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NEUTROPHILS RESPONSE IN DAIRY COWS DURING EARLY PREGNANCY

Santa Maria, RS
2021

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Doctoral thesis presented to the Postgraduate Program in Veterinary Medicine of the Federal University of Santa Maria (UFSM) as a partial requirement for obtaining the title of **Doctor in Veterinary Medicine**.

Advisor: Professor Alfredo Quites Antoniazzi

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TO

My family, especially Antônio and Roberto (in memoriam)

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All that is gold does not glitter,
Not all those who wander are lost;
The old that is strong does not wither,
Deep roots are not reached by the frost.

(J.R.R. Tolkien)

ABSTRACT

NEUTROPHILS RESPONSE IN DAIRY COWS DURING EARLY PREGNANCY

Candidate: Mariani Farias Fiorenza

Advisor: Prof. Alfredo Quites Antoniazzi

Studies highlighted the role of the immune system during the establishment of pregnancy. Maternal immune response has to find a balance to avoid any damage to the conceptus and maintain its function in combating microbes. When pregnancy cannot achieve this balance, losses might occur. Intercommunication between mother and conceptus is fundamental during early pregnancy to dictate the outcome of pregnancy. During pregnancy, the embryo signals to the maternal system its presence mainly locally *via* interferon tau (IFNT), and any stress factor can disrupt the signaling. The maternal immune response can alter between anti- and pro-inflammatory type, but usually, in the first few days (*e.g.*, day 7), anti-inflammatory responses are established. IFNT concentration begins to be detected in circulation around day 12 of pregnancy in low concentrations, suggesting that maternal immune modulation does not occur by the direct effect of IFNT during this time. However, it's unclear how amplification of IFNT signal in circulation occurs. Based on that, the present study focuses on determining IFNT effects in polymorphonuclear (PMN) cells and if immune cells can amplify embryonic signals to other immune cells *in vitro*. And if the signaling in PMN can be disrupted by stressor factor, *i.e.*, heat stress *in vivo*. The first study demonstrated that IFNT could generate anti-inflammatory responses and upregulation of ISGs expressions in PMNs, subsequently amplifying and transferring IFNT signals to a new cell population via a cell-to-cell communication mechanism possibly mediated by IFNA. The second study demonstrated that heat stress modulates PMN responses with greater anti- and pro-inflammatory cytokines expressions in different early pregnancy days. These findings might provide a better understanding of the modulation of innate immune responses during maternal recognition of pregnancy.

Keywords: Neutrophils. Interferon tau. Tolerance. Hyperthermia. Inflammation.

LIST OF ABBREVIATIONS

ACTB	Beta-actin
AI	Artificial insemination
ARG1	Arginase-1
CAT	Catalase
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CL	Corpus luteum
CM	Conditioned media
COCs	Cumulus oocyte complexes
CTGF	Connective Growth Factor
CXCL	Chemokine (C-X-C motif) ligand
D	Day
DC	Dendritic cell
e.g.	*exempli gratia*
ECM	Embryo culture media
ESR1	Estrogen receptor
FBS	Fetal bovine serum
FOXP3	Forkhead box P3
GCSF	Granulocyte colony-stimulating factor
HDN	High-density neutrophils
HR	Heart rate
HS	Heat stress
HSP	Heat-shock protein expression
i.e.	*id est*
IFN	Interferon
IFNA	Interferon alpha
IFNAR1	Interferon receptor subunit 1
IFNAR2	Interferon receptor subunit 2
IFNB	Interferon beta
IFNG	Interferon-gamma
IFNO	Interferon omega
IFNT	Interferon tau
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ISG15	Interferon-stimulated gene 15
ISGs	Interferon-stimulated genes
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
JAK/STAT	Janus kinase-signal transducer and activator of transcription
LDN	Low-density neutrophils
MDA	Malondialdehyde
MRP	Maternal recognition of pregnancy
MX1	Myxovirus resistance protein 1
N1	Pro-inflammatory neutrophils type 1
N2	Anti-inflammatory neutrophils type 2
NCF1	Neutrophil cytosolic factor 1
NDN	Normal density neutrophils
NETs	Neutrophil extracellular traps

NK	Natural killer
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase complex
OAS1	2'-5'-oligoadenylate synthetase 1
OXTR	Oxytocin receptor
P4	Progesterone
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGF	Prostaglandin 2 alpha
PGs	Prostaglandin
PMN	Polymorphonuclear cells
RIA	Radioimmune assay
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Respiratory rate
RT	Rectal temperature
SEM	Standard error of the mean
SOD	Superoxide dismutase
STAT1	Signal transducer and activator of transcription 1
TGFB	Transforming growth factor-beta
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
THI	Temperature-humidity index
TNFA	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor

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1. INTRODUCTION

Pregnancy represents the most crucial period for species conservation; therefore, it is essential to understand the mechanisms that protect the dam and its offspring (LEBER; TELES; ZENCLUSSEN, 2010). The maternal recognition of pregnancy (MRP) period culminates with the maximum gestational losses in cows. The days with the most significant impact on reproduction change according to the individual; however, it is possible to affirm that high-production dairy cows concentrate their losses before Day 8 after fertilization (DISKIN; PARR; MORRIS, 2012; SARTORI et al., 2002; WIEBOLD, 1988). These embryonic losses derive from several factors and generate profound economic impact (DISKIN; PARR; MORRIS, 2012). The solution for this problem may have a substantial effect on the reproductive performance of the global herd.

In ruminants, the central MRP signaling molecule is interferon (IFN) tau (IFNT), which acts in a paracrine manner in the uterus, together with progesterone (P4), stimulating the production of histotrophe by the endometrial glands, providing nutrition to the embryo (BROOKS; BURNS; SPENCER, 2014; SPENCER et al., 2004, 2007). Besides, embryos with a larger trophoblast area produce more IFNT; and the amount of IFNT is proportional to the expression of interferon-stimulated genes (ISG) in immune cells (MATSUYAMA et al., 2012).

Polymorphonuclear cells (PMNs) are the first line of defense of the organism against an aggressor agent. For a long time, they were the immune system's main villains due to their characteristics (ELLIOTT et al., 2017). However, the first cells to migrate to the injury site showed remarkable plasticity to establish highly specialized processes, such as pregnancy (FRIDLENDER et al., 2009). IFNT modulates PMN responses by inducing ISGs and immune response-related genes (WALKER et al., 2010) to modulate the maternal immune response. Pregnancy-related factors can modulate PMN phenotype to maintain embryonic and fetal development (KROPF et al., 2007; SSEMAGANDA et al., 2014). Besides that, immune cells exchange factors in communicating and amplifying a signal and modulating the response according to the situation; during pregnancy, *e.g.*, PMNs can induce a modification in lymphocytes phenotype to a more tolerogenic type to help gestation development (NADKARNI et al., 2016).

Pregnancy is a complex process; any stressful factor during the development may culminate in losses. Heat stress severely reduces fertility in cows (HANSEN; ARÉCHIGA, 1999), impairs

embryo development, consequently IFNT production (AMARAL et al., 2020), and alters the immune response of PMNs (LECCHI et al., 2016). These effects may lead to insufficient maternal-conceptus crosstalk resulting in pregnancy losses. Therefore, a successful pregnancy depends on the maternal immune system's ability to change and adapt to each specific developmental stage. Consequently, the aim of this research is: 1) to evaluate the response of PMNs to embryo-derived IFNT and if these cells can amplify and modulate the innate immune response *in vitro*; and 2) to assess *in vivo* embryo-derived IFNT modulation in immune factors under heat stress.

2. LITERATURE REVIEW

2.1 PREGNANCY

Pregnancy is the adequate period for the development of healthy offspring (SPONG, 2013). For the fetus to have a highly adaptive capacity at birth, many development stages shall happen (VEJLSTED, 2010), divided into three periods. In cows, the first stage includes fertilization until Day 13. The second stage is known as the embryonic period initiating on Day 14 until 45. The third stage is denominated fetal stage and goes from Day 46 until the end of the pregnancy, which can last 281 days (NEVES; OLIVEIRA; MACIEL, 2002; VEJLSTED, 2010).

During the embryonic period, pregnancy goes through a critical phase (DEGRELLE et al., 2005) named MRP (SHORT, 1969). In the MRP, the conceptus secretes factors acting autocrine, paracrine, and endocrine (GODKIN et al., 1984; OLIVEIRA et al., 2008; WANG et al., 2013). Although the first stages of uterine remodeling and implantation are programmed by maternal hormones regardless of the presence of the embryo (SANDRA et al., 2015), pregnancy requires conceptus-maternal crosstalk before implantation to generate an MRP signal and regulate gene expression of different cell types (FORDE; LONERGAN, 2017). Embryonic signals promote this communication, which varies according to mammal species, and IFNT is the MRP signal in ruminants (IMAKAWA et al., 1987; SHORT, 1969).

2.2 MATERNAL RECOGNITION OF PREGNANCY

MRP is known as the mechanism by which the embryo signals to the maternal system to help maintain pregnancy (GEISERT et al., 1988; ROBERTS et al., 2008; SHORT, 1969; SPENCER et al., 2007; VALLET et al., 1988), by inhibiting luteolysis process (MARTAL et al., 1998; ROBERTS et al., 2008). MRP occurs early to maintain CL for luteal production of P4 throughout pregnancy in cows (BAZER et al., 1986; THATCHER et al., 1986). Early pregnancy recognition befalls through the secretion of IFNT (IMAKAWA et al., 1987) by elongating trophoblastic cells (FARIN; IMAKAWA; ROBERTS, 1989) and ceases at the beginning of implantation (DEMMERS; DERECKA; FLINT, 2001).

The embryonic signal was known as trophoblastin or trophoblast protein 1 due to the embryonic extracts' ability to prolong the CL's life (BARTOL et al., 1985; GODKIN et al., 1982; HELMER et al., 1987; MARTAL et al., 1979). Studies discovered that trophoblastin had similarities with type I IFNs, such as alpha (A), beta (B), epsilon (E), kappa (K), omega (O) (PESTKA; KRAUSE; WALTER, 2004). The international Society of Cytokines and Interferons later designated it as IFNT (IMAKAWA et al., 1987), IFN produced exclusively by ruminant trophoblast cells (LEAMAN; ROBERTS, 1992).

Type I IFNs belong to a family of cytokines that have a critical role in linking innate and adaptive responses to protect and immunomodulate the organism against viral infection (GONZÁLEZ-NAVAJAS et al., 2012). IFNT has intense antiviral, antiproliferative, and immunomodulatory activities (ROBERTS, 1989). Also, IFNT protects the uterine environment and embryo against viral infections (NISWENDER et al., 2000) and help in the development of tolerance of the maternal response to the semi allogenic concept, *i.e.*, half of its genetic material is from paternal inheritance (BILLINGHAM; BRENT; MEDAWAR, 1953). IFNT gene has a homology of 70% with IFNO, 50% with IFNA, and 25% with IFNB in cattle (LEAMAN; ROBERTS, 1992). IFNT is distinguished from other IFNs by its trophoblast-specific, time-specific, and constitutive transcriptional control variables (EZASHI; IMAKAWA, 2017). Nearly every cell type, including leukocytes, can produce another type I IFNs. Depending on the stimulus and the responding cell type, the signaling pathways that lead to the induction of type I IFNs vary but finally lead to the activation of some common signaling molecules (HÄCKER et al., 2006). Viruses or double-stranded RNA induce IFNA and IFNB expressions for just a few hours. IFNT, in contrast, is not influenced by viruses or double-stranded RNA and is produced for several days (FARIN et al., 1991). Besides that, IFNT shows antiproliferative and antiviral activities with less toxicity than IFNA (PONTZER et al., 1988; SUBRAMANIAM et al., 1995). The onset of gene expression appears to be programmed independently of the uterine environment, but endometrial tissue may influence higher production (KERBLER et al., 1997). The expression of IFNT begins on the fourth day of development *in vitro* (YAO et al., 2009). Protein expression can be detected on the seventh, with peak production on Day 20, extending until Day 25 of gestation when it ceases (FARIN et al., 1990; GODKIN; BAZER; ROBERTS, 1984; GUILLOMOT et al., 1990).

Furthermore, the metabolism, transport, and density of prostaglandins (PGs) and their receptors also appear to be influenced by IFNT (AROSH et al., 2004), suggesting that inadequate

endometrial response to IFNT may be one of the reasons for gestational failure (ASSELIN; LACROIX; FORTIER, 1997). IFNT has an autocrine (trophoblast cells) (BROOKS; SPENCER, 2015; IMAKAWA et al., 2002; WANG et al., 2013), paracrine (endometrium luminal epithelium) (SPENCER; BAZER, 1996), and endocrine manner (extrauterine cells) (OLIVEIRA et al., 2008).

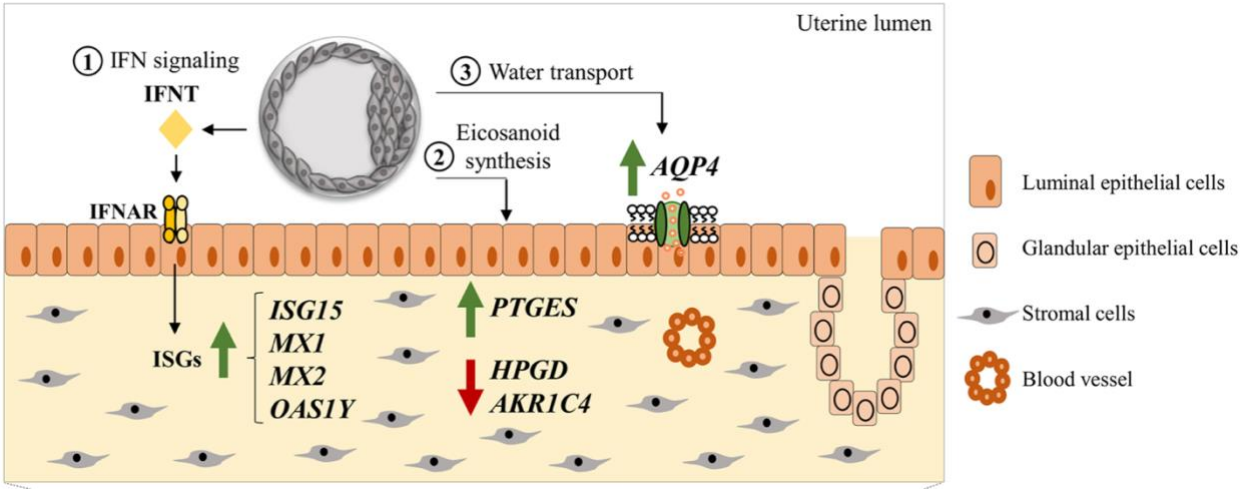
2.3 AUTOCRINE, PARACRINE AND ENDOCRINE SIGNALING

The autocrine effect of IFNT in trophoblast cells promotes embryonic development (BROOKS; SPENCER, 2015; IMAKAWA et al., 2002; WANG et al., 2013). Embryos express IFN receptor (IFNAR) subunit 1 (IFNAR1) and IFNAR2 (IMAKAWA et al., 2002), and inhibition of IFNT or IFNAR1 and IFNAR2 *in vivo* delay embryo development and induces malformation (BROOKS; SPENCER, 2015). As beneficial effects, trophoblast cells have high ISG and connective growth factor (CTGF) expression when cultured with IFNT, helping cell proliferation and embryo elongation (WANG et al., 2013). Thus, it is possible to suggest that the autocrine actions of IFNT involve the promotion of trophoblast differentiation and proliferation (BROOKS; SPENCER, 2015; IMAKAWA et al., 2002; WANG et al., 2013).

IFNT acts on the endometrium in a paracrine manner to suppress the transcription of estrogen receptor (ESR1) and oxytocin receptor (OXTR) and prevent the endometrium from producing luteolytic pulses of PGF without inhibiting its basal production (Figure 1) (SPENCER; BAZER, 1996). Also, IFNT induces the expression of classical ISGs in the endometrium (HANSEN et al., 1999), such as 2'-5'-oligoadenylate synthetase 1 (OAS1), myxovirus resistance protein 1 (MX1), interferon-stimulated gene 15 (ISG15), among others (HANSEN et al., 1999; NAGAOKA et al., 2003a; OTT et al., 1998). For instance, OAS1 can control antiviral responses and help cell growth and differentiation (JOHNSON et al., 2001). MX1 has well-known antiviral properties (LEE; VIDAL, 2002). ISG15 plays an essential role during the establishment and implantation of pregnancy in different cell types. Compared to wild-type mice, ISG15 knockout mice had significant pregnancy losses, indicating a critical role for ISG15 in pregnancy (ASHLEY et al., 2010). Some ISGs may assist the migration of immune cells to the endometrium and stimulate trophoblast cells' growth (NAGAOKA et al., 2003a, 2003b). IFNT also stimulates the expression of non-classical ISGs in the luminal, superficial glandular, and glandular epithelium of the endometrium (BROOKS; BURNS; SPENCER, 2014). The genes expressed are related to

proliferation, migration, fixation, and nutrient transport (SPENCER et al., 2004). Therefore, the paracrine effects of IFNT, through different signaling pathways, stimulate several biological processes during pregnancy development (CHOI et al., 2001; PLATANIAS, 2005; SPENCER et al., 2004).

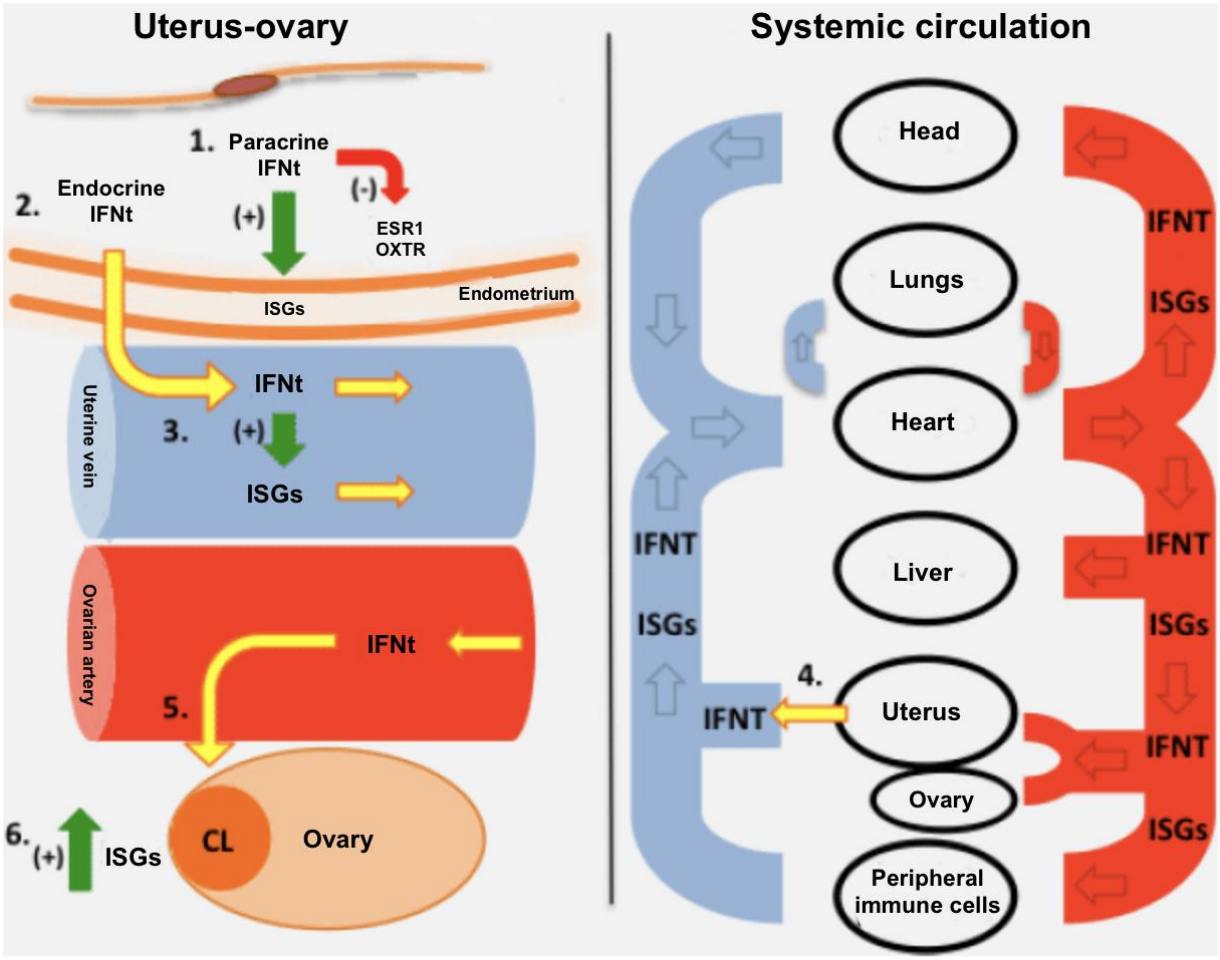
Figure 1. Paracrine mechanism of IFNT.



Source: (SPONCHIADO et al., 2017).

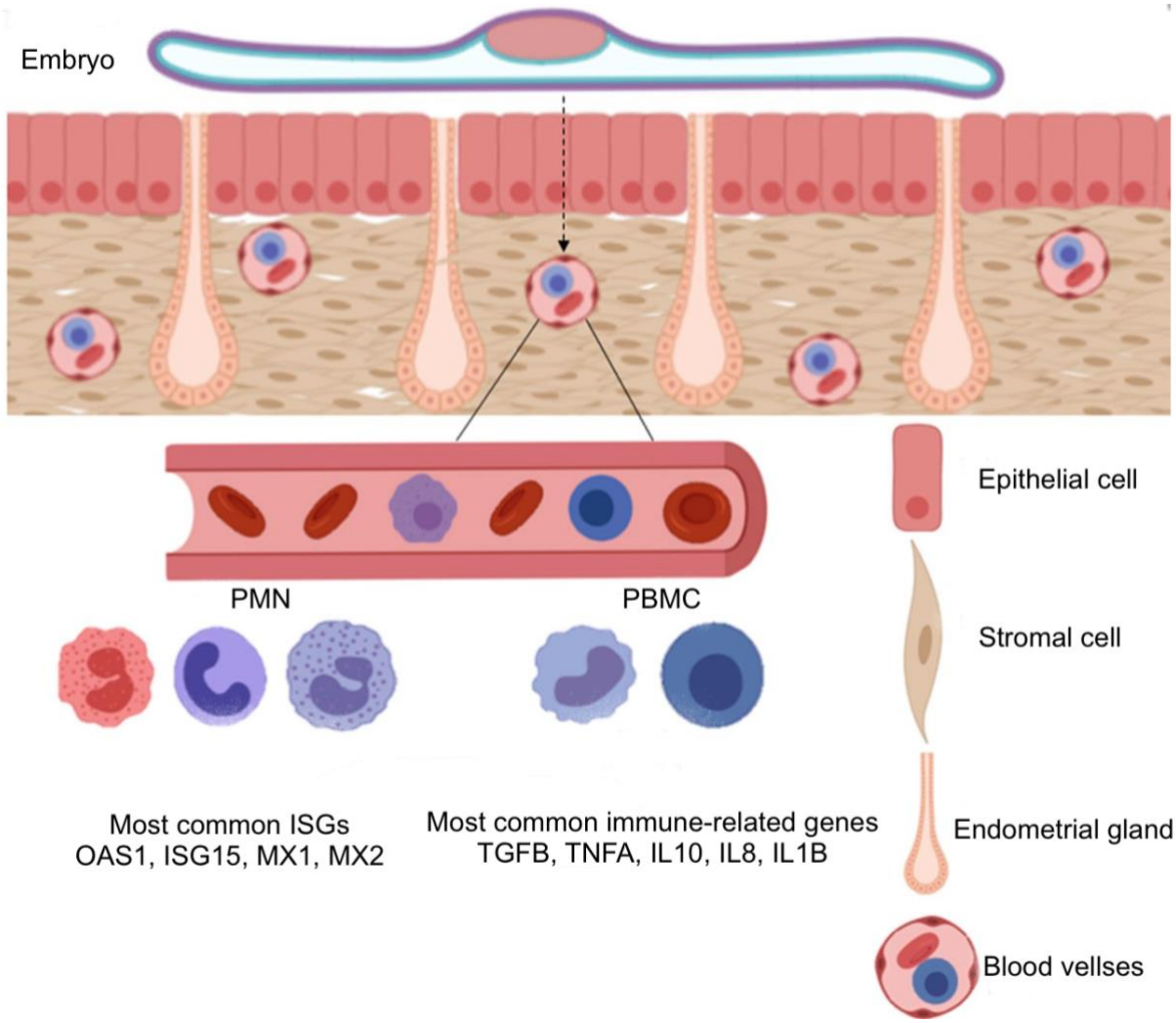
One of the main factors in the conceptus-maternal crosstalk is IFNT, resulting in the rescue of CL, immune cell activation, and recruitment (BAI et al., 2012; BAZER et al., 2015; FAIR, 2016; HANSEN; SINEDINO; SPENCER, 2017). The first report indicating the endocrine activity of IFNT was in females' uterine vein sera 15 days after fertilization (SCHALUE-FRANCIS et al., 1991; BOTT et al., 2010; OLIVEIRA et al., 2008; ROMERO et al., 2015). IFNT enters into the uterine vein and, as a result, stimulates the expression of multiple ISGs and immune-related genes in blood cells to help maintain pregnancy (GREEN et al., 2010; OLIVEIRA et al., 2008; SHIRASUNA et al., 2012; TALUKDER et al., 2017, 2018, 2019). The IFNT signal regulates the expression of interleukin (IL) 8 (IL8) and ISG15 so that their effects allow PMNs to infiltrate the CL during maternal recognition of pregnancy (Figure 2a; 2b) (SHIRASUNA et al., 2015). Collectively, in addition to the modulatory effects on embryo development, endometrial and luteal environments, IFNT also coordinates the maternal immune response during the MRP (HANSEN; SINEDINO; SPENCER, 2017).

Figure 2a. Endocrine mechanism of IFNT in different tissues.



Source: (ANTONIAZZI et al., 2011).

Figure 2b. Endocrine mechanism of IFNT in immune cells.



Source: (ROCHA et al., 2020).

2.4 EARLY PREGNANCY AND THE IMMUNE SYSTEM

Mammals have developed highly complex mechanisms against different disease forms throughout their evolution, detecting and destroying foreign biological material within their organisms (SCHJENKEN; TOLOSA, 2012). These are essential mechanisms that need to be precisely regulated to develop a sufficient and adequate pathogen response while limiting the host organisms' potential harm. For that purpose, the immune system has been adapting for millions of years (ENTRICAN, 2002).

A successful mammalian pregnancy is partly dependent on the release and action of various cytokines and other immunomodulators by conceptus-maternal unit (BILLINGHAM; BRENT;

MEDAWAR, 1953), as well as crosstalk between innate and adaptive immune cells (ARCK; HECHER, 2013). During gestational development, immunological patterns change regulated by conceptus signaling to boost communication with the maternal system (MOR; CARDENAS, 2010). When the whole pregnancy is analyzed, these patterns can alternate between a pro- or anti-inflammatory state. These changes rely on specific mediators (MOR; ABRAHAMS, 2002; ROMERO et al., 2006), like IFNT during the MRP (OTT et al., 2014).

Early pregnancy might be an anti-inflammatory or Th2 type environment, and a sudden shift to a pro-inflammatory Th1 type immune response could lead to complications (REINHARD et al., 1998; WEGMANN et al., 1993). However, current research argues against this notion and has shown a Th1 type environment in healthy pregnancies (GERMAIN et al., 2007; GUPTA et al., 2005). Extensive gene expression changes occur in bovine cells; many of these are ISGs and immune response genes (BAUERSACHS et al., 2006; WALKER et al., 2010). The expression of anti-inflammatory factors, such as IL10 and transforming growth factor-beta (TGFB) and pro-inflammatory like tumor necrosis factor-alpha (TNFA), were reported in cattle (RASHID et al., 2018; SHIRASUNA et al., 2012; TALUKDER et al., 2017). The secretion of IL10 by a diverse set of maternal and conceptus cells helps to orchestrate normal pregnancy. IL10 is a significant player in directing cell differentiation towards a Th2 phenotype (THAXTON; SHARMA, 2010), inhibiting the production of PGs and cytokines and regulating macrophage activation (SVENSSON et al., 2011). TGFB play a significant role in controlling apoptosis and cell survival at specific stages of pregnancy (SHOONER et al., 2005), inhibiting proliferation and differentiation of lymphocytes and the activation of other leukocytes (LETTERIO; ROBERTS, 1998), and inducing differentiation of neutrophils towards an anti-inflammatory phenotype (MISHALIAN et al., 2013). TNFA can be associated with inflammatory mechanisms related to implantation, placentation, and pregnancy outcome (ALIJOTAS-REIG et al., 2017). Also, TNFA levels were higher in the CL of pregnant than in non-pregnant cows, perhaps to help CL formation and maintenance (SAKUMOTO; HAYASHI; TAKAHASHI, 2014).

The expansion of the endometrial stromal macrophage and dendritic cell (DC) populations as early as Day 13 of pregnancy characterizes the maternal immune response to the developing embryo in cattle (MANSOURI-ATTIA et al., 2012). The initiation and maintenance of peripheral tolerance were associated with immature DCs (DIETL et al., 2006) and their presence in large numbers in the uterine decidua during the establishment of healthy pregnancies in women

(TIRADO-GONZÁLEZ et al., 2010). IFNT administration had dramatic effects on the recirculation and redistribution of immune cells by reducing the peripheral circulation of T helper, B cells, and gamma delta T cells without changing the number of T cytotoxic cells (TUO et al., 1999). The number and recruitment of peripheral cells with T regulatory phenotype to the endometrium also increased; complementary abnormal pregnancy is associated with T regulatory cell function inhibition. T regulatory cells secrete IL4 and induce tolerance to paternal alloantigen, helping develop the pregnancy (ALUVIHARE; KALLIKOURDIS; BETZ, 2004). Besides, IFNT and IFNA dose-dependent effects decreased lymphocyte proliferation (SKOPETS et al., 1992). They changed the number, distribution, and activity of natural killer (NK) cells on Day 16 of pregnancy (OLIVEIRA et al., 2013). Many studies provided the importance of immune cells during pregnancy; however, only recently, studies established the PMNs in healthy pregnancies.

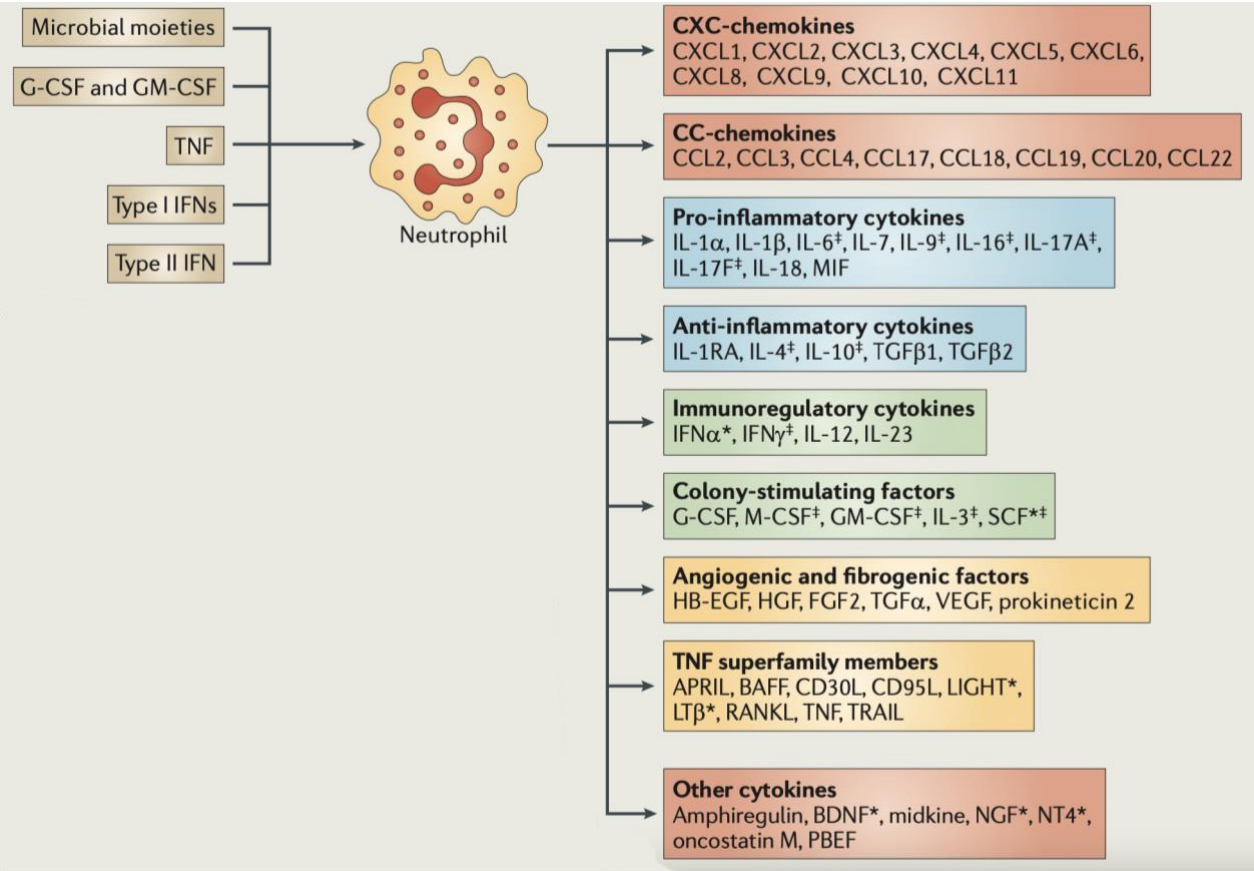
2.5 NEUTROPHILS DURING PREGNANCY

Neutrophils are the main population of immune cells that provide the first defense line during infection to ensure the physiologic state's return (MAYADAS; CULLERE; LOWELL, 2014). These cells migrate to the damaged area and phagocyte, degranulate and destroy the foreign body (BASU et al., 2000; PILLAY et al., 2013). The classical denomination of neutrophils is short-lived cells with three main primary activities: the production and release of 1) granules, 2) oxidative molecules (HEIFETS, 1982), and 3) neutrophil extracellular traps (NETs) (TAKEI et al., 1996). Once neutrophils migrate into damaged tissues, a complex bidirectional interaction with immune and non-immune cells starts (MANTOVANI et al., 2011). Neutrophils modulate the immune system, regulate hematopoiesis, angiogenesis, and wound healing (KOLACZKOWSKA; KUBES, 2013; MANTOVANI et al., 2011; PRUIJT et al., 2002; TECCHIO; CASSATELLA, 2014).

The capacity of PMNs to orchestrate inflammatory and immune responses is dependent on their release of molecules, including cytokines, as well as their ability to interact with other innate and adaptive immune cells (ARCK; HECHER, 2013; LOOD et al., 2020). PMNs can produce numerous cytokines, which can be anti- and pro-inflammatory cytokines (Figure 3) (MANTOVANI et al., 2011) essential role in pregnancy. Pregnant cows PMNs presented an anti-inflammatory response with greater expression of TGFB, IL10, and forkhead box P3 (FOXP3) (TALUKDER et al., 2019). Depending on their activation status (NADKARNI et al., 2016),

neutrophils can regulate B cell antibody production (PUGA et al., 2012), T cell suppression (PILLAY et al., 2012), DC antigen presentation (VAN GISBERGEN et al., 2005), NK activation, survival, proliferation, cytotoxic activity, and IFN gamma (IFNG) production (COSTANTINI; CASSATELLA, 2011). PMNs can regulate Th1 and Th17 recruitment via the release of C-C motif chemokine ligand (CCL) 2 (CCL2), chemokine (C-X-C motif) ligand (CXCL) 9 (CXCL9), and CXCL10 or CCL2 and CCL20, respectively (PELLETIER et al., 2010). Also, PMNs can induce T regulatory cells with a proangiogenic phenotype that aids pregnancy development (NADKARNI et al., 2016).

Figure 3. Cytokine's repertoire of PMNs.



Source: (MANTOVANI et al., 2011).

Polymorphonuclear cells contribute to conception, implantation establishment, and embryo protection (GIAGLIS et al., 2016). During pregnancy, oxidative burst and intracellular hydrogen peroxide production by PMNs were significantly decreased (CROUCH; CROCKER; FLETCHER, 1995). The crucial role in pregnancy was detected when PMNs depletion led to placental development impairment and reduced the number of viable offspring (HIGASHISAKA et al., 2018). PMNs may present two types of phenotypes in pregnancy (SSEMAGANDA et al., 2014). These PMNs polarize from one type to another, depending on the stimuli, being classified as low-density neutrophils (LDN), representing anti-inflammatory response type (N2), or high-density neutrophils (HDN), representing pro-inflammatory response type (N1) (FRIDLENDER et al., 2009). LDN promotes tissue growth through cytokine secretion, increased angiogenesis, and extracellular matrix modulation (GRANOT; JABLONSKA, 2015). Besides, the LDN can be immature, derived from myeloid cells and mature cells (SAGIV et al., 2015). These PMNs have as characteristic the high expression of arginase-1 (ARG1), CCL2, CCL5, and vascular endothelial

growth factor (VEGF), and the ability to inhibit T cell functions (FRIDLENDER et al., 2009). Conversely, HDN limits cellular proliferation (FINISGUERRA et al., 2015). The HDN phenotype has a hyper-segmented nucleus, high expression of CCL3, cluster of differentiation (CD) 54 (CD54), inducible nitric oxide synthase (iNOS) and TNFA, and the ability to activate T cytotoxic cells (FRIDLENDER et al., 2009). PMNs polarization might be via TGFB, granulocyte colony-stimulating factor (GCSF), and IFNB. TGFB and GCSF modulate the anti-inflammatory phenotype (CASBON et al., 2015; FRIDLENDER et al., 2009; WAIGHT et al., 2011), while IFNB acts as a regulator of a pro-inflammatory phenotype (JABLONSKA et al., 2010; WU et al., 2015). During human and rodent pregnancies, changes in lineage markers such as CD15 and ARG1, maturation, and activation markers such as CD16, CD62L, CD54, and CD63 demonstrate the type of response of PMNs (KROPF et al., 2007; SSEMAGANDA et al., 2014). Normal density neutrophils (NDN) and LDN are present in peripheral circulation, umbilical cord, and placenta (SSEMAGANDA et al., 2014). NDN has a higher expression of CD16 and ARG1, while LDN had a higher expression of CD15 and a lower expression of CD62L. Regulation of phagocytosis, oxidative burst, and degranulation in PMNs might be via CD16 (MISHALIAN et al., 2013; SALMON; EDBERG; KIMBERLY, 1990). ARG1 greater expression may contribute to maternal immune response suppression (KROPF et al., 2007). LDN greater expression of degranulation marker CD15 (NAKAYAMA et al., 2001), and lower expression of CD62L could indicate less migration and activation (VENTURI et al., 2003).

Besides that, problems like delayed apoptosis of neutrophils in normal pregnancy may contribute to pregnancy-associated neutrophilia and pregnancy-induced inflammatory changes (GILBERT, 2011). Therefore, tight control of neutrophils' function in pregnancy should happen since enhanced inflammatory responses lead to pregnancy complications.

2.6 HEAT STRESS AND IMMUNE RESPONSE DURING PREGNANCY

The immune status and productivity of animals can be affected by several environmental factors; heat stress dominates as a negative effect on animal health performance around the globe (DAHL; TAO; LAPORTA, 2020). Animals can acclimate to environmental heat by reducing heat gain and increasing heat dissipation (FUQUAY, 1981). High temperature alters follicular development (ROTH, 2015), steroidogenesis (BRIDGES; BRUSIE; FORTUNE, 2005), decreases

the quality of females and male gametes (AL-KATANANI; PAULA-LOPES; HANSEN, 2002; LUCIO et al., 2016). Heat stress has the potential to disrupt the process of interactions between the uterus and the embryo, consequently compromising the establishment and maintenance of pregnancy (THATCHER; MEYER; DANET-DESNOYERS, 1995). Heat stress affects cleavage rate and the cleaved embryos' ability to become a blastocyst during oocyte maturation, fertilization, and embryo development and reduces IFNT expression on Day 7 embryo. In the early stages of embryo production, reactive oxygen species (ROS) rates increased on Day 7 embryos under heat stress, specifically on oocyte maturation and fertilization and first-day zygote stages (AMARAL et al., 2020).

Besides that, heat stress can adversely affect animal growth and the immune competence to certain bacterial or viral infections (GOLIGORSKY, 2001). It may influence the weight of both primary and secondary lymphoid organs, profiles of circulating leukocytes, and antibody response (LIEW et al., 2003). The number of leukocytes appears to differ in terms of results. In summer, the mean neutrophil count was significantly higher than in winter (NAIK et al., 2013), while (NARAYAN; SINGH; SHARMA, 2007) observed a reduction in the neutrophil count during summer in cows. ROS production and phagocytosis in PMN exposed to high temperatures are impaired, which may explain the more significant occurrence of infections during hot weather periods (LECCHI et al., 2016).

Heat stress is associated with reducing Th1 cytokines and increasing Th2 cytokines, suppressing cellular-mediated immunity (LACETERA et al., 2005). It also impairs the cellular immune response by increasing the cortisol concentration (SGORLON et al., 2012). During periods of acute stress, cortisol production acts as an immune system stimulant, but its secretion has caused immune suppression during chronic stress (JU et al., 2014). The anti-inflammatory properties of corticosteroids decrease phagocytic cell activity and alter lymphocyte function (CAROPRESE et al., 2009). Chronic heat stress negatively affects the immune system. It increases infection susceptibility by increasing the number of T regulatory cells and greater expression of IL10 and TGFB associated with adaptive immune response suppression (MENG et al., 2013).

Therefore, heat stress environment alters essential reproductive functions and modifies embryonic development and immunological status, resulting in poor communication between mother and fetus, leading to gestational losses.

3. CHAPTER I – NEUTROPHILS RECOGNIZE AND AMPLIFY IFNT SIGNALS DERIVED FROM DAY 7 BOVINE EMBRYO FOR STIMULATION OF ISGS EXPRESSION *IN VITRO*: A POSSIBLE IMPLICATION FOR THE EARLY MATERNAL RECOGNITION OF PREGNANCY

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Neutrophils recognize and amplify IFNT signals derived from day 7 bovine embryo for stimulation of ISGs expression *in vitro*: A possible implication for the early maternal recognition of pregnancy

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Abstract

Previously, we reported that the presence of multiple day 7 (D7) bovine embryos in the uterus induces systemic immune responses in circulating polymorphonuclear neutrophils (PMNs), but with unknown mechanism. Thus, this study aimed to investigate the direct impact of D7 bovine embryo on PMNs' immune responses *in vitro* and whether these PMNs can amplify and transfer embryo signals further to another PMN population. PMNs were directly stimulated by embryo culture media (ECM) or interferon tau (IFNT) (10 ng/ml) followed by evaluating mRNA expression by real-time PCR and phenotypic analysis by flow cytometry. To test whether PMNs can transfer embryo signals to a new PMN population, PMNs triggered by ECM or IFNT, were thoroughly washed and diluted to remove any media components, and again were incubated in fresh culture media for 3 h, from which culture supernatants were collected and used as PMN conditioned media (CM) to stimulate a new PMN population. Similar to ECM, IFNT directly stimulated expressions of IFNs (*IFNA*, *IFNG*), interferon-stimulated genes (*ISGs*; *OAS1*, *ISG15*, *MX1*), *STAT1*, *TGFB* and *IL8*, and downregulated *TNFA* in PMNs. Flow cytometrical analyses demonstrated that IFNT stimulated expressions of pregnancy-related phenotypic markers, CD16 and arginase-1 (ARG1), in PMNs. Most importantly, PMN CM induced *ISGs* and *STAT1 mRNA* in fresh PMNs. Since IFNT directly upregulated *IFNA* expression in PMNs, the impact of IFNA on PMNs' immune responses was further tested. Stimulation of PMNs with IFNA, especially at a low level (1 pg/ml), induced IFNT-like immune responses comparable to those induced by PMN CM. Together, these findings indicated that D7 bovine embryos induce direct anti-inflammatory responses with upregulation of *ISGs* expressions in PMNs mainly *via* IFNT. Additionally, PMNs can amplify and transfer embryo signals to a new PMN population in a cell-to-cell communication mechanism possibly mediated in part by IFNA. Such a novel immunological crosstalk might contribute to embryo tolerance and pregnancy establishment in cattle.

Keywords: PMNs, Interferon-tau, Embryo, ISGs, innate immunity, bovine

1. Introduction

Pregnancy establishment requires a transient modulation of the innate and adaptive maternal immunity for tolerating the semi-allogenic embryo and supporting embryo development and implantation [1]. Thus, various immunological interactions between conceptus and mother have been established, resulting in a shift in Th1/Th2 balance [2]. Interferon tau (IFNT) is the main intermediator for maternal recognition of pregnancy in ruminants which is exclusively produced by embryo trophoblast cells as early as D7 [3], detected in the circulation during conceptus elongation period (day12-15) [4], and reaches the highest production around day 18 [5], when clear effects on circulating immune cells have been reported [6–8].

IFNT is a member of the Type I IFN family [9], which possesses antiviral, antiproliferative and immune modulatory properties [10]. IFNT stimulates ISGs expressions in the uterus [11], corpus luteum [12], polymorphonuclear neutrophils (PMNs), and peripheral blood mononuclear cells (PBMCs) [3, 6, 8, 11] *via* janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway. Similarly, other type I IFNs, such as IFNA and IFNB, can induce ISGs expressions [13]. During early pregnancy in ruminants, ISGs modulate the uterine innate immunity [3] and stimulate hyperplasia of the endometrial glands, stromal remodeling, and development of the uterine vasculature [14].

PMNs are the main population of circulating leukocytes comprising the first line of the innate immune defenses [15]. PMNs also regulate adaptive immune responses, including antibody production, T-cell suppression, and antigen presentation by dendritic cells [15]. Additionally, PMNs play a crucial role in pregnancy recognition. Thus, they are the first immune cells to sense embryo implantation in the uterus [7]. When a portion of IFNT enters the general circulation, it directly upregulates ISGs expressions in PMNs [6, 8, 16]. Furthermore, IFNT indirectly modulates PMN response by inducing the expression of interleukin 8 (IL8), a chemoattractant for PMNs, supporting luteal function by enhancing neutrophilic migration [17]. In human gestation, two types of phenotypically distinct neutrophils were detected [18]. Additionally, PMNs

induced T regulatory cells with a proangiogenic phenotype to support fetal development [19]. Pregnancy can modulate PMNs expressions of some phenotypic markers such as CD15, CD16, CD63, CD62L, CD54, and arginase-1 (ARG1) [18, 20].

Previously, we reported that IFNT derived from D7 embryo induces anti-inflammatory responses with upregulation of ISGs expressions locally in uterine epithelial cells and PBMCs [11] and these signals were amplified and transferred to circulating PBMCs and PMNs with the presence of multiple D7 embryos but with unknown mechanism [3]. Furthermore, it is believed that resident immune cells in the uterus can recognize the presence of the embryo, amplifying, and transferring embryo signals to effector cells and these cells enter the circulation priming other cells to affect their functions [21]. However, the immunological interactions between D7 bovine embryos and local PMNs and their impact on PMN functions and communication is still unclear. Therefore, this study aimed to investigate the direct impact of IFNT, derived from D7 embryos, on PMNs immune responses and phenotyping. Additionally, we tested whether PMNs, as the first responder cells in the innate immune system, can amplify and transfer embryo-derived IFNT signals to a new PMN population through a cell-to-cell communication mechanism.

2. Material and Methods

2.1 Ethics statement

All experimental procedures described in this manuscript were conducted following the Guiding Principles for the Care and Use of Research Animals Promulgated by Obihiro University of Agriculture & Veterinary Medicine, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments of Obihiro University (Permit number 27-74).

2.2 Experimental model

Initially to investigate the direct impact of the D7 embryo on PMNs immune responses, blood-isolated PMNs were stimulated with ECM, or IFNT (10 ng/ml) for 3 h. Then, PMNs were thoroughly washed and diluted (dilution factor >6.26×10^6 times), to remove any media components, and again were incubated in fresh culture media for further 3 h. The culture supernatants, referred as PMN conditioned media (CM), were collected and kept for the next use to test whether PMNs can transfer IFNT signals to a new PMN population through their culture medium (Fig. 1A). Next, to mimic a cell-to-cell communication system *in vitro*, the PMN CM was used to stimulate a new PMN population for 6 h (Fig. 1B). Cells were analyzed for gene expression by real-time PCR. Also, to characterize PMNs phenotyping, the expression of surface markers such as CD15, CD16, CD63, CD62L, CD54 and the intracellular marker, ARG1, were analyzed by flow cytometry.

2.3 Production of ECM *in vitro*

The *in vitro* maturation (IVM) and fertilization (IVF) were conducted as previously described [22]. Briefly, bovine cumulus oocyte complexes (COCs) were maturated *in vitro* for 22 h in 199 media (HP-M199; Research Institute for the Functional Peptides) containing 10 ng/ml epidermal growth factor (E4127, Sigma-Aldrich) and 10% FBS. IVF was performed by co-culturing of 15 *in vitro*-matured COCs and sperm ($5x10^6$/ml, Day 0) in 100 µl droplets of IVF-100 medium (Research Institute for the Functional Peptides) under mineral oil for 6 h. Presumptive zygotes (n = 25-30) were shifted to 400 µl droplets of BO-IVC medium (IVF-bioscience) under mineral oil at 38.5 °C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for *in vitro* culture (IVC) until D7 where IVC media were collected and kept in - 80 °C as embryo culture conditioned media (ECM). The average blastocyst rates were 35.25 ± 10.16 %.

2.4 Isolation of PMNs

Isolation of PMNs were conducted as previously described [17]. Briefly, heparinized blood from multiparous Holstein cows was collected, diluted with PBS, and slowly layered over ficoll-paque solution (Axis-Shield) and centrifuged for 35 min at 1000 *g*. PMNs were collected from the lower red layer, then mixed with hemolysis buffer (NH_4Cl 155 mM, $KHCO_3$ 9.9 mM, EDTA 96.7 uM) and centrifuged for 5 min at 500 *g* to purify from red blood cells. Subsequently, the cell pellet was washed and suspended in RPMI-1640 medium (Sigma-Aldrich), supplemented with 0.1% heat-inactivated, exosome-depleted, fetal bovine serum (System Bioscience). The purity of collected cells was > 95% as evaluated by forward scatter, and side scatter parameters of flow cytometry and the viability was around 95% as assessed by Trypan blue staining and Caspase 3 mRNA expressions throughout the whole *in vitro* cultivation.

2.5 Stimulation of PMNs

PMNs (7×10^6 cells) were stimulated either with ECM or IFNT at 10 ng/ml (recombinant bovine IFNT (rbIFNT) 2B; Zenoaq) for 3 h. Next, cells were washed 4 times with PBS (1:50) and diluted (> 6.25×10^6 times) to remove any media component, and again cultured in freshly prepared media for additional 3 h. Later on, PMN culture supernatant was collected and kept in -80 °C as PMN CM for further use in the stimulation of new sets of PMNs (cell-to-cell communication system). The cells were either lysed using Trizol reagent (Thermo Fisher Scientific) and stored at - 80 °C for RNA extraction, or stained with specific antibodies for flow cytometry.

2.6 Quantitative real-time PCR

RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed following the protocol described [22]. Succinctly, total RNA was extracted using the Trizol reagent (Thermo Fisher Scientific). Total RNA concentration was measured using a spectrophotometer (Eppendorf, Munich, Germany), and used for cDNA synthesis as previously described [22]. Quantitative real-time PCR of

targeted genes (Supplementary table 1) was performed using SYBR Green PCR Master Mix (Bio-Rad Laboratories) by using CFX Connect™ Real Time PCR detection system (Bio-Rad Laboratories). The amplification program was set up according to the protocol [22]. The calculated cycle threshold values were normalized using *ACTB* as the internal reference gene by applying the Delta-Delta comparative threshold method to quantify the fold change between samples.

2.7 Flow cytometry

The expression of some phenotypic markers in PMNs was determined using monoclonal or polyclonal antibodies: CD16-AF647 (MCA5665A647, AbD Serotec), CD15-PE (ABIN6945662, Antibodies Online), CD63-FITC (MCA2042GA, AbD Serotec), CD62L-FITC (MCA1649F, AbD Serotec), CD54-PE (ABIN192140, Antibodies Online), and ARG1-FITC (ab96183, Abcam) according to the manufacturer's protocol. PMNs (1×10^6) were incubated with fluorochrome labeled antibodies in PBS containing 1% of bovine serum albumin and kept for 30 min in the dark (4 °C). Then, labeled cells were washed once, fixed with 0.5% paraformaldehyde, and analyzed using the SA3800 Spectral Analyzer (Sony Biotechnology, Tokyo, Japan). For unconjugated antibodies, cells were incubated with fluorochrome-labeled secondary antibodies for further 30 min. For intracellular staining of ARG1, Leucoperm™ (Bio-Rad Laboratories) was used following manufacturer instructions. The Live/Dead cells were distinguished by adding propidium iodide (PI; 2 µg/ml, Calbiochem).

2.8 ELISA

Specific ELISA kits were used for determination of IFNA and IFNB (Biomatik; EKL58039, EKL58048, respectively) concentrations in PMN culture media, following the manufacturer's instructions. An ELISA microplate reader (Labsystem Multiskan MS 352) was used to detect the optical density (OD)

value at 450 nm wavelength. The standard curves were prepared in the range of 1000 – 15.6 pg/ml for IFNA and IFNB.

2.9 Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) of 4–5 independent experiments. Statistical analyses were done using GraphPad Prism 9.0 software (La Jolla, CA, USA) to analyze differences among groups by performing One-way ANOVA followed by Tukey's test (>two groups) or Student's t-test (two groups). Data were considered to be statistically significant at $p < 0.05$.

3. Results

3.1 ECM induced *ISGs* expression in PMNs

The direct stimulation of PMNs with ECM obtained from D7 embryos upregulated gene expressions of *IFNs* (*IFNA*, and *IFNG)*, *ISGs* (*ISG15*, *OAS1*, and *MX1*), *STAT1*, *TGFB*, and *IL8* in PMNs, while *TNFA* was downregulated (Fig. 2A). Additionally, culturing of a new PMN population in PMN CM, obtained by culturing of ECM-triggered PMNs in fresh media for further 3 h, resulted in upregulation of mRNA expression of *ISGs* and *STAT1* in PMNs (Fig. 2B).

3.2 IFNT induced *ISGs* expressions in PMNs

Likewise, results revealed that the direct stimulation of PMNs with IFNT (10 ng/ml) stimulated the expression of *IFNs*, *ISGs*, *STAT1*, *TGFB* and *IL8*, and downregulated *TNFA* (Fig. 3A1), demonstrating similar gene expression patterns with ECM. Moreover, Real-time PCR analysis of cells cultured in PMN

CM, obtained by culturing of IFNT-triggered PMNs in fresh media for further 3 h, stimulated *ISGs* and *STAT1* mRNA expressions (Fig. 3B1).

3.3 IFNT induced expressions of phenotypic markers, CD16 and ARG1, in PMNs

We analyzed the expression of surface markers, CD16, CD15, CD63, CD62L and CD54, and the intracellular marker ARG1 in PMNs stimulated with either IFNT (Fig. 5A.2) or IFNT-triggered PMN CM (Fig. 3B.2). Direct stimulation with IFNT induced expressions of CD16 and ARG1. IFNT-triggered PMN CM increased CD16 expression in PMNs. No differences were found in CD15, CD63, CD62L, and CD54 expressions compared to control.

3.4 IFNA induced *ISGs* expression in PMNs

Since direct stimulation of PMNs either by ECM or IFNT stimulated mRNA expressions of other type I IFNs, such as IFNA and IFNB which can induce ISGs expressions [13], we hypothesized that PMNs can transfer embryo signals partially *via* induction of other type I IFNs. However, we could not quantify very low levels of IFNA and IFNB proteins in PMN culture media using ELISA, which might be due to the short time-period of cultivation as protein accumulation takes longer than upregulation of cytokine genes. The well-known short half-life of PMNs (max. 8 h) and their high tendency to undergo rapid apoptosis during *in vitro* culture [15, 17] restricted the extension of PMN cultivation time over than 6 h. PMNs were stimulated with the most upregulated IFNs, IFNA, at 1, 10, 100 or 1000 pg/ml (rbIFNA; Kingfisher Biotech) for 6 h and gene expressions were analyzed using real-time PCR. The results showed that IFNA induced IFNT-like responses inducing mRNA expressions of *ISGs*, *STAT1*, *IFNA* and *TGFB* in PMNs (Fig. 4).

4. Discussion

The present study provides *in vitro* evidence that the D7 embryo-derived IFNT generates anti-inflammatory responses with upregulation of *ISGs* expressions in PMNs which subsequently amplify and transfer IFNT signals to a new PMN population through the cell-to-cell communication mechanism possibly mediated by IFNA. One could argue that this effect might be due to the contamination of PMN CM by IFNT residues. In the present model, PMNs were thoroughly washed and diluted ($> 6.25\times10^6$ times) to remove any media components before the preparation of PMN CM and therefore the expected IFNT residues in such CM would be extremely low (around 1-2 fg/ml). Moreover, the addition of a specific IFNT neutralizing antibody, that exclusively recognizes IFNT but not IFNA, into PMN CM did not disrupt their immune responses in new PMNs (data not shown) confirming that IFNT was completely removed from PMN CM. Besides, this study reports novel findings for the ability of IFNT to induce a phenotypic differentiation of PMNs populations towards N2 anti-inflammatory neutrophils through upregulation of CD16 and ARG1 expressions in PMNs. These findings might lead a new hypothesis for the novel mechanism by which D7 embryo, *via* IFNT, can modulate the local innate immune responses and communicate with other immune cells for embryo tolerance and pregnancy establishment in cattle.

The current results showed that the direct stimulation of PMNs with D7 embryo culture media induced upregulation of *IFNs*, *ISGs*, and *STAT1* and generated anti-inflammatory responses through upregulation of the anti-inflammatory cytokine, *TGFB*, and downregulation of the pro-inflammatory cytokine, *TNFA*, possibly to support pregnancy tolerance, since TNFA can impair embryo development and implantation [23]. Additionally, the expression of the PMNs chemoattractant, *IL8*, was upregulated. Likewise, PMNs' immune responses to IFNT were relatively similar to those induced by embryo culture media, indicating that D7 embryo-PMNs interactions were mediated mainly by IFNT. In parallel, similar immune responses were reported in uterine epithelial cells and PBMCs stimulated by IFNT *in vitro* [11]. Together, it seems that at the embryo-maternal interface, the pre-hatching bovine embryo secretes minute amounts of IFNT locally into the uterine lumen which potentially modulate the local uterine immunological milieu for

252 accepting this semi-allogenic embryo and induction of a state of immune tolerance essential for embryo
253 survival and pregnancy establishment.

254 In human, pregnancy can change the phenotyping of PMN populations in a mechanism that contributes
255 to the suppression of the maternal immune system [18]. In the present study, the direct stimulation with
256 IFNT induced CD16 and ARG1 expressions in PMNs suggesting a potential shift of PMNs phenotyping
257 into anti-inflammatory N2 neutrophils. This might be due to the upregulation of *TGFB* expression, a well-
258 known Th2 cytokine, which can induce differentiation of neutrophils towards an N2 pro-tumorigenic
259 phenotype [24]. The increased expression of ARG1 might impact maternal immune response and fetal
260 development [18]. The surface marker, CD16, is known to be involved in phagocytosis, respiratory burst,
261 and degranulation in PMNs [25]. Similarly, it has been shown that CD16 expression increased after
262 stimulation with IFN [26].

263 Cell communication networks are complex; constantly, immune cells exchange signaling molecules to
264 communicate with each other through different mechanisms including direct contact, soluble factors
265 exported from cells as cytokines, or extracellular membrane vesicles [15]. The current results showed that
266 PMN CM induced IFNT-like responses in PMNs, suggesting that PMNs can amplify and transfer embryo-
267 derived IFNT signals to a new PMN population through their culture medium. Given that direct stimulation
268 of PMN by either ECM or IFNT induced upregulation of mRNA expressions of other type of I IFNs, it is
269 possible that IFNT signals could be amplified in PMNs *via* other types of IFNs, especially IFNA.
270 Interestingly, direct stimulation of PMNs with very low levels of IFNA (1 pg/ml), below the detectable
271 range of ELISA kits, induced IFNT-like immune responses comparable to those induced by PMN CM,
272 suggesting that IFNA partially mediates the amplification of embryo-derived IFNT signals within PMNs
273 *via* a cell-to-cell communication mechanism. Some interferons are active in healthy pregnancy [14], and
274 administration of IFNA into the uterine lumen generated antiviral activity comparable to early pregnancy
275 [13]. The findings that IFNA expression was induced later on during pregnancy in cows [27], could be on
276 the line of the above hypothesis that other IFNs are involved in indirectly amplifying embryo signals.

Possibly, other cell communication mechanisms especially exosomes are involved in such PMN-to-PMN interaction due to their ability to transfer biomolecules including lipids, proteins, DNAs, mRNAs, and microRNA between the cells either locally or systemically [28] which require further investigations.

Altogether, our findings demonstrate that D7 bovine embryo can modulate PMNs immune responses and induce functional polarization of neutrophils into anti-inflammatory subpopulations encouraging embryo tolerance. Of note, PMNs can amplify and transfer embryo signals into a new cell population partially mediated by IFNA. Such mechanisms of immune cell communications, might temporarily maintain immune tolerance by expanding embryo signals to the extrauterine environment until day 12-15 of pregnancy when IFNT is released into the circulation. Further investigations are needed to identify this hypothesis under the real *in vivo* conditions in cows.

Declaration of Conflict of Interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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Figures legends

Fig. 1. Schematic representation of the experimental design. (A) PMNs, isolated from blood, were stimulated with ECM or IFNT (10 ng/ml) for 3 h. After, cells were washed 4 times (1:50) in order to remove any media components from the cells. PMNs were re-incubated in freshly prepared media for further 3 h. (B) Supernatants were collected and used as PMN conditioned media (CM) for stimulation of a new PMNs population for 6 h in cell-to-cell communication experiment. Later on, cells were analyzed for gene expression by RT-PCR and phenotypic markers by FCM.

Fig. 2A. ECM induced *ISGs* mRNA expressions in PMNs. Fig. 2B. PMN conditioned media stimulated *ISGs* mRNA expressions in PMNs. Relative mRNA expressions of IFNs (*IFNA*, *IFNB1*, and *IFNG*), ISGs (*OAS1*, *ISG15*, and *MX1*), *STAT1*, anti-inflammatory cytokine (*TGFB*), and pro-inflammatory cytokines (*TNFA*, and *IL8*) in PMNs. Data are presented as mean ± SEM of 5 independent experiments. Different number of asterisks denote a significant variance between the different groups (*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001).

Fig. 3A.1 IFNT induced *ISGs* mRNA expressions in PMNs. Fig. 3B.1 PMN conditioned media stimulated *ISGs* mRNA expression in PMNs. Relative mRNA expressions of IFNs (*IFNA*, *IFNB1*, and *IFNG*), ISGs (*OAS1*, *ISG15*, and *MX1*), *STAT1*, anti-inflammatory cytokine (TGFB), and pro-inflammatory cytokines (*TNFA*, and *IL8*) in PMNs. **Fig. 3A.2. IFNT induced CD16 and arginase-1 (ARG1) expression in PMNs. Fig. 3B.2 PMN conditioned media increased CD16 expression.** Expression of phenotypic markers, CD54, CD62L, CD16, CD15, CD63, and ARG1 in PMNs. Data are presented as mean ± SEM of 4 independent experiments. Different number of asterisks denote a significant variance between the different groups (*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001).

Fig. 4. IFNA induced *ISGs* mRNA expressions in PMNs. Relative mRNA expressions of IFNs (*IFNA*, *IFNB1*, and *IFNG*), ISGs (*OAS1*, *ISG15*, and *MX1*), *STAT1*, anti-inflammatory cytokine (*TGFB*), and pro-

inflammatory cytokines (*TNFA*, and *IL8*) in PMNs. Data are presented as mean ± SEM of 4 independent experiments. Different number of asterisks denote a significant variance between the different groups (*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001).

Supplementary table 1. List of primers used in real-time PCR

Gene	Sequence of nucleotide (5'- 3')	Accession number
ACTB	F: TCACCAACTGGGACGACATG	NM_173979.3
	R: CGTTGTAGAAGGTGTGGTGCC	
IFNA	F: TCTGCAAGAGAAGAGACACAGC	NM_001017411.1
	R: TCTCCTGAAACTCTCCTGCAAG	
IFNB1	F: TGCCTGAGGAGATGAAGCAAG	NM_174350.1
	R: TGAGAATGCCGAAGATGTGC	
IFNG	F: AGAATCTGTGGGTTGTGCAC	NM_174086.1
	R: TCAAGTGCTGTCTGACATGC	
OAS1	F: TAGGCCTGGAACATCAGGTC	NM_001040606.1
	R: TTTGGTCTGGCTGGATTACC	
ISG15	F: TCTGAGGGACTCCATGACGG	NM_174366
	R: TTCTGGGCGATGAACTGCTT	
MX1	F: GTACGAGCCGACTTCTCCAA	NM_173940.2
	R: ATGTCCACAGCAGGCTCTTC	
STAT1	F: CTCATTAGTTCTGGCACCAGC	NM_001077900.1
	R: CACACGAAGGTGATGAACATG	
TNFA	F: CAAAAGCATGATCCGGGATG	NM_173966.3
	R: TTCTCGGAGAGCACCTCCTC	
TGFB	F: CTGCTGAGGCTCAAGTTAAAAGTG	NM_001166068.1
	R: CAGCCGGTTGCTGAGGTAG	
IL8	F: CCTCTTGTTCAATATGACTTCCA	NM_173925
	R: GGCCCACTCTCAATAACTCTC	

Fig. 1

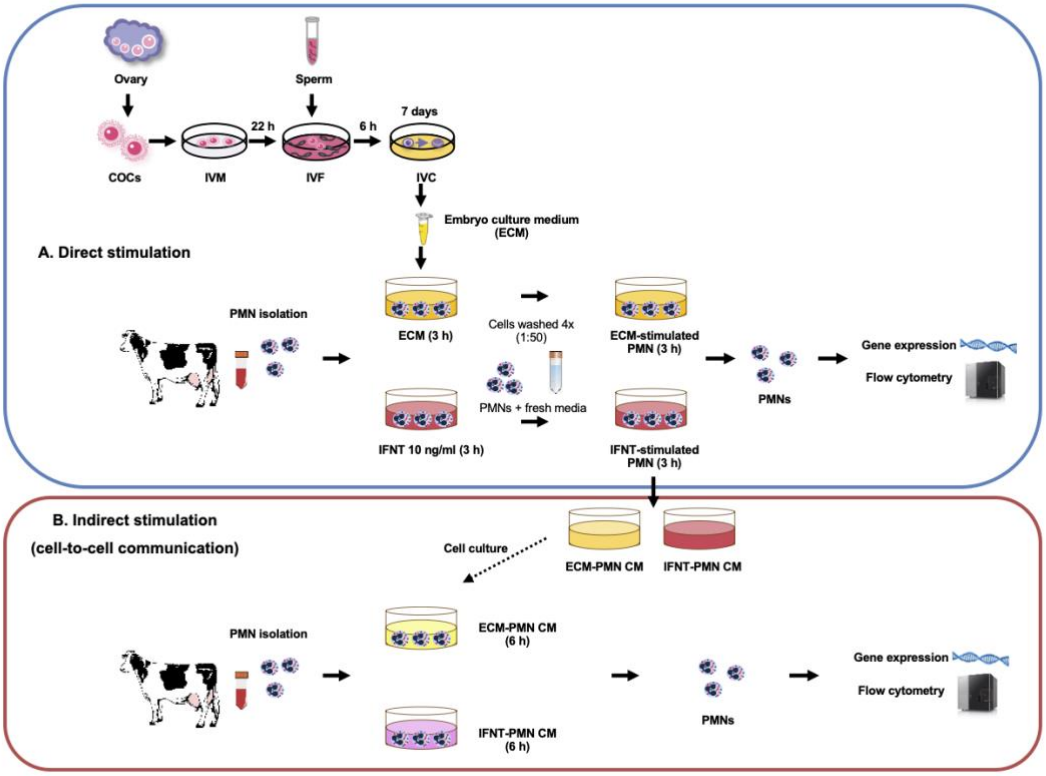
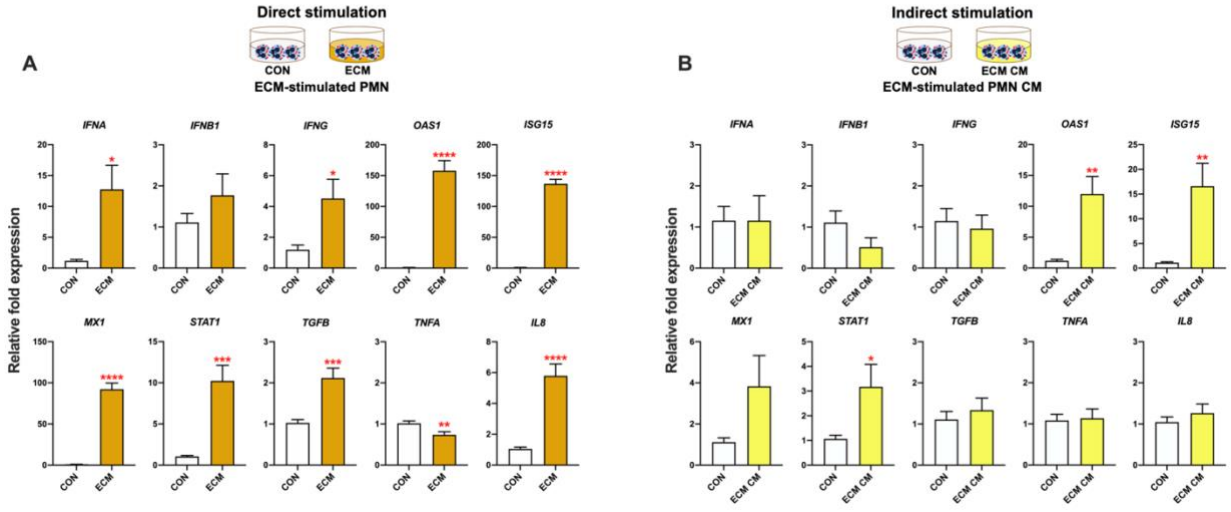

Ovary
Sperm
22 h
6 h
7 days
COCs
IVM
IVF
IVC
Embryo culture medium (ECM)
A. Direct stimulation
PMN isolation
ECM (3 h)
Cells washed 4x (1:50)
ECM-stimulated PMN (3 h)
PMNs
Gene expression
Flow cytometry
PMNs + fresh media
IFNT 10 ng/ml (3 h)
IFNT-stimulated PMN (3 h)
B. Indirect stimulation
(cell-to-cell communication)
Cell culture
ECM-PMN CM
IFNT-PMN CM
PMN isolation
ECM-PMN CM (6 h)
IFNT-PMN CM (6 h)
PMNs
Gene expression
Flow cytometry

Fig.2


A
Direct stimulation
CON
ECM
ECM-stimulated PMN
B
Indirect stimulation
CON
ECM CM
ECM-stimulated PMN CM
Relative fold expression
IFNA
IFNB1
IFNG
OAS1
ISG15
MX1
STAT1
TGFB
TNFA
IL8

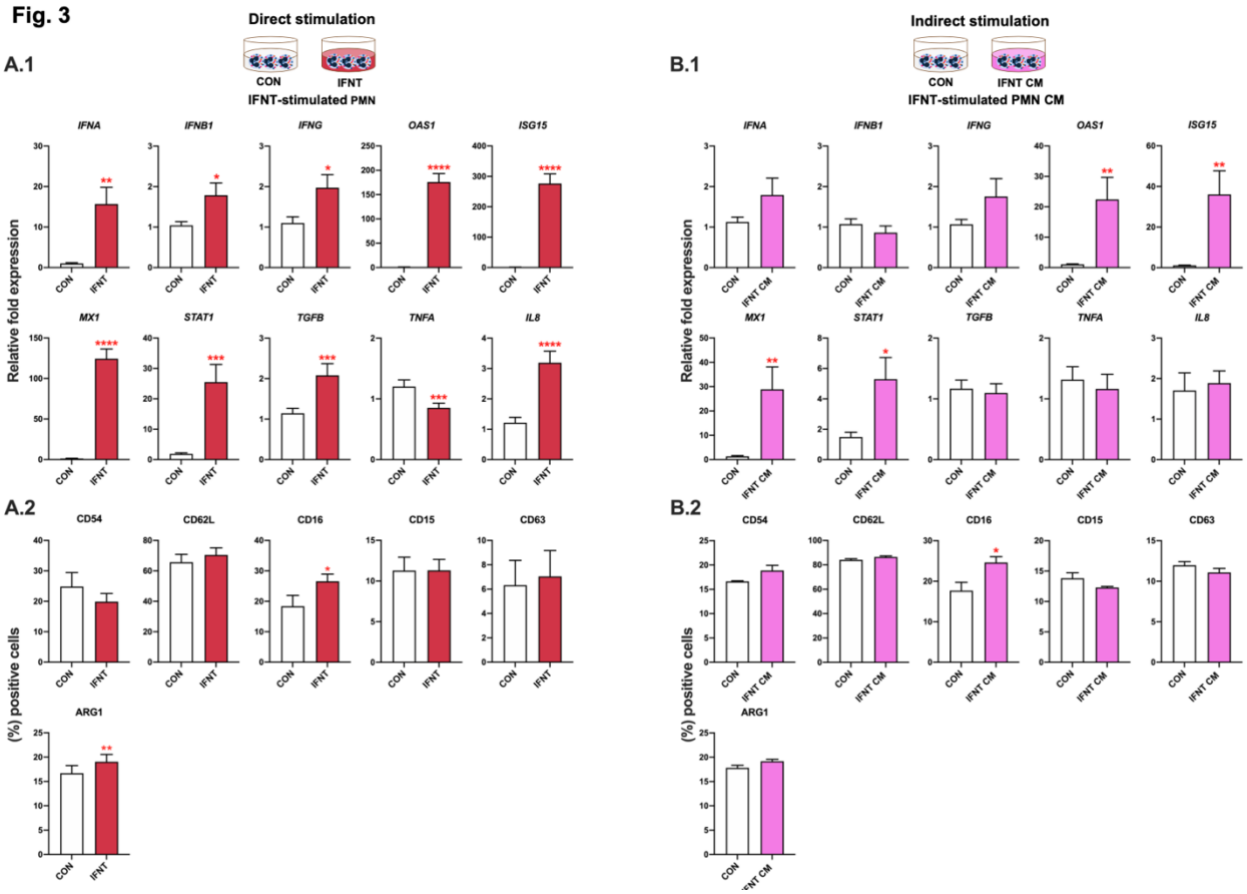


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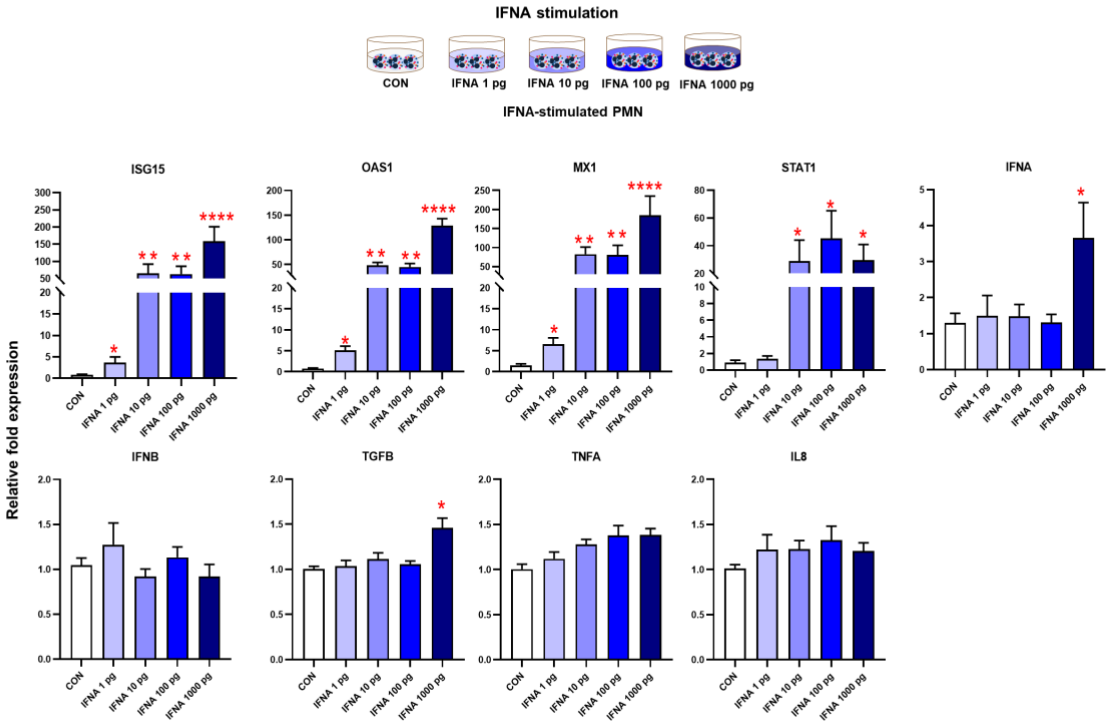
Fig. 4



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4. CHAPTER II – HEAT STRESS MODULATES POLYMORPHONUCLEAR CELLS RESPONSE IN EARLY PREGNANCY OF COWS: II. PRO- AND ANTI-INFLAMMATORY MARKERS

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Heat stress modulates polymorphonuclear cells response in early pregnancy of cows: II. pro- and anti-inflammatory markers

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Abstract

Environmental high temperature impairs several immune functions, which might lead to early pregnancy loss. In the present study, gene expression of PMNs derived from cows was characterized during comfort or heat stress situations using anti-inflammatory (*TGFB*, *ARG1*, *CCL2*), pro-inflammatory (*TNFA*), growth factor (*VEGF*), and oxidative burst (*iNOS*, *NCF1*) markers. Cows had their estrous cycle synchronized and randomly assigned to a comfort or heat stress group. Blood samples were collected at artificial insemination (AI; D0) and D10, 14, and 18 following AI. Thirty to sixty days post-AI ultrasound examination was performed in all animals to confirm pregnancy. Results are presented as mean ± SEM. Pregnant cows under comfort situation had upregulation of *NCF1* gene expression on D10 and 14. All the other genes did not differ between pregnant vs. non-pregnant in comfort situation. Comparing pregnant vs. non-pregnant cows under heat stress situation, pregnant cows had gene expression upregulation of *TNFA* on D10, *iNOS* in all Days, *TGFB* on D14, and *ARG1* D18. Comparison of pregnant cows either in comfort or heat stress demonstrated higher expression of *TGFB* on D18 and *NCF1* on D14 and 18 in cows under comfort environment. The results showed that PMNs response to heat stress acquired both pro-and anti-inflammatory characteristics on different Days, suggesting that immune response adapts to stress situations during pregnancy.

Keywords: hyperthermia, inflammation, pregnancy, innate immune

1. Introduction

During the summer, heat stress (HS) disrupts several reproductive processes, resulting in a significant decline in dairy cows' conception rate worldwide (de Rensis and Scaramuzzi, 2003). Pregnancy loss in dairy herds is an essential factor that influences reproductive performance negatively and occurs more frequently in the first 30 days of pregnancy (Ayalon, 1978; Wiltbank et al., 2016). One of the leading causes of early pregnancy losses is HS (Wolfenson et al., 2000; de Rensis and Scaramuzzi, 2003). Exposure to higher temperature suppresses the immune response in dairy cows, increases the risk of mastitis (Giesecke, 1985; Smith et al., 1985; Waage et al., 1998; Olde Riekerink et al., 2007), and somatic cell count (Bertocchi et al., 2014), impairs follicular development and steroidogenesis, reduces gametes quality (Al-Katanani et al., 2002; Bridges et al., 2005; Roth, 2015; Lucio et al., 2016), misbalances reactive species production, and antioxidant capacity, leading to oxidative stress (Slimen et al., 2014; Soysal et al., 2017).

Nevertheless, the effects of HS on immune functions are not apparent. The immune function might be enhanced, suppressed, or not affected by HS depending on several variables (Lacetera, 2019). In dairy cows, severe HS impairs immune functions. Both *ex vivo* and *in vitro* studies have shown that high temperatures alter peripheral mononuclear blood cells (PBMCs) proliferation capacity (Lacetera et al., 2005). One survey by Lecchi et al., (2016) (Lecchi et al., 2016) reported that phagocytosis and reactive oxygen species (ROS) production in polymorphonuclear cells (PMN) exposed to severe high temperature were impaired, concluding that HS might induce higher infection rates. Still, these adverse effects could be reverted via cooling of PMN derived from heat-stressed dairy cows (do Amaral et al., 2011).

The success of pregnancies depends on an immune challenge: the unresponsiveness to the semi-allogeneic fetus *in utero* and the responsiveness to pathogens (Kropf et al., 2007). n

ruminants, maternal recognition of pregnancy is the signaling period derived from the conceptus to the maternal system from Days 10-20 of pregnancy (Bazer et al., 1986). The primary signaling molecule involved in the embryo-maternal interaction is interferon tau (IFNT) (Short, 1969; Farin et al., 1990). IFNT exerts functions in several tissues and cells, including immune cells (Green et al., 2010; Shirasuna et al., 2012; Kizaki et al., 2013; Rashid et al., 2018; Talukder et al., 2019). Immune cell's response to IFNT can be classically characterized by the expression of interferon-stimulated genes (ISGs) (Oliveira and Hansen, 2008; Shirasuna et al., 2012; Kizaki et al., 2013), but also by the expression of pro-and anti-inflammatory genes (Talukder et al., 2017, 2018, 2019; Rashid et al., 2018).

Pregnant cows' PMNs had higher expression of anti-inflammatory markers such as transforming growth factor-beta (TGFB), interleukin 10 (IL10), and forkhead box P3 (FOXP3), and suppressed pro-inflammatory marker tumor necrosis alpha (TNFA) (Talukder et al., 2019). The phenotype of PMNs also can be modulated in pregnancy (Ssemaganda et al., 2014). The two types of phenotypically distinct neutrophils are low-density neutrophils (LDN) or high-density neutrophils (HDN). LDN cells have high expression of arginase-1 (ARG1), C-C motif chemokine 2 (CCL2), C-C motif chemokine 5 (CCL5), and vascular endothelial growth factor (VEGF) and the ability to suppress T cell functions, conferring anti-inflammatory (N2) properties to these PMNs. Alternatively, HDN has a higher expression of C-C motif chemokine 3 (CCL3), cluster of differentiation 54 (CD54), inducible nitric oxide (iNOS), and TNFA and can activate $CD8^+$ cells, which characterized them as pro-inflammatory (N1) cells (Fridlender et al., 2009). PMNs showed decreased microbial killing and chemotaxis capacity (Crouch et al., 1995; Crocker et al., 1999), however, increased activation status during pregnancy (Luppi et al., 2002). Healthy pregnancies overcome oxidative stress through the upregulation of antioxidation machinery (Wang et al., 1991; Qanungo and Mukherjea, 2000). The reactive oxygen species (ROS) and reactive nitrogen species

(RNS) constitute the oxidant system, which are indispensable molecules for embryo implantation, uterine and cervical modification during the last gestation phase. Conversely, the balance of oxidative stress is fundamental in periods where their levels can reach high concentrations, such as early and late pregnancy (Sciorsci et al., 2020). IFNT is one of the molecules that could reduce oxidants generation without affecting its basal levels (Hara et al., 2014).

Based on the effects of pregnancy on innate immune response, the present study hypotheses that early gestation modulates PMN response towards a more anti-inflammatory state (N2). However, this modulation is disrupted by heat stress in dairy cows to a pro-inflammatory state (N1). This study aims to describe PMN profile during normothermia or hyperthermia using anti-inflammatory (*TGFB, ARG1*, *CCL2*) and growth factor (*VEGF*) markers that confer a more tolerant phenotype, and pro-inflammatory (*TNFA*) and oxidative burst (*iNOS*, neutrophil cytosolic factor 1; *NCF1*) markers that confer a more suppressive phenotype.

2. Materials and methods

2.1. Chemicals

Unless otherwise indicated, chemicals and reagents were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Cattle and herd management

The study was conducted on a commercial dairy farm in Southern Brazil. Thirty-two Holstein cows in lactation from the same herd were included in this study. The cows were 3 to 6

years old; body condition score greater than 2.5 (1=thin and 5=obese on a scale of 1 to 5), absent of any detectable reproductive and clinical disorder during the study period. Cows were milked twice a day and fed complete ration and corn silage, *ad libitum* access to water.

2.3. Experimental design, synchronization protocol, and artificial insemination (AI)

The experiment took place over two separate seasons. Samples from the comfort group (n=15) were collected in September (late winter/early spring) when in Southern Brazil, the temperature-humidity index (THI) is about 65-70. Samples were collected in January (Summer) from the heat-stressed group (n=17), characterized by high temperatures associated with high humidity when THI is approximately 80-85. Synchronization of the estrus cycle was initiated by the insertion of an intravaginal device containing 1.9 g of progesterone (CIDR, Zoetis, São Paulo, Brazil), administration of 2 mg (i.m.) of estradiol benzoate (Sincrodiol, Ourofino, Minas Gerais, Brazil) and 2 ml (i.m.) of gonadorelin, an analog of GnRH (Cystorelin, Boehringer Ingelheim, São Paulo, Brazil) 11 days before AI (Day -11). Four days before AI (Day -4) was administered 0.5 mg (i.m.) of sodium cloprostenol, a synthetic prostaglandin F2α analog (PGF; Sincrocio, Ourofino). Two days before AI (Day -2) intravaginal device was removed, and the animals received 0.5 mg (i.m.) of PGF and 1 mg (i.m.) of estradiol cypionate (ECP; E.C.P. Zoetis). Only animals that exhibited estrus 48 hours after intravaginal device removal were included in the experiment (Comfort group n=12; Heat Stressed group n= 13). AI was performed 48 hours (D0) after intravaginal device removal. The semen was obtained from a commercial company stored in liquid nitrogen, and thawing was performed at 36 °C for 30 seconds for subsequent AI.

2.4. Physiological parameters and environmental data

155

156 Respiratory rate (RR), heart rate (HR), and rectal temperature (RT) were evaluated at 3 p.m.
157 on D10, 14, and 18 following AI. RR was expressed in breaths per minute (bpm) and was obtained
158 using a timer to count respiratory movements for 30 seconds, multiplied by 2 to obtain the number
159 of breaths per minute. HR was expressed in beats per minute (bpm) and was obtained using a
160 flexible stethoscope (Standard, Bic Med, São Paulo, Brazil) placed directly into the left thoracic
161 region under one of the auscultation *foci* for 30 seconds, multiplied by 2 to obtain the number of
162 heart beats per minute. RT was measured with a large animal clinical thermometer inserted 3 cm
163 depth into the rectum and held to maintain contact with the mucosa for one minute. Ambient
164 temperature and relative humidity (RH) were recorded at 4 p.m. on D0, 10, 14, and 18. The THI
165 was calculated using the mathematical equation proposed by (Ferguson et al., 1994): $THI = (0.8 \times Dbt) + [(RH/100) \times (Dbt - 14.4)] + 46.4$; where Dbt = dry bulb temperature, and RH = relative
166
167 humidity.

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169 2.5. Corpus luteum (CL) diameter and progesterone analysis

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171 Corpora lutea diameter (mm) was measured on D18 following AI through ovarian
172 ultrasonography (Mindray DP10 with a 6.5 MHz linear transducer). The progesterone
173 concentration was determined in plasma by chemiluminescent assay kit (ADVIA Centaur,
174 Siemens) also on D18 following AI. The sensitivity of the assay was 0.15 ng/mL of progesterone

175

176 2.6. Blood sample collection

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Blood was collected from the coccygeal vein using a 21G needle coupled to a vacuum collection system (BD Vacutainer®) in 4 ml EDTA-containing tubes. The collections were performed at D0, D10, 14, and 18 following AI to isolate leukocytes and progesterone concentration.

2.7. Isolation of polymorphonuclear (PMN) peripheral blood cells

Isolation of PMNs was performed as follows. After blood collection, 2 ml of whole blood was briefly diluted in an equal part of 0.9% NaCl and then added on top of 3 ml of Ficoll-Paque PREMIUM®. Centrifugation was performed at 400xg for 15 minutes at room temperature to obtain layers of plasma, PBMCs, and PMN. Samples were stored in a cryotube at -80 °C for subsequent total RNA extraction. Slides smears were stained using a rapid stain (Diff-Quik Differential Stains Set; Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations to determine population purity. An experienced clinical pathologist examined the slides. A differential cell count was done by identifying 100 consecutive leukocytes using a 100x objective. Samples above 95% of specific cell type (PMN) (Kizaki et al., 2013) were included in this study.

2.8. RNA extraction, reverse transcription, and real-time PCR

According to the manufacturers' recommendations, total RNA was extracted from the PMN cells using Tri Reagent (BD). Quantification and estimation of RNA purity were performed using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA; Absorbance 260/280nm ratio). RNA was treated with DNAse Amplification Grade (Thermo Fisher, Waltham, MA, USA) for 15 minutes at 27 ºC to neutralize any DNA molecules. DNAse was inactivated with 1 µl EDTA

for 10 minutes at 65 ºC. Reverse transcription was performed adding iScript cDNA synthesis Kit (BioRad, Hercules, CA, USA) for 5 minutes at 25 ºC followed by 30 minutes at 42 ºC and 5 minutes at 85 ºC. Quantitative polymerase chain reaction (qPCR) was conducted in a thermocycler (BioRad, Hercules, CA, USA) using cDNA, forward and reverse bovine specific primers, and GoTaq® Master Mix (Promega Corporation, Madison, USA). Amplification was performed with initial denaturation at 95 ºC for 5 minutes, followed by 40 cycles of denaturation at 95 ºC for 15 seconds and annealing/extension at 60 ºC for 30 seconds. Serial dilutions of cDNA templates were used to generate a standard curve, and efficiency between 90 and 110% was considered. Samples were run in duplicates, and the results of all genes were expressed relative to the geometric mean of Beta-actin (*ACTB*) reference gene, according to (Pfaffl, 2001). The genes assessed in this study are presented in Table 1.

2.9. Pregnancy diagnosis by ultrasound scanning

In both groups, ultrasound examination (Mindray DP10 with a 6.5 MHz linear transducer) evaluated uterine conditions to select only animals without any evident pathology. The pregnancy rate was determined by dividing the number of pregnant cows 30- and 60-days following AI by the total number of cows artificially inseminated (P/AI).

2.10. Statistical analysis

Continuous data were checked for normality using the Shapiro-Wilk test. Gene expression data were analyzed by repeat measures for within-group analysis and standard least squares for between-group (comfort vs. heat stress and pregnant vs. non-pregnant cows) analysis. All data was

performed using the JMP7 Software (SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± standard error of the mean (SEM), and $P<0.05$ was considered significant.

3. Results

3.1. Physiological, reproductive, and environmental parameters

To determine the experimental heat stress model, calculations of summer and late winter/early spring THI were made. The indices were different during summer and late winter/early spring in the experimental period; thus, cows in the summer (higher THI) were considered under HS compared to late winter/early spring (lower THI). HS affected RT, HR, and RR in dairy cows, which were evident at all time points (days along the season). Effect of season on estrous occurrence and pregnancy rate were not different between groups. The estrous occurrence rate was 80% in the comfort group and 76.47% in the heat-stressed group. The pregnancy rate was 50% in the comfort group and 38.46% in the heat-stressed group. CL diameter on D18 following AI was significantly larger in pregnant cows when compared to non-pregnant cows in both comfort and heat-stressed groups. No differences in CL diameter in pregnant cows of both groups were found. Progesterone concentration was lower in heat-stressed pregnant cows when compared to pregnant cows of the comfort group. In non-pregnant cows, the CL diameter and progesterone concentration did not differ between groups, confirming the experimental model.

3.2. PMNs gene expression of non-pregnant or pregnant cows in comfort season

Relative gene expression of anti-inflammatory (*TGFB*, *ARG1*, *CCL2*), growth factor (*VEGF*), pro-inflammatory (*TNFA*), and oxidative burst (*iNOS, NCF1*) markers were evaluated in pregnant or non-pregnant cows in comfort on D10, 14, and 18 after AI (Fig 1). Expression of *TGFB* (Fig 1B), *ARG1* (Fig 1F), *VEGF* (Fig 1D), *TNFA* (Fig 1A), *CCL2* (Fig 1C), *iNOS* (Fig 1E) did not differ ($p>0.05$). Expression of *NCF1* upregulated on D10 and 14 in pregnant cows (Fig 1G).

3.3. PMNs gene expression of non-pregnant or pregnant cows in heat stress season

Relative gene expression of anti-inflammatory (*TGFB*, *ARG1*, *CCL2*), growth factor (*VEGF*), pro-inflammatory (*TNFA*), and oxidative burst (*iNOS, NCF1*) markers were evaluated in pregnant or non-pregnant cows in heat stress on D10, 14, and 18 after AI (Fig 2). There was upregulation of *TNFA* on D10 (Fig 2A), *iNOS* in all days (Fig 2E), *TGFB* on D14 (Fig 2B), and *ARG1* D18 (Fig 2F) in pregnant cows.

3.4. PMNs gene expression of pregnant cows in comfort or under heat stress seasons

Relative gene expression of anti-inflammatory (*TGFB*, *ARG1*, *CCL2*), growth factor (*VEGF*), pro-inflammatory (*TNFA*), and oxidative burst (*iNOS, NCF1*) markers were compared in PMNs of only pregnant cows in comfort or heat-stressed environment on D10, 14 and 18 after AI (Fig 3). Among anti-inflammatory markers, *TGFB* (Fig 3B) was upregulated on D18 in cows in comfort. Oxidative burst marker *NCF1* (Fig 3G) was upregulated on D14 and 18 on comfort cows. All other genes did not differ.

4. Discussion

The present study provides *in vivo* evidence that HS modulates PMN response not exclusively to pro- or anti-inflammatory response with higher expression of *TNFA* (D10) and *iNOS* (D10, 14, and 18), while still providing protective function with upregulation of *TGFB* (D14) and *ARG1* (D18). Surprisingly, pregnant cows in comfort had an upregulation of *NCF1* (D10 and 14). Comparing pregnant cows in comfort to heat stress, comfort cows had upregulation of *NFC1* (D14 and 18) and *TGFB* (D18). These findings suggest that TGFB and ARG1 expression modulate PMNs response under HS, preventing excessive damage during pregnancy. Previous reports have shown that during a healthy pregnancy, immune cells have an anti-inflammatory and tolerant-like response. However, it is still not clear how heat stress modulates immune response during maternal recognition of pregnancy in cows.

Evaluation of ISGs and IFN signaling pathway gene expression and oxidative stress is present by (Amaral et al., 2021; submitted manuscript). Expression of ISGs and type I IFN pathway genes in PMNs of pregnant cows under comfort situation increased time-dependently, reaching a peak on D18, while non-pregnant cows kept baseline expression. The expression of ISGs and type I IFN pathway in PMNs from heat-stressed cows did not differ between non-pregnant and pregnant cows on all days. Malondialdehyde (MDA) levels were more significant in both non-pregnant and pregnant cows under heat stress. At the same time, the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) did not have a proportional increase, indicating that heat stress leads to oxidative stress (Amaral et al., 2021; submitted manuscript).

Under physiologic conditions, the expression of TNFA did not differ in pregnant cows (Shirasuna et al., 2012; Manjari et al., 2016), corroborating with the presented results of comfort cows. TNFA is a central inflammation regulator associated with inflammatory mechanisms linked to the outcome of implantation, placentation, and pregnancy. Exposure to daily heat increased

297 TNFA (Hop et al., 2018), and complications during pregnancy were associated with higher TNFA
298 levels (Azizieh and Raghupathy, 2015). Innate immune cells are one of the primary producers of
299 TNFA, and pregnancy disorders can occur by an increase of pro-inflammatory cytokines, primarily
300 TNFA (Toder et al., 2003). PMNs of heat-stressed cows had greater TNFA expression only on
301 D10, which may be due to heat stress and IFNT concentration. On D10, there is a lower blood
302 concentration of IFNT by RIA than on D14 and 18 (Romero et al., 2015), and IFNT can reduce
303 TNFA expression in immune cells *in vitro* (Talukder et al., 2017).

304 Anti-inflammatory marker *TGFB* was upregulated on D14 in pregnant cows under HS. The
305 inflammatory response to HS is usually characterized by changes in cytokine levels, reflecting a
306 change in systemic immunity from Th1 to Th2 (Kelley et al., 1982). Several studies suggest that
307 TGFB might induce heat-shock protein expression (HSP) 70 and HSP90 under hyperthermia
308 conditions (Takenaka and Hightower, 1992; Cao et al., 1999; Weber et al., 2000). The
309 overexpression of TGFB influenced by hyperthermia shows a similar pattern with the present
310 study. The current findings suggest that IFNT may improve HS effects as TGFB expression
311 increases in cells and tissues during cattle pregnancy (Talukder et al., 2017, 2018, 2019; Rashid et
312 al., 2018). Protection against harmful effects of HS may be associated with greater expression of
313 TGFB (Flanders et al., 1993). Therefore, *TGFB* upregulation in response to HS might be a part of
314 a complex process that leads to cellular defense against other stressful conditions (Weber et al.,
315 2000). The evaluation between pregnant cows under comfort or HS conditions demonstrates that
316 comfort cows had greater *TFGB* expression on D18, suggesting pregnancy-derived signals,
317 possibly IFNT, modulate PMN response to a tolerant environment. Results of pregnant vs. non-
318 pregnant cows in comfort were not different from *TGFB*, *TNFA*, *CCL2*, *VEGF*, *iNOS*, and *ARG1*
319 expressions; this may be due to the number of animals used, which could be a limitation of the
320 present study.

PMNs can generate large amounts of reactive species such as superoxide and nitric oxide (NO) through the activity of NADPH oxidase complex (NOX) and nitric oxide synthase (NOS) as antimicrobial factors (Vazquez-Torres et al., 2000). Synthesis of NO is by three different isoforms of NO synthases, including iNOS (Hickey, 2001), and its production occurs in a redox reaction between arginine and oxygen to control blood flow, promote angiogenesis, maintain cellular redox state, cellular immunity, and survival (Levine et al., 2012). The present study has shown an upregulation of *iNOS* on D10, 14, and 18 in heat-stressed pregnant cows. Immune cells and tissues express iNOS in response to pro-inflammatory mediators, like TNFA, interleukin (IL) 1 beta (IL1B), and interferon-gamma (IFNG) (Marks-Konczalik et al., 1998; Obermeier et al., 1999). Cows under heat stress acclimatization also had higher levels of *iNOS*. The upregulation of *iNOS* in PMNs of cows under HS may be due to the imbalance between antioxidants and oxidants' system during heat stress demonstrated by (Amaral et al., 2021; submitted manuscript).

Moreover, *ARG1* expression was greater on D18 in heat-stressed pregnant cows. ARG1 is an essential regulatory enzyme for the availability of arginine (Bronte et al., 2003; Rodriguez et al., 2007; Munder, 2009). Arginine is a substrate for both ARG1 and iNOS, distinctive anti- and pro-inflammation markers, respectively (Meurs et al., 2002; Maarsingh et al., 2006; el Kasmi et al., 2008). ARG1 can establish an anti-inflammatory environment and inhibits pro-inflammatory responses to decrease HS severity (Bronte et al., 2003; Gobert et al., 2004; Lu et al., 2004; Chatterjee et al., 2006; Munder, 2009). IL4, IL10, and mainly, TGFB can induce the activity of ARG1 (Gobert et al., 2004; Chatterjee et al., 2006), which directs the transportation and metabolism of arginine (Chatterjee et al., 2006). Besides that, ARG1 and iNOS, both upregulated on D18 of pregnant heat-stressed cows, can impair T cell activation (Müller et al., 2009; Puga et al., 2012). The greater expression of ARG1 on D18 HS pregnant cows could be characteristics of maternal immune suppression.

Surprisingly, pregnant cows in comfort had upregulation of *NCF1* on D10 and 14. Comparing comfort vs. heat-stressed pregnant cows, comfort cows had higher expression of *NFC1* on D14 and D18. Physiological functions and healthy early development of the placenta have higher expression of NCF1 (Hernandez et al., 2019). NCF1 is a component of the leukocyte NOX that mediates ROS generation, an essential factor in host defense (Sumimoto, 2008). NOX is detected mainly in neutrophils and is a primary physiological source of phagocytic ROS (Sumimoto, 2008). In the absence of iNOS, NCF1 enhances neutrophil responses. It triggers defense mechanisms (Zhong et al., 2020), suggesting upregulation of *NCF1* without *iNOS* modulation in pregnant comfort cows aids the responsiveness of innate immune cells without compromising the pregnancy.

In conclusion, the present study demonstrated that heat stress modified anti- and pro-inflammatory status and oxidative burst response in pregnant dairy cows. PMNs answer varies between pro-and anti-inflammatory type during heat stress condition, possibly maintaining inflammation while preventing extensive damage in pregnant cows.

Declaration of interest

The authors have nothing to declare.

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Table 1. List of primers used for real-time PCR.

Gene	Forward (5’ – 3’)	Reverse (5’ – 3’)	Accession number
ACTB	TCACCAACTGGGACGACATG	CGTTGTAGAAGGTGTGGTGCC	NM_173979.3
TGFB	CTGAGCCAGAGGCGGCGGACTAC	CTGTGCGAGCTAGACTTCATTTTG	NM_001166068.1
ARG1	CCAGAAGAAGTGACTCGAACAG	GGTGGGCTAAGGTAATCAATAGG	NM_001046154.1
TNFA	CAAAAGCATGATCCGGGATG	TTCTCGGAGAGCACCTCCTC	NM_173966.3
CCL2	TGCAGACCCCAAGCAGAAAT	AGAGGGCAGTTAGGGAAAGC	NM_174006.2
VEGF	ATTTTCAAGCCGTCCTGTGT	TATGTGCTGGCTTTGGTGAG	NM_001316955.1
iNOS	GATCCAGTGGTCGAACCTGC	CAGTGATGGCCGACCTGATG	NM_001076799.1
NCF1	CAAGAACAACGCTGCGGACAT	TGGCCAGAGCCATTTGGGAA	NM_174119.4

Fig 1. Anti-, pro-inflammatory and oxidative burst gene expression in polymorphonuclear cells on D10, 14, and 18 post-AI of pregnant and non-pregnant cows in comfort condition. A) *TNFA*; B) *TGFB*; C) *CCL2*; D) *VEGF*; E) *iNOS*; F) *ARG1*; and G) *NCF1*. Values are presented as mean ± SEM. The asterisk represents the difference at $p<0.05$ between pregnant and non-pregnant cows.

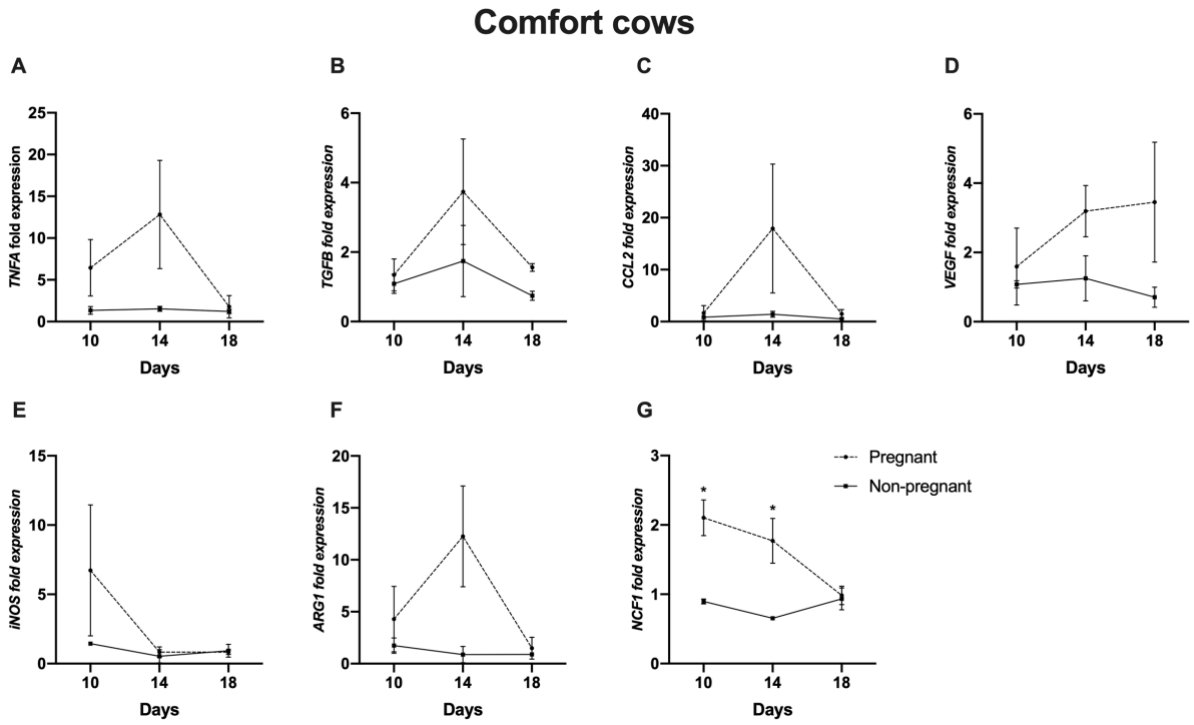


Fig 2. Anti-, pro-inflammatory and oxidative burst gene expression in polymorphonuclear cells on D10, 14, and 18 post-AI of pregnant and non-pregnant cows in heat stress condition. A) *TNFA*; B) *TGFB*; C) *CCL2*; D) *VEGF*; E) *iNOS*; F) *ARG1*; and G) *NCF1*. Values are presented as mean ± SEM. The asterisk represents the difference at $p<0.05$ between pregnant and non-pregnant cows.

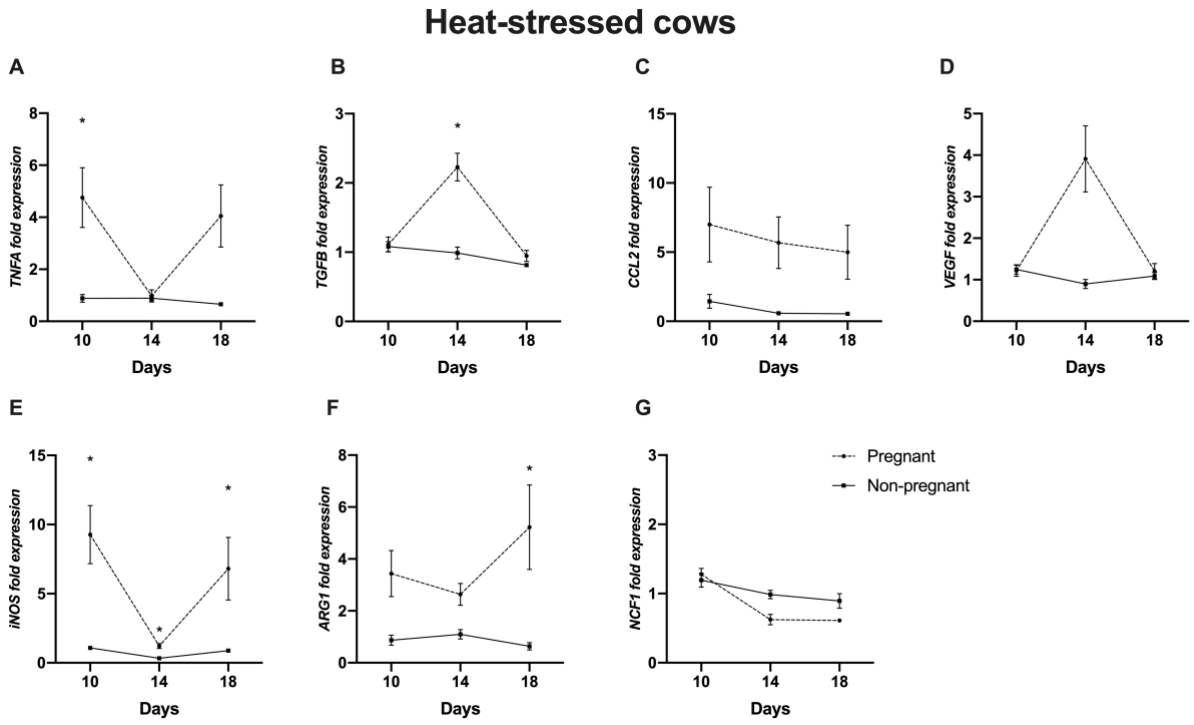
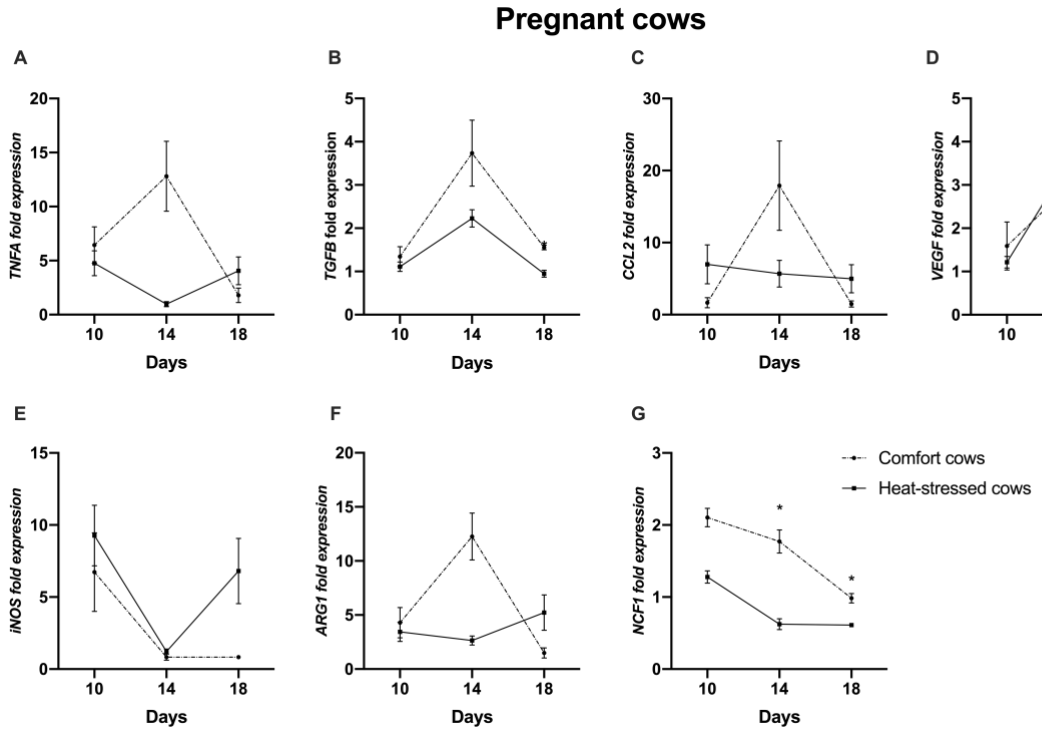


Fig 3. Anti-, pro-inflammatory and oxidative burst gene expression in polymorphonuclear cells on D10, 14, and 18 post-AI of pregnant cows in comfort or heat stress condition. A) *TNFA*; B) *TGFB*; C) *CCL2*; D) *VEGF*; E) *iNOS*; F) *ARG1*; and G) *NCF1*. Values are presented as mean ± SEM. The asterisk represents the difference at $p<0.05$ between pregnant and non-pregnant cows.



5. THESIS DISCUSSION

Several studies had demonstrated the importance of the immune system during pregnancy. Immune cell residents in the uterus recognize the embryo's presence, amplify and signal through the bloodstream to other cells to induce systemic embryo tolerance (BAUERSACHS et al., 2012). Modulation of the immune response in pregnancy needs to find a fine-tune not to disrupt the balance between maternal and conceptus crosstalk, together with the capacity to offer protection against pathogens (AL-KATANANI; PAULA-LOPES; HANSEN, 2002; BRIDGES; BRUSIE; FORTUNE, 2005; LUCIO et al., 2016; ROTH, 2015). A healthy pregnancy is characterized by the different number of immune cells present in diverse tissues that can signalize and modulate each other responses (SLIMEN et al., 2014; SOYSAL et al., 2017). Importantly, pregnancy status should be evaluated according to a specific stage, *e.g.*, MRP, implantation, placentation, term. Immunological responses vary during these stages from anti- to pro-inflammatory (AMARAL et al., 2020; HICKMAN et al., 2013). The results presented herein demonstrated that: 1) embryo derived IFNT can modulate PMNs into anti-inflammatory response encouraging embryo tolerance. Additionally, PMNs can amplify and transfer embryo signals into a new cell population, possibly by IFNA; 2) heat stress can modulate anti- and pro-inflammatory response and oxidative burst response to maintain inflammation status while preventing extensive damage in pregnant cows.

Several studies had also demonstrated that maternal immune response shift to an anti-inflammatory response type (ALHUSSIEN et al., 2018). Pregnant ruminants exclusively produced IFNT modulate the immune response by increasing anti-inflammatory cytokines, such as IL10 and TGFB, and reducing pro-inflammatory cytokine expression TNFA (AZIZIEH; RAGHUPATHY, 2015; HOP et al., 2018). IFNT derived from Day 7 embryo induces anti-inflammatory response and upregulation of ISGs expressions in PBMC (TODER et al., 2003). It seems that IFNT induces immune tolerance essential for embryo survival and development. Pregnancy can change blood PMNs phenotype to contribute to the maternal immune system's modulation (KELLEY et al., 1982). In the first study, direct stimulation with IFNT stimulated *ISGs*, *TGFB* and *IL8* gene expression, and CD16 and ARG1 proteins in PMNs, demonstrating a shift into an anti-inflammatory response. This shift might be due to upregulation of *TGFB* expression, well known Th2 cytokine, which can induce differentiation of neutrophils towards an N2 phenotype

(FLANDERS et al., 1993). Increased expression of ARG1 impacts maternal immune response and fetal development (SSEMAGANDA et al., 2014). CD16 is involved in phagocytosis, oxidative burst, and degranulation (HUIZINGA et al., 1989). Stimulation of the new PMNs population with conditioned media generates an IFNT-like response suggesting that PMNs can amplify and transfer embryo-derived IFNT signals to a new cell population through their culture media type I IFNs. Some interferons are actively expressed in a healthy pregnancy (BAZER, 2013). Type I IFNA expression is detected later on in pregnant cows (SHIROZU et al., 2016). Administration of IFNA into the uterine lumen generated antiviral activity comparable to early pregnancy (BAUERSACHS et al., 2012).

Heat stress affects reproductive features, like steroidogenesis, folliculogenesis, and gametogenesis (AL-KATANANI; PAULA-LOPES; HANSEN, 2002; BRIDGES; BRUSIE; FORTUNE, 2005; LUCIO et al., 2016; ROTH, 2015). Also, heat stress induces the excessive production of reactive species, which might lead to oxidative stress (SLIMEN et al., 2014; SOYSAL et al., 2017). Heat stress can modify pregnant cows' immune response; however, few studies approached the effects of heat stress, specifically during MRP (AMARAL et al., 2020; HICKMAN et al., 2013) and a lesser focus PMNs response (ALHUSSIEN et al., 2018). The second study results have shown that heat stress-induced inflammation with higher expression of *TNFA* and *iNOS*, while still providing protective function with upregulation of *TGFB* and *ARG1* in heat-stressed cows during early pregnancy. Pro-inflammatory cytokines have a vital role linked to pregnancy outcome depending on the stage of detection (AZIZIEH; RAGHUPATHY, 2015; HOP et al., 2018). Excessive production of pro-inflammatory cytokines can lead to early pregnancy termination (TODER et al., 2003). On the other hand, pregnant cows in comfort had an upregulation of *NCF1*. Comparing pregnant cows under comfort or heat stress conditions, comfort cows had upregulation of *NFC1* and *TGFB*. The inflammatory response to heat stress is usually characterized by changes in cytokine levels, reflecting a change in systemic immunity from Th1 to Th2 (KELLEY et al., 1982). Protection against harmful effects of heat stress may be associated with higher expression of anti-inflammatory cytokines (FLANDERS et al., 1993), suggesting that TGFB and ARG1 expression moderate PMNs response stimulated by heat stress to prevent excessive damage during pregnancy. Notably, the expression of *NCF1* in pregnant comfort cows might suggest that PMNs defense mechanisms are kept without damaging pregnancy.

Based on the results obtained with the two studies presented here, different aspects of the maternal immune response during pregnancy can be further analyzed. The first study has demonstrated that IFNT can modulate PMNs response under physiological conditions, and these cells can amplify IFNT signal towards a more tolerant response. The second study has shown that PMNs response does not behave in an anti- or pro-inflammatory response under stressor conditions during MRP.

6. CONCLUSION

The results presented in this thesis may conclude that IFNT modulates the response of PMNs to a more tolerant (anti-inflammatory; N2) pattern. These cells can amplify IFNT signal to other immune cells population through IFNA, possibly to enhance embryo signals for tolerance to the extra-uterine environment until IFNT enters the circulation around day 12 of pregnancy. However, when stressful factor-like heat stress is involved, this response ceases to be exclusively anti-inflammatory. It fluctuates between pro-and anti-inflammatory, potentially maintaining inflammation while preventing extensive damage in pregnant cows.

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