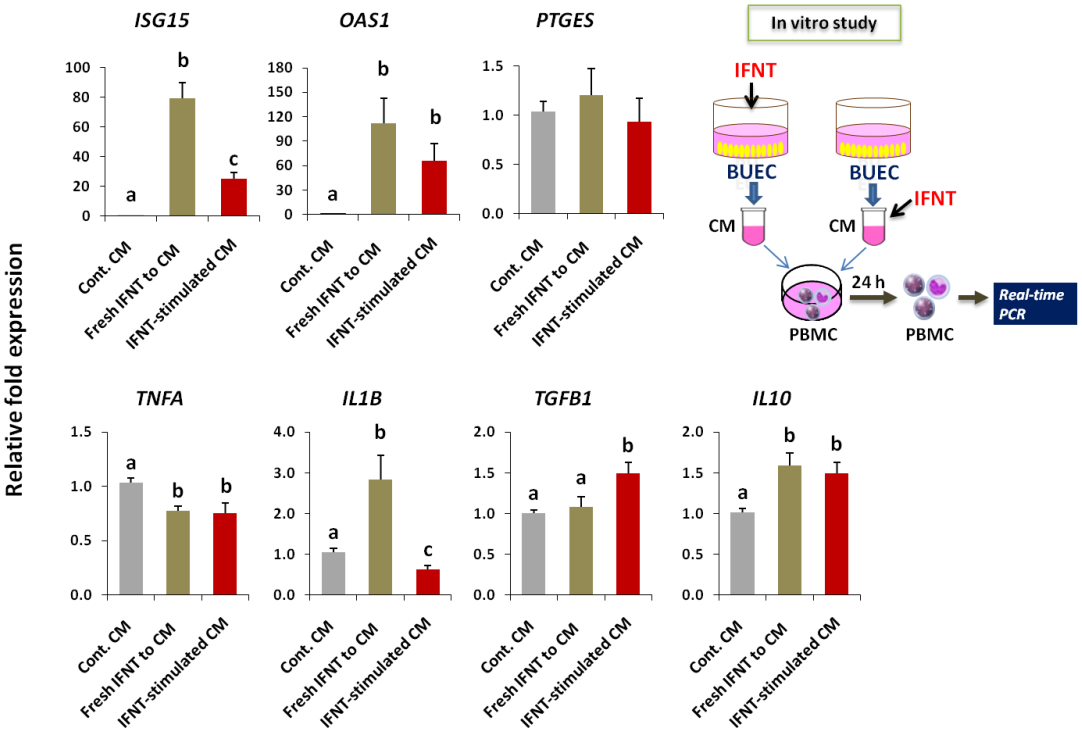

Fig. 2-6. IFNT alone on direct stimulation to PBMCs also affected immune gene expression as the IFNT-stimulated BUEC-CM. CM from BUEC culture without IFNT stimulation served as the control. Relative mRNA expression of interferon-stimulated genes, *PTGES*, pro-inflammatory cytokines and anti-inflammatory cytokines. Data are presented as Mean ± SEM of three independent experiments performed in triplicates. Different letters denote significant difference at $P<0.05$ level, when compared to the controls.

3.5. Summarized view of the results

Results from both *in vivo* and *in vitro* studies showed an anti-inflammatory response in PBMCs to embryos and IFNT, respectively. However, some dissimilarity in gene expressions were also observed particularly in cytokines expression (Fig. 2-7). These differences might be due to action of IFNT plus other factors derived from embryos (*in vivo* study), factors from BUECs in response to IFNT (*in vitro* study), and effect of IFNT alone on PBMCs.

Study	ISGs		Pro-inflammatory		Anti-inflammatory		*PTGES*	Response
	ISG15	*OAS1*	*TNFA*	*IL1B*	*TGFB1*	*IL10*		
In vivo study (UF-Embryo)	↑	↑	↓	↓	=	↑	=	Anti-inflammatory
In vitro study (IFNT–stimulated BUEC-CM)	↑	↑	↓	↓	↑	↑	=	Anti-inflammatory
In vitro study (Fresh IFNT to BUEC-CM)	↑	↑	↓	↑	=	↑	=	Anti-inflammatory

Fig. 2-7. Summarized results from *in vivo* and *in vitro* studies on PBMCs gene expressions. Upward and downward directed arrows indicate up-regulated and down-regulated gene expression, respectively. Equal signs indicate no changes in gene expression between control and treatment groups. Different colors denote different groups of genes.

4. Discussion

This study showed that IFNT secreted from Day-7 embryo in the uterus generated anti-inflammatory response in immune cells. A complete block of these effects in immune cells by the specific antibody indicates the specific action of IFNT in UF from Day-7 pregnant cows. CM from IFNT (100 pg/ml)-stimulated-BUECs culture (*in vitro*) demonstrated very similar response in immune cells gene expressions to those by UF from

Day-7 pregnant cows. These findings strongly suggest that IFNT secreted by Day-7 embryos mediates this immune crosstalk with uterine epithelium as well as immune cells.

IFNT protein has been detected in the trophectoderms of *in vitro* derived non-hatched bovine blastocysts on Day-7 [39]. It is also reported that Day-7 bovine blastocyst, developed either *in vivo* or *in vitro*, produces IFNT at around 100 to 1000 pM/day as measured by antiviral cell protection assay [40]. The present study showed that UF from day-7 pregnant cows induced *ISGs* in PBMCs. This finding indicates that Day-7 multiple blastocysts secrete small but significant amount of IFNT into uterine fluid which activates interferon-signaling cascades in PBMCs. This finding is supported by those in the previous study [17], where *ISGs* expression was induced only around single bovine embryo in the endometrium of uterine horn. It is possible that type 1 interferon other than IFNT could also induce *ISGs* expression upon stimulation. In my study, the immune neutralization of IFNT in UF, using a specific IFNT antibody that exclusively recognizes IFNT but not IFNA (data not shown), confirmed the presence of IFNT in UF from Day-7 pregnant cows and its specific action on PBMCs gene expressions. Together, these results suggest that IFNT acts as one of the major factors for immunological crosstalk between Day-7 embryo and immune cells in the cow.

I used UF from pregnant cows having multiple embryos in the uterus in order to amplify the signals including IFNT from embryos so that changes in PBMCs can reach up to the detectable level, although it does not happen under physiological condition in cattle. In a previous study, it has been demonstrated that proteomic changes in UF on Day-8 pregnancy in cows by single embryo is similar to those of cyclic cows, whereas multiple embryos (n=30-60) produce distinct changes in compare to cyclic cows [41].

Successful pregnancy requires modulation of the maternal immune system that predominantly shifted from Th1 to Th2 cytokines at feto-maternal interface [42]. It is reported that IFNT regulates bovine granulocyte chemotactic protein -2 secretions in the endometrium, thereby regulating cytokine networks in the uterus in pregnant cows [43]. In this study, UF from pregnant cows suppressed pro-inflammatory cytokine and stimulated anti-inflammatory cytokine expression, which was mainly, mediated by IFNT present in UF from pregnant cows. A recent study showed that IFNT plays an anti-inflammatory role in *S. aureus*-induced endometritis in mice, through suppression of the NFkB pathway and inhibition of TNFA and IL1B production [32]. It was also shown that IFNT induced IL10 and suppressed IL1B secretion in human macrophages [33]. Therefore, the results of this study suggest that embryo-derived IFNT generates anti-inflammatory (Th2) immune environment in the uterus for immune cell tolerance to early embryo.

Progesterone is a potent immunosuppressive molecule to inhibit the release of pro-inflammatory cytokine from the leukocytes in mouse uteri [44]. In this study, I measured progesterone concentration in UF by enzyme immunoassay (EIA). I found very low concentration (<100 pg/ml) of progesterone in UF of both control and pregnant groups (data not shown). Thus, the possibility that progesterone affected modulation of PBMCs gene expressions in this study is unlikely.

CM from IFNT-stimulated BUECs culture also induced similar anti-inflammatory immune responses in PBMCs as those with UF from Day-7 pregnant cows. Moreover, a recombinant IFNT given to CM (control) also generated an anti-inflammatory immune response in PBMCs *via* suppression of *TNFA* and stimulation *IL10* expression, however, did not suppress *IL1B* as did by CM from IFNT-stimulated BUEC culture. These very similar,

but not identical, effects of each model suggest that BUEC secretes some factors in response to IFNT that play a role in induction of anti-inflammatory response in PBMCs.

Altogether, the findings of the present study support the hypothesis that IFNT from Day-7 blastocyst modulates local uterine immune environment towards an anti-inflammatory response, which could play a pivotal role in immunological acceptance of the blastocyst in the bovine uterus. Further study is needed to investigate the impact of exogenous IFNT (at very low concentration) infusion in the uterus on such immune regulation in the cow.

Chapter II

Interferon-tau (IFNT) infusion in the uterus modulates the uterine immune-environment towards anti-inflammatory in immune cells in cattle

1. Introduction

In my first study (as described in 'Chapter I'), I investigated the effect of uterine flush (UF) from Day-7 donor pregnant cows with multiple embryos on the expression of interferon-stimulated genes (*ISGs*) as well as immune-related genes in PBMCs. The finding of this study showed *in vivo* evidence that IFNT secreted from Day-7 embryo generates an anti-inflammatory immune response in the uterus. In this study, I used multiple embryos in the uterus in order to amplify the IFNT signal so that changes by IFNT can reach up to the detectable level. Indeed, it does not happen under the physiological condition in cattle, this model showed evidence that IFNT among other signaling molecules responsible for such immune regulation. Therefore, the basis of this finding, IFNT infusion in the uterus along with embryo transfer would be one of the effective tools for improving the fertility in dairy cattle. However, it is needed to investigate the impact of IFNT along on regulation of local immune-environment in the uterus in *in vivo* condition.

The unattached bovine embryo that suspended in the uterine fluid releases several bioactive signaling molecules along with IFNT as include pregnancy-associated glycoprotein (PAG) and prostaglandins E2 [9, 45, 46]. Similarly, uterine endometrium also secretes several protein molecules (histroph) for growth and development of the embryo [3]. The molecules that released from the embryo and uterine endometrium affect uterine gene expression and contents of UF and thereby directly affect local immune cells to regulate local uterine immunity. A recent study in dairy heifer demonstrated that conceptus increased tolerogenic molecules around the endometrium that are likely to alter the functional state of uterine myeloid lineage cell to tolerogenic phenotype [22].

To avoid the possible involvement of other signaling molecules from the embryo in such immune regulation in *in vivo* condition, I used uterus with exogenous IFNT infusion model to observe the impact of IFNT alone in the uterus in the cow. I hypothesized that exogenous IFNT infusion in the uterus at a very low concentration can regulate similar immune-environment of the uterus to those of IFNT from Day-7 embryos in cattle. However, such information is necessarily required for developing the proper strategy to improve fertility through embryo transfer program (ET) at Day-7. Therefore, I aimed to investigate the effect of exogenous IFNT infused uterine flush (UF) from Day-9 of a cyclic cow on immune-related genes expression in peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Research Animals and under the approval of Animal Experiments Ethics Committee, Obihiro University of Agriculture and Veterinary Medicine, Japan (Permit number 25–101).

2.2. Experimental model

I used an *in vivo* study model (Fig. 3-1) to investigate the role of IFNT-infused uterine flush (UF) on gene expression in immune cells. First, cows (n=5) were synchronized to estrus and remained non-inseminated. On Day-6 fresh RPMI-1640 media (100 ml) was infused into the uterus (ipsilateral horn), incubated for 24 h and UF was collected (served as control-1) on Day-7. A similar procedure was done again for infusion of fresh media on Day-7 and collection of UF (served as control-2) on Day-8. Immediately after collection of UF, an exogenous IFNT (100 pg/ml) with the same volume of RPMI-1640 media (100 ml) was

infused into the uterus and UF was collected after 24 h incubation (served as IFNT-infused UF) on Day-9. Subsequently, PBMCs were cultured in UF with or without IFNT infusion for 24 h and gene expressions were analyzed.

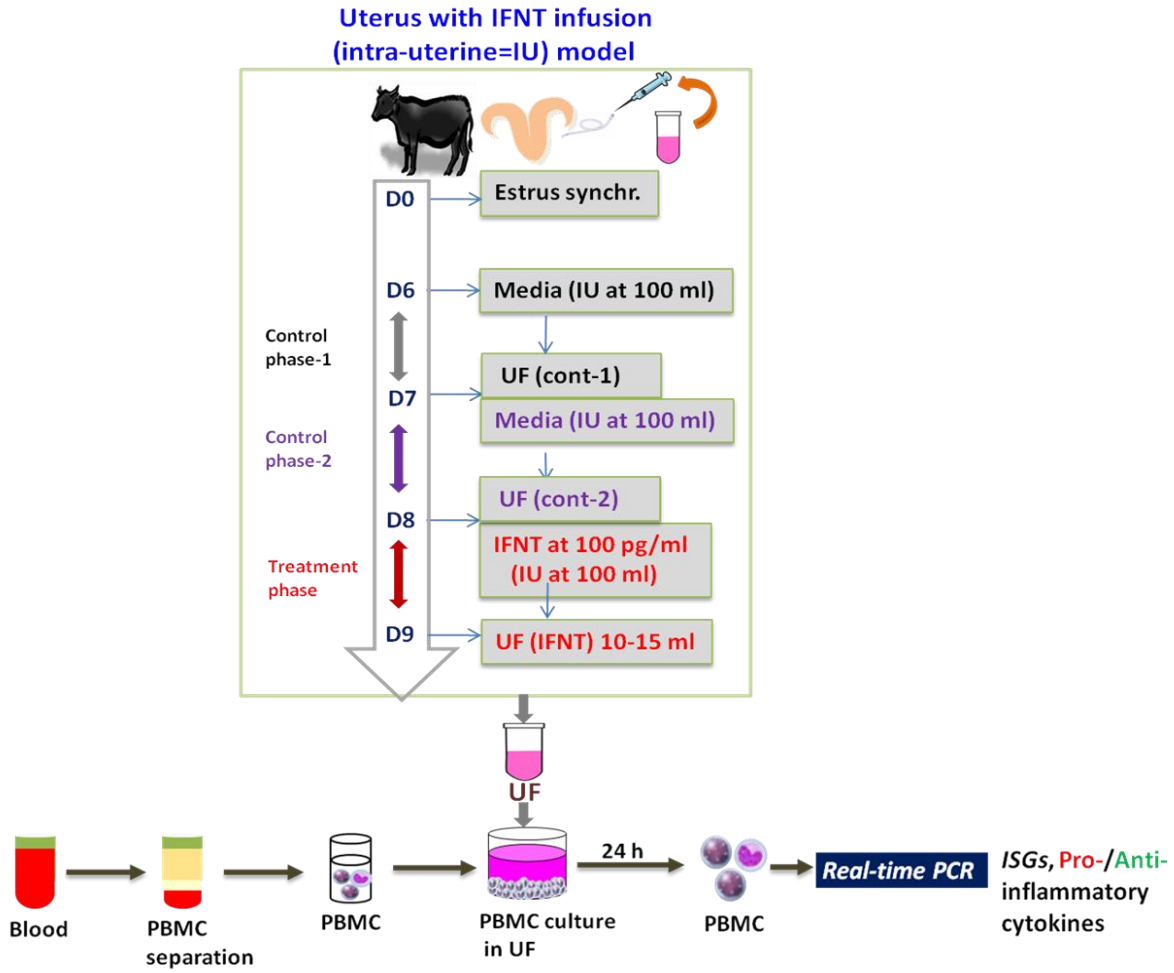

Fig. 3-1. Schematic representation of the experimental model; Cows were synchronized to the estrus and remained non-inseminated. On Day-6 fresh RPMI-1640 media (100 ml) was infused into the uterus incubated for 24 h and UF was collected (served as control-1) on Day-7. A similar procedure was done again for infusion of fresh media on Day-7 and collection of UF (served as control-2) on Day-8. Immediately after collection of UF, an exogenous IFNT (100 pg/ml) with the same volume of media (100 ml) was infused into the uterus and UF was collected after 24 h incubation (served as IFNT-infused UF) on Day-9. Subsequently, PBMCs were cultured in UF with or without IFNT infusion for 24 h and gene expressions were analyzed.

2.3. Animal preparation and estrus synchronization

Reproductively normal multiparous Holstein cows (n=7; average body weight 550±15 kg) were randomly selected in university farm (Field Center of Animal Science and

Agriculture of Obihiro University, Obihiro, Japan). All experimental procedures complied with the guidelines for the care and use of agricultural animals at Obihiro University. Cows were provided with balanced ration and ad-libidum water supply throughout the whole experimental period. Estrus synchronization and exact day of ovulation was done as previously described [47] with minor modifications. At first, cows having large follicle received a single intramuscular GnRH (100 µg fertirelin acetate/ cow, Suporunen, Kyoritsu seiyaku, Tokyo, Japan) injection followed 7 days later by PGF2α injection (25 mg, tromethamine dinoprost/cow; Veterinary Pronalgon F, Pfizer, Tokyo, Japan) and again administered GnRH injection at 48 h after PGF2α. Only the animals (n=5) that possessed a pre-ovulatory follicle after the last GnRH application were used in the study. The entire animals remained non-inseminated and ovulation was confirmed using a trans-rectal ultrasonic device equipped with a 5.0-MHz linear transducer (HS-101V, Honda Electronics, Toyohashi, Japan).

2.4. Intra-uterine infusion of media/IFNT and collection of UF

Synchronized cows were monitored until Day-10, on Day-6 of the estrus cycle, 100 ml of fresh RPMI-1640 medium (Sigma-Aldrich) was infused in the ipsilateral horn. After washing the perineum with lactacyd soap and drying with paper towels, an embryo flush balloon catheter was inserted into the uterine horn (Ipsilateral). Then fresh sterile RPMI-1640 medium (100 ml) normalized with room temperature was infused with the help of 50 ml syringe (Nipro Co. Ltd, Japan). A measure was taken during infusion of medium to prevent possible contamination. Uterine flush (UF) was collected after 24 h of uterine infusion according to the protocol as described [48]. Briefly, at first fluid in the uterine horn was localized by ultrasound image and then aspirated into a syringe through the catheter. If no fluid was obtained by aspiration, 10- 20 ml of sterile same media was flushed into the uterine

horn, gently mixed and then aspirated into a syringe. Collected UF was kept on ice; after centrifugation at 1000 x g for 15 min at 4°C, the supernatant fraction was obtained and stored at −80°C for further use. The same procedure was applied again for infusion of fresh RPMI-1640 media on Day-7 and for recombinant bovine IFNT (bIFNT 2B, specific activity = 4.15 × 10^7 U/mg; Zenoaq, Koriyama, Japan) at a dose 100 pg/ml mixed with the same volume of media (100 ml) on Day-8. Subsequently, UF was collected after 24 h of infusion on Day-8 and Day-9 respectively. The dose of IFNT (100 pg/ml) was chosen from our previous *in vitro* study [18] where a similar magnitude of *ISG15* mRNA stimulation was observed in BUECs with this dose as with the embryo. UF collected on Day-7 & 8 after infusion of fresh media on was considered as control UF-1 & 2 respectively and UF collected on Day-9 after infusion of IFNT containing media was considered as IFNT-infused UF.

2.5. Isolation of PBMCs

PBMCs were isolated as previously described [15] with minor modifications. Blood was collected during early luteal phase in heparinized blood tube (20 ml) and mixed with an equal volume of PBS-/-, slowly layered over Ficoll-paque solution (Lymphoprep, Axis Shield, Norway), and centrifuged at 1000 x *g* for 35 min at 10 °C. The white buffy coat was collected as PBMCs, mixed with twice volume of hemolysis buffer (NH_4Cl 155 mM, $KHCO_3$ 9.9 mM, EDTA 96.7µM) for 3 minutes, and centrifuged at 500 x *g* for 5 min at 10 °C to remove red blood cells. After centrifugation, the cell pellet was washed twice with PBS-/-. The purity of PBMCs as evaluated by flow cytometry was >98% and the viability as assessed by Trypan blue staining was 98%.

2.6. Culture of PBMCs in UF

PBMCs (5 × 10^6 cells) were cultured in a 12-well plate in 1ml UF from cow infused with IFNT or in UF infused with fresh media (control 1& 2) and for 24 h in a humidified

atmosphere at 38.5 °C in 5% CO_2. After 24 h of incubation, the supernatant was removed and the cells were collected, lysed using Trizol, and then stored at -80 °C until RNA extraction.

2.7. RNA extraction and cDNA synthesis

RNA extraction from PBMCs using the Trizol reagent (Thermo Fisher Scientific) was performed according to the previously described protocol [36]. Extracted RNA was detected by ultraviolet (UV) spectroscopy (optical density, 260 nm) and the concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany) at 260 and 280 nm absorbance values. The total extracted RNA was stored in RNA storage solution (Ambion, Austin, TX, USA) at –80 °C until cDNA production. The cDNA synthesis was carried out as previously described protocol [37]. Briefly, DNase treatment step was performed using RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA) to remove residual genomic DNA and other contaminations. The extracted RNA (1 µg in 7 µl) was incubated for 30 min at 37 °C with 1 unit of the 10× RQ1 RNase-free DNase reaction buffer and 2 µl of the 1 µg/µl RNase-free DNase. To terminate the reaction, 1 µl of the RQ1 DNase Stop solution (20 mM EDTA) was added to the sample, and the mixture was incubated for 10 min at 65 °C. First-strand cDNA synthesis was conducted according to the commercial protocol described in the Super Script II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The mixture was prepared using 1.5 µl of 50 ng/µl random primer (Invitrogen, Carlsbad, CA, USA), 1.5 µl of 10 mM PCR Nucleotide Mix (dNTP; Roche Diagnostics, Indianapolis, IN, USA), and 4 µl of H_2O to obtain a total volume of 18 µl per sample. This mixture was then incubated at 65 °C for 5 min in a thermal cycler (Bio-Rad, Munich, Germany). The samples were kept on ice while the second mixture, which consisted of 3 µl of 0.1M dithiothreitol (DTT, Invitrogen, Carlsbad, CA, USA), 1.5 µl of 40 units/µlRNasin Ribonuclease Inhibitor (Promega, Madison, WI, USA), and 6 µl of 5× First-Strand Buffer (Invitrogen, Carlsbad, CA, USA), was added to

each tube. The samples were incubated for 2 min at 42 ℃, and 0.2 µl of 200 units/µl of Super Script II Reverse Transcriptase was added to each tube. The thermal cycler was programmed at 25 ℃ for 10 min, 42 ℃ for 50 min, and then 70 ℃ for 15 min. The synthesized cDNA was stored at -30 ℃.

2.8. Real-time PCR

Quantitative real-time PCR of specific target genes (Table 3-1) was performed using QuantiTect SYBR Green PCR Master Mix (QIAGEN GmbH, Hilden, Germany) by an iCycler iQ (Bio-Rad Laboratories, Tokyo, Japan). The amplification program was set up according to a previously described protocol [37]. Briefly, this program was run with first activation step (15 min at 9 ℃), followed by 40 cycles of PCR (15 sec denaturation at 95 ℃, 30 sec annealing at 55–58 ℃, and 20 sec extension at 72 ℃). At the end of the program, the melting curve was evaluated to check the specificity of the amplification. The cycle threshold values that are calculated after ending of this program were normalized using *B-actin* as the internal standard by applying the Delta-Delta comparative threshold method [38] to quantify the fold change between samples.

Table 3-1. List of the primers used in real-time PCR

Gene		**Sequence of nucleotide (5'→3')**	**Accession no.**	**Fragment size (bp)**
B-actin	Forward	TCACCAACTGGGACGACATG	NM_173979.3	51
	Reverse	CGTTGTAGAAGGTGTGGTGCC		
ISG15	Forward	TCTGAGGGACTCCATGACGG	NM_174366	51
	Reverse	TTCTGGGCGATGAACTGCTT		
OAS1	Forward	TAGGCCTGGAACATCAGGTC	NM_001040606.1	105
	Reverse	TTTGGTCTGGCTGGATTACC		
PTGES	Forward	AAAATGTACGTGGTGGCCGT	NM_174443.2	51
	Reverse	CTTCTTCCGCAGCCTCACTT		
TNFA	Forward	CAAAAGCATGATCCGGGATG	NM_173966.3	51
	Reverse	TTCTCGGAGAGCACCTCCTC		
IL1B	Forward	AATCGAAGAAAGGCCCGTCT	NM_174093.1	51
	Reverse	ATATCCTGGCCACCTCGAAA		
TGFB1	Forward	CTGCTGAGGCTCAAGTTAAAAGTG	NM_001166068.1	90
	Reverse	CAGCCGGTTGCTGAGGTAG		
IL10	Forward	GAGATGCGAGCACCCTGTCT	NM_174088.1	51
	Reverse	GGCTGGTTGGCAAGTGGATA		

2.9. Statistical analysis

Data are presented as Mean ± SEM. Statistical analysis was performed using SPSS software version 14.0 (SPSS Inc., Chicago, USA). Student's t-test was applied to compare the data between two groups. *P* values <0.05 were considered to be statistically significant.

3. Results

Uterine flush (UF) infused with exogenous rbIFNT regulated immune gene expression towards anti-inflammatory condition in PBMCs

I investigated whether UF infused with rbIFNT has regulated immune gene expression in PBMCs. UF infused with exogenous rbIFNT affected the mRNA expression of PBMCs during 24 h culture when compared with UF infused with fresh media. In comparison with the UF (Day-8) infused with fresh media, the UF infused with rbIFNT significantly down-regulated of pro-inflammatory cytokines (*TNFA, IL1B)* and up-regulated anti-inflammatory cytokine (*TGFB1)* and *PTGES* in PBMCs. Moreover, Fresh media infused UF at two subsequent days (Day-7 & 8) did not affect the gene expression in PBMC except *PTGES* expression that suppressed by UF of Day-8 (Fig. 3-2).

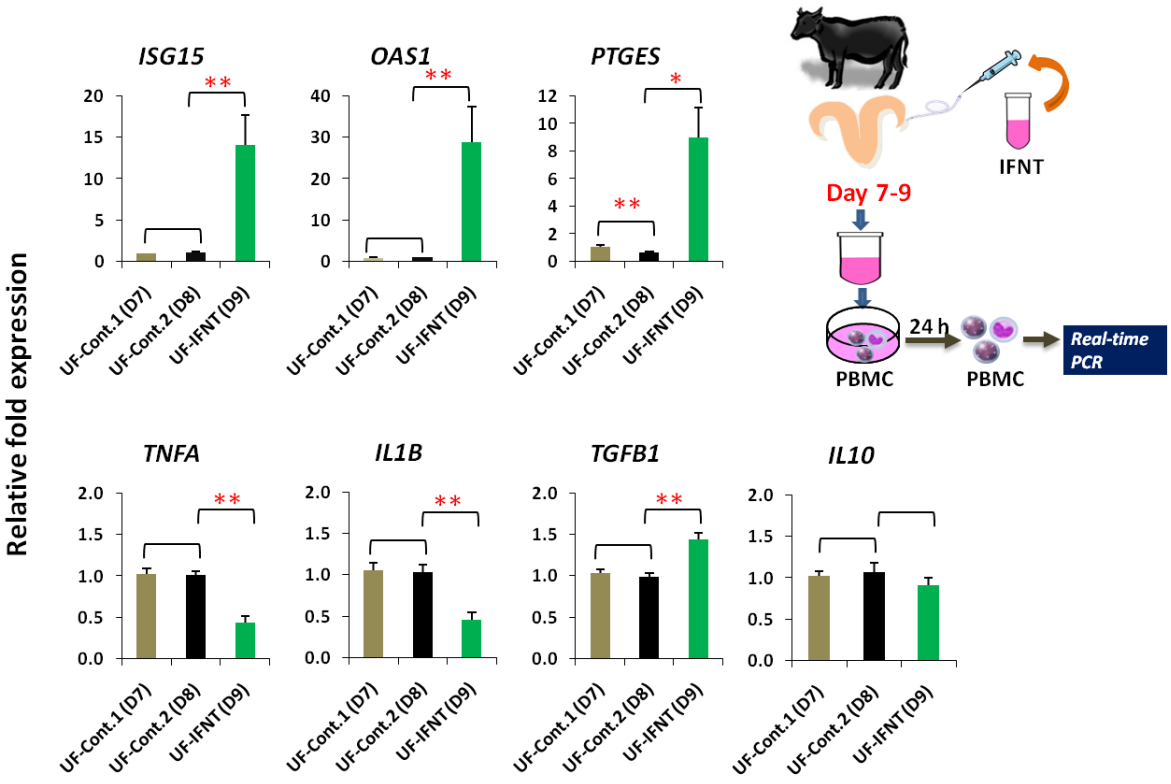

Fig. 3-2. Uterine flush (UF) infused with exogenous rbIFNT regulated immune gene expression towards anti-inflammatory condition in PBMCs. UF from cow without IFNT infusion served as the control. Relative mRNA expression of IFN-stimulated genes (*ISG15, OAS1*), an enzyme involved in prostaglandin E synthesis (*PTGES*), pro-inflammatory cytokines (*TNFA, IL1B*) and anti-inflammatory cytokines (*TGFB1, IL10*). Data are presented

as Mean ± SEM of five independent experiments preformed in triplicates. All mRNA expression levels were normalized to β-actin. Asterisks denote significant difference: * P<0.05, ** P<0.01, when compared to the control.

4. Discussion

Embryo-derived IFNT generates an anti-inflammatory immune response in the uterus on Day-7 of pregnancy (as described in 'Chapter I'). To avoid the possible involvement of other signaling molecules from the embryo in such immune regulation, I used uterus with exogenous IFNT infusion model to observe the impact of IFNT alone in the uterus. This study showed that uterine flush (UF) infused with exogenous rbIFNT induced the similar anti-inflammatory response in PBMCs to those of IFNT from Day-7 embryo. This finding strongly suggests that despite many molecules other than IFNT from the embryo, IFNT alone can mediate the uterine immune-environment towards anti-inflammatory in immune cells.

This study revealed that UF infused with exogenous rbIFNT significantly activated the interferon-stimulated genes (*ISGs*) in PBMCs. That indicates that IFNT present in UF activates interferon-signaling cascades in PBMCs. IFNT is also known to induce the expression of interferon-stimulated genes (*ISGs*) in the endometrium [13], CL [14] and peripheral blood mononuclear cells (PBMCs) [15] during early pregnancy in cows. This result suggests that IFNT along with uterine secretion actively influence gene expressional changes in immune cells.

In this study, the UF infused with exogenous rbIFNT clearly down-regulated *TNFA & IL1B* (Th1 cytokine) expression in PBMCs. That is in agreement with our previous finding (as described in Chapter I) in which I demonstrated that UF from Day-7 pregnant cow significantly down-regulated pro-inflammatory cytokines (*TNFA, ILIB*) expression in

immune cells, mediated chiefly by embryo-derived IFNT. In this study, IFNT along with uterine secretion actively takes part in the suppression of pro-inflammatory cytokines (*TNFA & IL1B*) expression in PBMCs. Our recent *in vitro* study has also demonstrated similar findings in which embryo (Day 5-9) derived IFNT suppresses pro-inflammatory cytokine expression in immune cells [18]. In addition, a recent study showed that IFNT plays an anti-inflammatory role in *Staphylococcus aureus*-induced endometritis in mice, through suppression of the nuclear factor kappa B (NFkB) pathway and inhibition of TNFA and IL1B production [32]. Thus present finding suggests exogenous IFNT incubation in the uterus suppress pro-inflammatory immune response in the immune cells.

In the present study, the UF infused with exogenous rbIFNT clearly up-regulated anti-inflammatory cytokine (*TGFB1*) and prostaglandin E synthetase enzyme-producing gene (*PTGES*) in PBMCs. A similar result has found in our very recent study that embryo-derived IFNT on Day-4 stimulated *TGFB1 & PTGES* expression in immune cell and thereby induces anti-inflammatory response [49]. TGFB is a potent anti-inflammatory cytokine, higher expression of *TGFB1* in PBMC by UF infused with IFNT suggesting IFNT favors more anti-inflammatory condition in the uterus. It has been reported that IFNT can induce PGE2 synthesis in cow [50] as well as bovine endometrial cells [51] during early pregnancy. PGE2 molecule is known as a potent immune suppressor and actively inhibits the proliferation of lymphocyte in culture [52]. PGE2 has also reported earlier as a key regulator for generation of anti-inflammatory response in the bovine oviduct in vitro [37]. It is well known that both TGFB and PGE2 work synergistically to enhance the differentiation of naïve T cells (Th0) to regulatory T cells for immune suppression and tolerance in mice [53, 54]. Thus present finding showed more anti-inflammatory condition in the uterus in response to IFNT stimulation.

Altogether, the results of the present study support the hypothesis that exogenous IFNT infusion instead of embryo-derived IFNT in the uterus can also regulate the uterine immune environment towards anti-inflammatory in immune cells which could play an important role for acceptance of the Day -7 bovine blastocyst in the uterus. It is likely that IFNT at a low dose (100 pg/ml) along with embryo may help in driving the environment for tolerance of embryo in the uterus, thus it could be applied along with embryo transfer technology to improve fertility.

General Discussion and Conclusion

The maternal immune system is very important for embryo-maternal crosstalk during pregnancy and indeed disrupt immune response may lead to early embryonic rejection. The bovine embryo following successful fertilization needs to start a bio-molecular dialogue with the maternal immune system to set up a modified immune response to the embryo. Before implantation, the embryo has to suspend on the uterine fluid and releases several bioactive signaling molecules that initiate dialogue with mother. Interferon-tau (IFNT), a trophectoderm derived embryonic signal molecule is to be considered as one of the main factors in embryo-uterus crosstalk during pregnancy. IFNT is regarded as a molecule responsible for the process of maternal recognition of pregnancy (MRP) and maintains pregnancy through rescuing corpus luteum from lysis [9, 10]. Apart from its anti-luteolytic function, IFNT is being reported as an immune suppressive & anti-proliferative to the immune cell. Moreover, local uterine immune cells [20], as well as circulatory immune cells [28], responded to conceptus-derived IFNT suggesting that there might be IFNT mediated immune regulation exists in the uterus during early pregnancy. Till now, there is little or no information regarding direct evidence of IFNT involvement in immune modulation during early pregnancy in the cow.

The bovine blastocyst that developed in the FRT is a semi-allogenic and expresses the paternal antigen of MHC molecule I on Day-7 of pregnancy [5, 6]. Thus, it may attack by the maternal immune system and could be one of the main reasons for the early embryonic loss. However, the semi-allogenic blastocyst avoids such immune attack and establishes pregnancy in the uterus. The bio-molecular mechanism by which embryo avoids the maternal immune attack is nearly unknown. Recent studies from ours [18] and others [17] suggest that Day-7 bovine embryo starts to communicate with the uterine epithelium through interferon-tau (IFNT) signaling. However, immune modulatory role of IFNT in the uterus just after the

embryo moves from the oviduct is unclear. I hypothesized that Day-7 embryo secretes very small amount of IFNT in the uterus which then communicates locally with the uterine epithelium for generation of an anti-inflammatory response in immune cells. It is, however, difficult to collect a number of local immune cells from the uterus for molecular analysis, so that an interaction between the embryo, IFNT, and immune cells has not yet been investigated.

Therefore, in Chapter I, I used both *in vivo* and *in vitro* model to investigate the effect of uterine flush (UF) from Day-7 pregnant cow with multiple embryos on interferon-stimulated genes (*ISGs)* as well as immune-related genes in peripheral blood mononuclear cells (PBMCs). And also further examined on whether or not IFNT directly regulated this immune-related gene expression in PBMCs through the use of bovine uterine epithelial cells (*in vitro*). The uterine flush (UF) with multiple embryos was collected from Day-7 donor pregnant cows and peripheral blood mononuclear cells (PBMCs) were then cultured in UF. Transcripts detected in PBMCs revealed that UF from pregnant cows down-regulated pro-inflammatory cytokines (*TNFA, IL1B)* and up-regulated anti-inflammatory cytokine (*IL10)* expression, with activation of interferon-stimulated genes (*ISGs; ISG15, OAS1)* as compared with UF from non-pregnant cows. An addition of specific anti-IFNT antibody to the UF inhibited the effect on PBMCs, indicating that IFNT is a major factor for such immune modulation. The observation that conditioned media from bovine uterine epithelial cells both stimulated with IFNT *in vitro* and supplemented with fresh IFNT induced similar PBMCs gene expression, confirming that IFNT directly acts on this immune crosstalk. This study shows that IFNT secreted from Day-7 embryo *in vivo* generates anti-inflammatory response in immune cells, which may provide immunological tolerance to accept the embryo.

It could be argued that the present study used multiple embryos in the uterus for the collection of UF that does not happen under the physiological condition in cattle. In this study, multiple embryos were used in order to amplify the signals including IFNT from embryos so that changes in PBMCs can reach up to the detectable level. In a previous study, it has been demonstrated that proteomic changes in UF on Day-8 pregnancy in cows by single embryo is similar to those of cyclic cows, whereas multiple embryos (n=30-60) produce distinct changes in compare to cyclic cows [41].

Importantly, in my first study (as described in 'Chapter I'), I demonstrated *in vivo* evidence that IFNT secreted from Day-7 embryo generates an anti-inflammatory immune response in the uterus. To avoid the possible involvement of other signaling molecules from the embryo in such immune regulation, in Chapter II, I used uterus with exogenous IFNT infusion model to observe the impact of IFNT alone in the uterus. However, such information is necessarily required for developing the proper strategy to improve fertility through embryo transfer program (ET) at Day-7. Therefore, I aimed to investigate the effect of exogenous IFNT infused uterine flush (UF) from a Day-9 cyclic cow on the expression of immune-related genes in peripheral blood mononuclear cells (PBMCs). Exogenous IFNT (100 pg/ml) with RPMI-1640 media (100 ml) was infused into the uterus on Day-8 of estrus cycle and UF was collected after 24 h incubation (served as IFNT-infused UF) on Day-9. Subsequently, PBMCs were cultured in UF with or without IFNT infusion for 24 h and gene expressions were analyzed. Gene analysis revealed that UF infused with rbIFNT down-regulated pro-inflammatory cytokines (*TNFA, IL1B)* and up-regulated anti-inflammatory cytokine (*TGFB1)* and *PTGES* expression, with activation of interferon-stimulated genes (*ISGs; ISG15, OAS1)* as compared with UF infused with fresh media (Control UF). This result indicates that

exogenous IFNT alone or co-action with other factors secreted from uterine epithelial cells can mediate local anti-inflammatory immune condition for immune cells.

In conclusion, the results of the present study support the hypothesis (Fig. 4) that IFNT secreted from Day-7 bovine blastocyst modulates local immune environment towards an anti-inflammatory response in immune cells, which could play a pivotal role in the immunological acceptance of the blastocyst in the uterus. Furthermore, exogenous IFNT instead of embryo-derived IFNT can also induce such immune regulation in the uterus during this period of pregnancy. Therefore, it is likely that IFNT at a low dose (100 pg/ml) along with embryo may help in driving the environment for tolerance of embryo in the uterus, thus it could be applied along with embryo transfer technology to improve fertility.

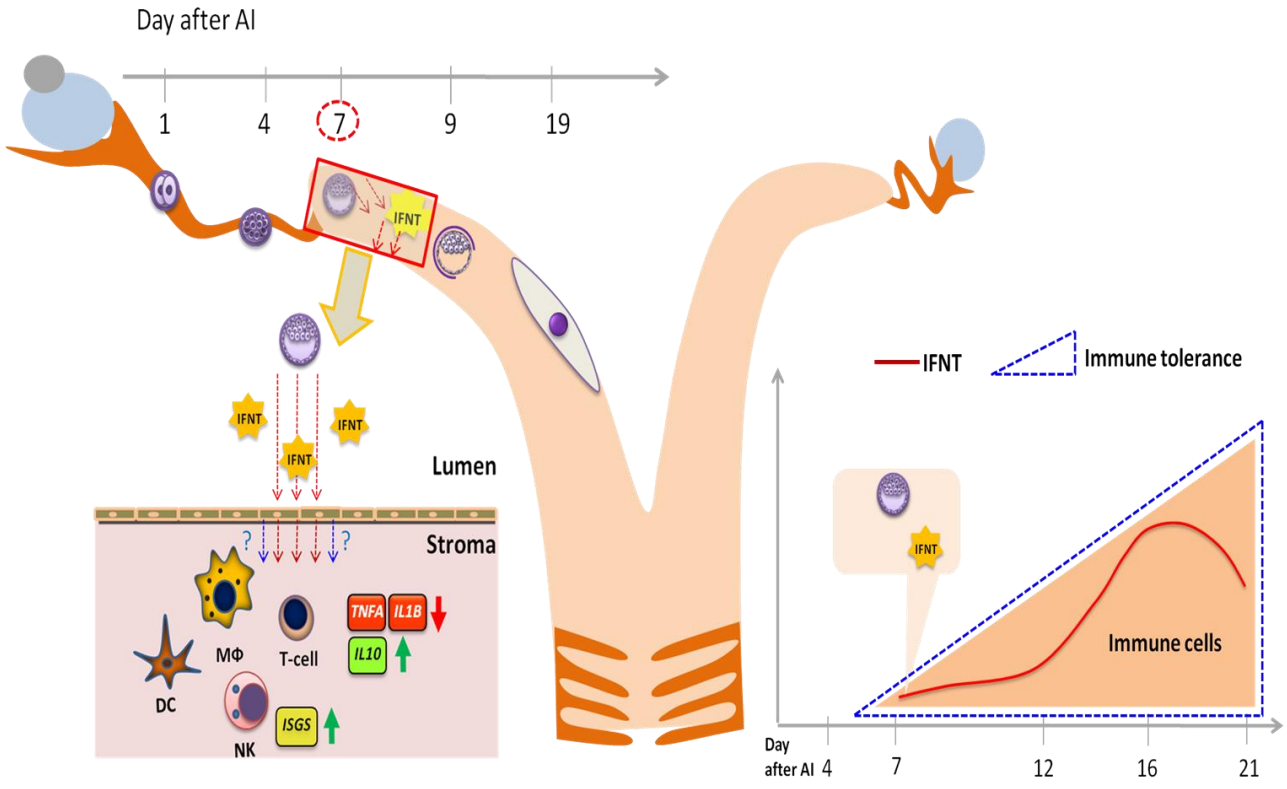

Fig. 4. Hypothetical representation of the modulation of local immune-environment by IFNT secreted from Day-7 bovine blastocyst in the uterus.

Summary

Immune modulation in the uterus at the embryo-maternal interface is critically important for establishing successful pregnancy in cattle. Any disruption of the immune response in the uterus may lead to early embryonic rejection. The early bovine embryo needs to start a bio-molecular dialogue with the maternal immune system to set up a modified immune response to the embryo. The unattached embryo suspending in the uterine fluid releases several bioactive signaling molecules that initiate dialogue with mother. Interferon-tau (IFNT), a trophectoderm derived embryonic signal molecule, is to be considered as one of the main factors in embryo-uterus crosstalk during early pregnancy. IFNT is a maternal recognition signal and maintains pregnancy by protecting corpus luteum from lysis. Apart from its anti-luteolytic function, IFNT is being reported as an immune suppressive molecule to the immune cell. Moreover, local uterine immune cells, as well as circulatory immune cells, responded to conceptus-derived IFNT suggesting that there might be IFNT mediated immune regulation exists in the uterus. Till now, there is little or no information regarding direct evidence of IFNT involvement in immune modulation at the period just after the embryo moves from the oviduct to the uterus. However, the semi-allogenic bovine blastocyst avoids the maternal immune attack and establishes pregnancy in the uterus in cattle. Recent studies suggest that Day-7 bovine embryo starts to communicate with the uterine epithelium through IFNT signaling. Therefore, I hypothesized that Day-7 bovine embryo secretes very small amount of IFNT in the uterus which then communicates locally with the uterine epithelium for generation of an anti-inflammatory response in immune cells. To examine the hypothesis, the present study aimed to investigate the effect of uterine flush (UF) from Day-7 pregnant cow (*in vivo*) on interferon-stimulated genes (*ISGs)* as well as immune-related genes in peripheral blood mononuclear cells (PBMCs). Further, the current study examined whether

or not IFNT directly regulated this immune-related gene expression in PBMCs through the use of bovine uterine epithelial cells (*in vitro*) and cow uterus (*in vivo*).

In Chapter I, in *in vivo* study, the effect of UF from Day-7 pregnant cow with multiple embryos on gene expressions in PBMCs was investigated. First, cows were superovulated by using routine hormonal treatment for superovulation regimen of embryo transfer program. After estrus (Day-0), cows were either inseminated (n=12; pregnant group) or remained non-inseminated (n=5; control group). On Day-7, the first UF was collected (20-25 ml) and pregnancy was confirmed with the presence of multiple embryos in UF. Subsequently, PBMCs were then cultured in UF of all groups and gene expressions were analyzed. Transcripts detected in PBMCs revealed that UF from pregnant cows down-regulated pro-inflammatory cytokines (*TNFA, IL1B)* and up-regulated anti-inflammatory cytokine (*IL10)* expression, with activation of interferon-stimulated genes (*ISGs; ISG15, OAS1)* as compared with UF from non-pregnant cows. An addition of specific anti-IFNT antibody to the UF inhibited the effect on PBMCs, indicating that IFNT is a major factor for such immune modulation. In *in vitro* study, the effect of IFNT-stimulated BUEC-CM on immune-related gene expressions in PBMCs was analyzed. First, bovine uterine epithelial cells (BUECs) after first passage were stimulated with IFNT (100 pg/ml) for 24 h and conditioned media (CM) was collected (IFNT-stimulated CM). CM from BUECs without IFNT stimulation served as controls and CM supplemented with IFNT (100 pg/ml) served as recombinant IFNT-CM. Subsequently, PBMCs were cultured in IFNT-stimulated CM (*in vitro*, indirect) or CM supplemented with IFNT (*in vitro*, direct) and gene expressions were analyzed. The observation that conditioned media from BUEC both stimulated with IFNT *in vitro* and supplemented with fresh IFNT induced similar gene expression in PBMCs, confirming that IFNT directly acts on this immune crosstalk.

In Chapter II, the effect of IFNT-infused UF on immune-related gene expressions in PBMCs was investigated. First, cows (n=5) were synchronized to estrus and remained non-inseminated. On Day-6 fresh RPMI-1640 media (100 ml) was infused into the uterus, incubated for 24 h and UF was collected (served as control-1) on Day-7. A similar procedure was done again for infusion of fresh media on Day-7 and collection of UF (served as control-2) on Day-8. Immediately after collection of UF, an exogenous IFNT (100 pg/ml) with the same volume of RPMI-1640 media (100 ml) was infused into the uterus and UF was collected after 24 h incubation (served as IFNT-infused UF) on Day-9. Subsequently, PBMCs were cultured in UF with or without IFNT infusion for 24 h and gene expressions were analyzed. Gene analysis revealed that UF infused with IFNT down-regulated pro-inflammatory cytokines (*TNFA, IL1B)* and up-regulated anti-inflammatory cytokine (*TGFB1)* and *PTGES* expression, with activation of interferon-stimulated genes (*ISGs; ISG15, OAS1)* as compared with UF infused with fresh media (control-2, Day-8). This result indicates that exogenous IFNT alone or co-action with other factors secreted from uterine epithelial cells can mediate local anti-inflammatory immune condition for immune cells.

Altogether, the results of the present study support the hypothesis that IFNT secreted from Day-7 bovine blastocyst modulates local uterine immune environment towards an anti-inflammatory response in immune cells, which could play a pivotal role in the immunological acceptance of the blastocyst in the uterus. Furthermore, exogenous IFNT instead of embryo-derived IFNT can also induce such immune regulation in the uterus during this period of pregnancy. Therefore, it is likely that IFNT at a low dose (100 pg/ml) along with embryo may help in driving the environment for tolerance of embryo in the uterus.

The present study has provided new insight into the molecular mechanism by which a semi-allogenic Day-7 blastocyst escapes from attack by the maternal immune system and is accepted in the uterus in cows. The findings of this study could be useful to make a further plan of IFNT (100 pg/ml) infusion in the uterus at the time of embryo transfer (ET) on Day-7, for assuring acceptable immune-environment in the uterus to accept the embryo. This could be one of the ways for achieving high reproductive performance in dairy cows.

Acknowledgement

All credits go to Almighty Allah (the Creator of the Universe) for His kind blessings upon me to complete this study.

Firstly, I would like to express my sincere gratitude to my main supervisor Prof. Dr. Akio Miyamoto for accepting me as a PhD student and for his scholastic supervision throughout my study and related research. His motivational guidance, useful criticisms, and enthusiastic encouragement had inspired me to upgrade my thinking ability for getting new ideas in the research. I am really grateful to him for his valuable instructions and patience during my study period, and also for taking care of my private life in Obihiro. I could not have imagined having a better advisor and mentor for my PhD study.

Besides my main supervisor, I would like to thank my co-supervisors Prof. Dr. Motozumi Matsui, Department of Clinical Veterinary Medicine for his generous help, valuable guidance and suggestions throughout this study.

I am particularly grateful to Dr. Shingo Haneda Department of Clinical Veterinary Medicine for his technical support during uterine flush (UF) collection. I wish to acknowledge his great effort especially in managing the cows for estrus synchronization and follicular detection by ultrasonographic imaging.

My grateful thanks are also extended to Prof. Dr. Kazuhiko Imakawa, The University of Tokyo for kindly donating valuable reagents (IFNT and antibody) and to Prof. Dr. Masayuki Shimada, Hiroshima University for his kind support and suggestions at the final stage to conclude the project.

My sincere thanks go to Dr. Toshiro Takedomi, Head of the Taketomi Reproduction Clinic, Obihiro and to Dr. Satoru Moriyasu, Research leader, Animal Biotechnology Group, Hokkaido Research Organization, Japan for their kind help in uterine flush (UF) collection from cow.

I would like to express my deep gratitude to the Obihiro University of Agriculture and Veterinary medicine for allocating scholarship for my doctoral study and also for providing nice academic facilities with quality staffs.

I am really grateful to Dr. Anup Kumar Talukder (My dearest younger brother) for his kind help in research work as well as in personal life in Obihiro. I will remain grateful to his contribution in my life. I am also thankful to Mr. Kazuhiro Morita (my Tutor) for his kind assistance in the laboratory and to my daily life. My sincere thanks also go to the laboratory Post doc fellow Dr. Marey (Egypt), PhD fellow Ahmed & Ezz (Egypt), Ihshan (Srilanka), Vernadyn (Philippines) and Zinnah (Bangladesh), MS fellow Haruhisha, Yura & Shiori, and undergraduate fellow Takehiro & Akane for their fruitful discussion and continuous support during the experiments.

I would like to offer my special thanks to my beloved wife Anjuara for her endless effort for taking care of sons (Arosh & Ayan) in my absence and for keeping patience for long waiting. I would like to acknowledge her encouraging mental support for both research and personal life in Obihiro specially, when I was getting failure in the research. Thank you so much dear for doing lot of sacrifices to build up my carrier. I also would like to express appreciation to my dear sons (Arosh & Ayan) for their sacrifice in father's love and care. I sincerely thank my beloved mother, brothers, sisters and all other relatives for their kind prayers and blessings for me.

Finally, I would like to dedicate this thesis to my beloved parents who allowed seeing this beautiful world and paid tremendous efforts since my birth. Their dedication for my education and for being developed as a good human being was exceptional. None other than my father (Late Md. Islam Uddin) would have been the happiest person to see that I am writing doctoral thesis. May Almighty always keep his soul in the best place of heaven!

Thank you all.

Md. Bazlar RASHID

2018

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和 文 要 約

子宮内での初期胚と母体の間の免疫調節は、ウシの妊娠成立に極めて重要である。子宮内での免疫応答の混乱は初期胚の拒絶反応に繋がるかもしれない。ウシ初期胚は母体の免疫システムと生物活性を持つ分子による対話によって、自身への免疫応答を変更させる必要がある。インターフェロン・タウ（IFNT）は、初期胚の栄養膜細胞から分泌される分子であり、免疫細胞の機能を抑制することが解ってきた。したがって、ウシ子宮内に浮遊する初期胚が分泌する、ごく微量の IFNT が、局所の子宮上皮細胞や免疫細胞に作用して、着床に向けた母体の免疫システム調節に関わっている可能性がある。しかしながら、初期胚が卵管から子宮に降りてきでも間もない受精後 7 日目の、初期胚と子宮の免疫クロストークの存在とそのメカニズムについては知られていない。本研究では、受精後 7 日目のウシ初期胚からごく微量の IFNT が分泌されており、子宮上皮と免疫細胞は、そのシグナルを認識して、子宮内の免疫環境を着床に向けた寛容型にシフトすることに関わっているのではないかと考えた。この仮説を検証するために、以下に述べる生体モデルと細胞培養モデルを用いた。

第 1 章では、ウシの受精卵移植用の過剰排卵処置をした供卵牛を生体モデルとして活用した。受精後 7 日目の複数の受精卵回収の際の子宮灌流液を用いて、リンパ球と単核球からなるウシ PBMC を培養して、遺伝子発現を調べた。対照群として、同様に処理した人工授精を行わないウシを設定した。その結果、IFNT で刺激される遺伝子（*ISG15, OAS1*）の発現が増加し、同時に、炎症性サイトカイン遺伝子（*TNFA, IL1B*）が抑制され、寛容に関わる Th2 サイトカイン遺伝子（*IL10*）が刺激された。この作用は、ウシ IFNT 抗体で消失したことから、本作用は、主に IFNT によるものであることが伺われた。さらに、培養されたウシ子宮上皮細胞（BUEC）をウシ IFNT（100 pg/ml）で刺激し、その培養上清で PBMC を培養したところ、上述の子宮灌流液と同様の PBMC への影響が確認された。加えて、IFNT（100 pg/ml）で直接 PBMC を刺激しても、同様の影響が見られた。以上の結果から、ウシ子宮内では、受精後 7 日目の初期胚から分泌されたごく微量の IFNT は、子宮局所の免疫クロストークに直接関わっていることが示唆された。

第 2 章では、IFNT（100 pg/ml）を発情周期の 8 日目のウシの子宮内に直接投与して、1 日後に得た子宮灌流液で PBMC を培養して、遺伝子発現を調べた。その結果、IFNT で刺激される遺伝子（*ISG15, OAS1*）の発現が増加し、同時に、炎症性サイトカイン遺伝子（*TNFA, IL1B*）が抑制され、寛容に関わる Th2 サイトカイン遺伝子（*TGFB1*）および *PTGES* が刺激された。以上の結果から、低濃度のウシ IFNT は、ウシ子宮内で免疫寛容にシフトするための調節因子として作用する可能性が示された。

以上の一連の知見から、ウシ子宮内の局所免疫システムは、受精後 7 日目の初期胚を認識する能力を備えており、着床に向けて初期胚を寛容する局所免疫環境を整えることが伺われた。さらに、初期胚から分泌される微量の IFNT が、その重要なコミュニケーション因子の 1 つであることが初めて示された。