The Role of Extracellular Vesicles in Immunomodulation During Bovine Pregnancy

Amber E. Thornton
Utah State University, amber.thornton@usu.edu

Follow this and additional works at: https://digitalcommons.usu.edu/etd2023

Part of the Animal Sciences Commons

Recommended Citation
Thornton, Amber E., "The Role of Extracellular Vesicles in Immunomodulation During Bovine Pregnancy" (2024). All Graduate Theses and Dissertations, Fall 2023 to Present. 103. https://digitalcommons.usu.edu/etd2023/103

This Thesis is brought to you for free and open access by the Graduate Studies at DigitalCommons@USU. It has been accepted for inclusion in All Graduate Theses and Dissertations, Fall 2023 to Present by an authorized administrator of DigitalCommons@USU. For more information, please contact digitalcommons@usu.edu.
THE ROLE OF EXTRACELLULAR VESICLES IN IMMUNOMODULATION
DURING BOVINE PREGNANCY

by
Amber E. Thornton

A thesis submitted in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE
In
Animal, Dairy, and Veterinary Science

Approved:

Heloisa M. Rutigliano, Ph.D., D.V.M
Major Professor

S. Clay Isom, Ph.D.
Committee Member

Irina Polejaeva, Ph.D.
Committee Member

D. Richard Cutler, Ph.D.
Vice Provost of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2023
ABSTRACT

The Role of Extracellular Vesicles in Immunomodulation
During Bovine Pregnancy

by

Amber E. Thornton, Master of Science
Utah State University, 2023

Major Professor: Heloisa M. Rutigliano, Ph.D., D.V.M.
Department: Animal, Dairy, and Veterinary Science

During pregnancy, the maternal immune system must be altered to allow
tolerance of the fetus. Our previous studies show an inflammatory response in the
uterus of cows carrying somatic cell nuclear transfer (SCNT) pregnancies due to
dysregulation in the proteins expressed by trophoblast cells. Between 30 and 90 days
the rate of pregnancy loss is 50-100% for SCNT compared to 2-10% for artificial
insemination (AI) pregnancies. Abnormal immunological crosstalk during placentation is
a major cause of this loss. The trafficking of extracellular vesicles (EVs), membrane-
bound cargo carriers, potentially represents a form of fetal-maternal crosstalk. The aims
of this study were to determine the role of trophoblast-derived EVs in bovine
pregnancies established by AI and SCNT. We hypothesized that EVs from SCNT
pregnancies will stimulate maternal leukocytes to express more pro-inflammatory mediators compared to cells treated with AI EVs.

Pregnancies were established by AI or SCNT (n = 6/group) and collected at 42±3 days, a time of major embryonic loss in cattle. Placental tissue was digested and cultured in EV-depleted medium for 21 days. EVs were isolated from trophoblast supernatant by size exclusion chromatography. Peripheral blood mononuclear cells (PBMCs) were collected from day 35-70 AI pregnant cows and isolated by density gradient centrifugation. PBMC populations were sorted for CD4+, CD8+, and CD14+ using flow cytometry and cultured with EVs for 24 hours. Reverse transcription quantitative polymerase chain reactions using primers for pro- and anti-inflammatory genes were performed on the collected cells using the Fluidigm BioMark system. Relative gene expression was evaluated. Experimental data were analyzed as a randomized block design using SAS® University Edition Version 3.8, where block was cow and experimental unit was the cell culture well. The interaction between treatment and block was investigated.

Our data reveal changes in the relative gene expression level in maternal leukocyte populations between AI EV-treated and SCNT EV-treated cells. Rather than establishing either a solely anti-inflammatory or pro-inflammatory phenotype, our findings suggest that EVs from both healthy and abortion-prone pregnancies may orchestrate a complex interplay between expression of anti- and pro-inflammatory genes to establish immunological balance at the fetal-maternal interface during early gestation.
The Role of Extracellular Vesicles in Immunomodulation
During Bovine Pregnancy

Amber E. Thornton

During pregnancy, the maternal immune system must be altered to protect the partially non-self fetus from attack. Our previous studies show an inflammatory response in the uterus of cows carrying somatic cell nuclear transfer (SCNT), commonly known as cloned, pregnancies due to abnormalities in proteins expressed by placental trophoblast cells. Between 30 and 90 days the rate of pregnancy loss is 50-100% for SCNT compared to 2-10% for artificial insemination (AI) pregnancies. Abnormal communication between the maternal and fetal systems during placentation is a major cause of this loss. The trafficking of extracellular vesicles (EVs), membrane-bound cargo carriers, potentially represents a form of fetal-maternal crosstalk. The aims of this study were to determine the role of trophoblast-derived EVs in bovine pregnancies established by AI and SCNT. We hypothesized that maternal immune cells treated with EVs from SCNT pregnancies will stimulate a more pro-inflammatory immune response than cells treated with AI EVs.

Cattle pregnancies were established by AI or SCNT (n = 6/group) and collected at 42±3 days, a time of major embryonic loss in cattle. Placental tissue was cultured in EV-free medium for 21 days. EVs were collected from trophoblast supernatant by size exclusion chromatography, a method of separating particles by size. Peripheral blood
mononuclear cells (PBMCs) were collected from day 35-70 AI pregnant cows and isolated by density gradient centrifugation. PBMC populations were sorted for CD4+ helper T cells, CD8+ cytotoxic T cells, and CD14+ macrophages/monocytes and cultured with EVs for 24 hours. Reverse transcription quantitative polymerase chain reactions using primers for pro- and anti-inflammatory genes were performed on the collected cells using the Fluidigm BioMark system to assess relative gene expression. Experimental data were analyzed as a randomized block design using SAS® University Edition Version 3.8, where block was cow and experimental unit was the cell culture well. The interaction between treatment and block was investigated.

Our data reveal changes in the relative gene expression level in maternal immune cell populations between AI EV-treated and SCNT EV-treated cells. Rather than inducing either a solely anti-inflammatory or pro-inflammatory immune response, our findings suggests that EVs from both healthy and abortion-prone pregnancies may mediate communication between the fetal and maternal systems to establish balance between the expression of anti- and pro-inflammatory genes during early gestation.
ACKNOWLEDGMENTS

First and foremost, I would like to express my deepest appreciation to my major advisor and committee chair, Dr. Heloisa Rutigliano, for her dedicated support, guidance, encouragement, and patience with me over the course of my degree. I couldn’t have chosen a better, more supportive advisor if I tried and am so thankful for all the opportunities she provided me with! I am also extremely grateful to my supervisory committee members, Dr. Clay Isom and Dr. Irina Polejaeva, for their insight and thoughtful suggestions to elevate the quality of this work to the highest degree possible. I would like to show my gratitude for Dr. Chris Davies, Dr. Aaron Thomas, Evan Peterson, Kaylyn Bauer, Kaatje Fisk, and all others who assisted me in completing this work. I want to sincerely thank the Utah State University School of Veterinary Medicine for supporting me with a graduate student assistantship. Additionally, this endeavor would not have been possible without the generous support from the USDA National Institute of Food and Agriculture.

Completing a thesis requires more than only academic support, and I am blessed with so many people who supported me outside of the University. My acknowledgements would be longer than my actual thesis if I listed them all, but I would like to specifically mention a few. I would like to thank my family—my parents Daniel and Angela, my siblings Megan and Tyler, and all my extended family—for encouraging and supporting me in all my endeavors throughout my entire life. Thank you to Chris, Alana, Kayt, and Andon Ward, who opened their home and hearts to me and gave me a family while my studies kept me 1,600 miles away from my own. I am appreciative of the Robinette family—Stuart, Jackie, Sara, Kari, Jeff, and others—for all their love and
kindness. I am grateful for all my friends for motivating me and filling my life with so much joy as I completed this thesis!

Finally, I would like to thank and praise God for being the unwavering constant presence in my life throughout these hectic years and for His endless mercy even when I don’t deserve it.

Amber E. Thornton
CONTENTS

Page

ABSTRACT ........................................................................................................... iii
PUBLIC ABSTRACT .......................................................................................... v
ACKNOWLEDGEMENTS ....................................................................................... vii
LIST OF TABLES .................................................................................................. x
LIST OF FIGURES ............................................................................................... xi
LIST OF ABBREVIATIONS ...................................................................................... xii

CHAPTER

I. LITERATURE REVIEW ......................................................................................... 1
   1. Introduction .................................................................................................... 1
   2. Bovine Placentation and Communication at the Fetal-Maternal Interface .......... 3
   3. Overview of the Immune System ...................................................................... 11
   4. Maternal Immune Response and Changes during Pregnancy ....................... 34
   5. Overview of Extracellular Vesicles ................................................................. 55
   6. Overview of MicroRNAs ................................................................................ 59
   7. Immunomodulatory Roles of Extracellular Vesicles in Pregnancy 
      Establishment and Maintenance ................................................................... 62
   8. Applications, Further Directions, and Challenges of Research ......................... 78

II. THE ROLE OF EXTRACELLULAR VESICLES IN IMMUNOMODULATION 
    DURING BOVINE PREGNANCY ..................................................................... 86
Abstract .............................................................................................................. 86
LIST OF TABLES

Table 1. Antibodies used for lymphocyte identification and sorting by fluorescence-activated cell sorting ................................................................. 117

Table 2. Genes, gene definitions, and primer sequences used for real time reverse transcription-polymerase chain reaction ........................................ 118
LIST OF FIGURES

Figure 1-A. Total particle concentration of size exclusion chromatography fractions...120
Figure 1-B. Particle size distribution in size exclusion chromatography fractions……120
Figure 2. CD4+ relative gene expression for significant genes........................................121
Figure 3. CD14+ relative gene expression for significant genes........................................122
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial insemination</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus luteum</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>Estrogen</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicle</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>IDO1</td>
<td>Indoleamine 2,3 dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated lymphoid tissue</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MV</td>
<td>Microvesicle</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>P4</td>
<td>Progesterone</td>
</tr>
<tr>
<td>PAG</td>
<td>Pregnancy associated glycoprotein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PGES</td>
<td>Prostaglandin E synthase</td>
</tr>
<tr>
<td>PGF2α</td>
<td>Prostaglandin F2 alpha</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophil</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>sIgA</td>
<td>Secretory IgA</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TFH</td>
<td>T follicular helper</td>
</tr>
<tr>
<td>TGC</td>
<td>Trophoblastic binucleate giant cell</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TH</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory</td>
</tr>
<tr>
<td>UTC</td>
<td>Uninucleate trophoblast cell</td>
</tr>
</tbody>
</table>
CHAPTER I
LITERATURE REVIEW

I. Introduction

Dairy cow fertility has been declining for decades (M. D. Mitchell et al., 2020; Turner et al., 2021). This presents a significant challenge to dairy farmers because impaired herd fertility jeopardizes the viability and reduces the profit margins of the operation, as the ability of a cow to deliver a healthy calf, return to cyclicity, and become pregnant again in quick succession is essential for milk production and the generation of a constant stream of replacement heifers (Ott, 2019; Turner et al., 2021). Although fertilization rates are typically high, embryonic loss in high producing lactating dairy cows is approximately 65% with 70 to 80% of this loss happening within the first month of pregnancy and 20 to 50% of this loss occurring within the first week of pregnancy (Diskin et al., 2011; Fair, 2015; Wiltbank et al., 2016). Factors that affect the reproductive performance of dairy cows are numerous, diverse, and complex. The maternal immune system plays a paramount role in determining reproductive success or failure in all species, including cattle (Fair, 2015). Dysregulation of maternal immune function has been identified as a major source of infertility and early embryo loss in cattle (Fair, 2015; Z. Wang et al., 2021).

In order for pregnancy to be established successfully, the maternal immune system has to be altered so that becomes tolerant of the semi-allograft that is the conceptus (Bai et al., 2021; Ott, 2019, 2020). In most instances, the overall objective of the immune system is to recognize non-self molecules, or antigens, in the host animal’s body and mount a response against the antigen to protect the host (Hato & Dagher,
Pregnancy, however, represents an “immunological paradox” in which the maternal immune system, which would normally function to eliminate non-self molecules in the host’s body, allows the semi-non-self conceptus with paternal antigens to persist in the maternal body. Not only does the maternal immune system tolerant the conceptus in healthy pregnancies, but it also works to establish an intrauterine environment that is supportive of the survival and development of the conceptus (Ott, 2019; Rocha et al., 2021).

During pregnancy, crosstalk between the fetal and maternal systems at the fetal-maternal interface of the placenta is critical to modulate the maternal immune system to protect the fetal allograft from immunological destruction (Talukder et al., 2020). Extracellular vesicles (EVs), small membrane-bound molecules released from all cell types, potentially represent an important method of this essential fetal-maternal communication (Buca et al., 2020; Das & Kale, 2020). EVs mediate cell-to-cell communication through the trafficking of a variety of cargo molecules, including nucleic acids, proteins, lipids, metabolites, signaling molecules, and more, between donor and target cells (Ciferri et al., 2021; Colombe et al., 2014). Research, though limited in cattle, has shown that EVs have many roles in regulating the maternal immune response to the conceptus during the establishment and maintenance of pregnancy, and, when EV secretion or cargo content is inadequate or abnormal, pathological reproductive conditions can occur as a result of maternal immune dysfunction (Abeysinghe et al., 2023; M. D. Mitchell et al., 2016; Nair & Salomon, 2018; Uyar et al., 2020).
II. Bovine Placentation and Communication at the Fetal-Maternal Interface

a. Bovine Placenta Anatomy and Classification

Cattle are “eutherian”, or placenta-having, species (Schlafer et al., 2000). The bovine placenta is made of three layers of extraembryonic membranes—the amnion, allantois, and chorion. The innermost layer, the amnion, is a fluid-filled sac that surrounds the fetal calf and supports embryonic development by creating a shock-absorbing and compression-resistant environment. The allantois forms from the embryonic gut and is continuous with the urogenital system of the developing calf. It serves as a site of waste transport and respiratory exchange for the embryo. The outermost layer of the placenta, the chorion, develops from the trophectoderm and nonvascular mesoderm germ layer and functions to enclose the amnionic sac and fetal calf. The chorion and allantois layers form the chorioallantois, or “fetal placenta.” The term “fetal membranes” describes both the chorioallantois and the amnion (Peter, 2013).

The bovine placenta is categorized as cotyledonary synepitheliochorial. The term “cotyledonary” denotes the presence of cotyledons, areas of trophectodermal protrusions localized to the fetal aspect of the placenta. In the dam’s uterus, there are approximately 100 evenly distributed caruncles, specialized crypts of the endometrium, which associate with cotyledons on the surface of the fetal placenta (Schlafer et al., 2000). When fetal membrane cotyledons project into and interlock with the endometrial caruncles, they create structures referred to as placentomes, which are semicircular elevations arranged in four rows that stretch lengthwise along the uterine horns.
Formation of placentomes greatly increases the surface area of the bovine placenta, which supports the continuously expanding requirements for nutrient, gas, and waste exchange between the dam and fetal calf (Peter, 2013). Due to the extensive intermeshing of fetal placenta cotyledons with endometrial caruncles, the surface area of contact between the cow and developing calf is estimated to be 130 square meters—analogous to the total surface area of a large room (Russe & Sinowatz, 1991).

The three characteristics of the bovine placenta that classify it as “synepitheliochorial” are the formation of a placentomal chorioallantoic structure, the existence of trophoblastic binucleate giant cells (TGCs) in the fetal placenta, and the development of fetomaternal hybrid syncytial plaques (Peter, 2013). As mentioned previously, placentome formation on the chorioallantois substantially increases the contact between the maternal endometrium and extraembryonic fetal membranes, which is important for exchange of materials between the cow and calf. TGCs make up approximately one fifth of the bovine trophoblast cell population and form from the fusion of trophectodermal cells with the uterine epithelium. As the TGCs migrate from the chorionic layer of cotyledon to the epithelial lining of the caruncles, they fuse with the uterine epithelium, which temporarily forms a three-nuclei hybrid of two separate cell types (Schlafer et al., 2000). The fusion of TGCs with epithelial cells causes TGCs to release cytoplasmic granules into maternal circulation and is coupled with the death of numerous columnar uterine epithelial cells, likely due to a change in the endometrial environment (Wathes & Wooding, 1980). TGC fusion with endometrial epithelial cells also forms fetomaternal hybrid syncytial plaques, which have up to eight separate nuclei at the junction of the fetal placenta and maternal endometrium. These plaques are
short-lived structures and replaced by uterine epithelial cells by day 40 of gestation in cattle (Peter, 2013).

b. Description of Trophoblast Cells and Placenta Histology

The trophectoderm forms during the blastocyst stage of embryonic development and is composed of ectodermal germ layer epithelium that forms a continuous layer over the surface of the fluid-filled blastocoel cavity. Specialized cells of the trophectoderm, known as trophoblast cells, remain as the outermost layer of the extraembryonic membranes and form the epithelial chorion layer which envelopes the fetus and fetal membranes in all domestic animal species. Trophoblast cells secrete several hormones and growth factors which are correlated with pregnancy maintenance and embryonic development, such as progesterone (P4), estrogen (E), bovine placental lactogen, pregnancy associated glycoproteins, and transforming growth factor (TGF) beta. There are two subpopulations of trophoblast cells present in the bovine placenta, each having different functions (Schlafer et al., 2000).

Uninucleate trophoblast cells (UTCs) compose 80% of the total trophoblast cells population from trophectoderm establishment until shortly before parturition. UTCs are polarized and irregular in shape with a single round nucleus and are anchored to the trophoblast basal lamina. They form tight junctions with neighboring UTCs to create the placental barrier, thus separating tissue spaces to establish compositionally distinct compartments. The apical or top surface of UTCs, which faces the epithelium of the caruncles, contains microvilli, which amplifies the surface area contact between the fetal membranes and maternal endometrium (Polei et al., 2020). Two subsets of UTCs, one that lines the fetal aspect of the placentomes between the cotyledonary villi and one that
covers the endometrial gland openings of the interplacentomal chorioallantois, have a phagocytic phenotype (Schlafer et al., 2000). As gestation approaches term, UTCs differentiate into the second subpopulation of trophectoderm cells, TGCs (Peter, 2013).

TGCs are nonpolarized, polyploid cells that make up approximately 20% of the total trophoblast cell population. Unlike UTCs, they are not attached to the trophoblast basal lamina but rather migrate throughout the extraembryonic membranes and across the placental barrier. TGCs translocate from the cotyledonary regions of the chorion to the epithelial layer of the caruncular crypts and around the interplacentomal areas of the fetal placenta (Schlafer et al., 2000). The cytoplasm of TGCs contains granules of fetal secretory glycoproteins associated with pregnancy success. After migration, TGCs fuse with caruncular epithelial cells to create transient fetomaternal hybrid syncytial plaques and release their cytoplasmic granules to the maternal compartment. After degranulation, these syncytial plaques undergo apoptosis and are taken up by other trophoblast cells. This subsequent loss of TGCs is compensated by the creation of additional TGCs by differentiation of UTCs (Polei et al., 2020).

There are two regionally distinct subsets of TGCs, cotyledonary and intercotyledonary. Both cotyledonary and intercotyledonary TGCs share similar characteristics during development, including having two nuclei, an expansive Golgi body, numerous rough endoplasmic reticulum (ER) cisternae, and a double lamellar apparatus. Although structurally similar, the cotyledonary and intercotyledonary populations have considerable differences in gene expression, metabolism, and glycoprotein production. Because the two TGC subsets are both provided the same fetal blood supply, maternal variables, such as glandular secretion from the
endometrium, likely cause the observed variation between cotyledonary and intercotyledonary TGCs (Wooding et al., 1996).

c. Bovine Placental Development

Placental development and implantation are elaborate processes involving many biochemical components and biological interactions. Formation of the placenta begins during the blastocyst stage of embryonic development, when trophoblast cells form an outer wall around the blastocoel, the fluid-filled central cavity of the blastocyst embryo. This layer of trophoblast cells, along with somatic mesoderm germ layer cells, encloses the developing calf throughout pregnancy and forms the chorion, the outermost layer of the placenta. After the initiation formation of the chorion, the blastocyst expands, the embryo continues to develop, and additional layers of the placenta take shape (Schlafer et al., 2000).

The innermost placenta layer, the amnion, forms as ruffles of chorionic trophoblast cells and somatic mesoderm envelope the embryo to create an enclosed space. (Schlafer et al., 2000). The fluid-filled amnionic sac provides the developing calf with a protective, compression-resistant, and shock-absorbing environment. By day 35 of gestation in cattle, the amniotic vesicle can be grossly identified by transrectal palpation or ultrasonography (Peter, 2013).

Once the amnion is formed and the embryonic calf develops into a fetus, its hindgut grows outward and infiltrates the loose tissues of the vascular mesoderm germ layer. The sac-like structure that forms from the outpocketing of the hindgut is the allantois, the second placental layer (Schlafer et al., 2000). The allantois is continuous with the fetal calf’s urogenital system. The allantois is infused with blood vessels that vascularize
the placenta to provide a rich blood supply for respiratory exchange between the fetal calf and cow. As an extension of the fetus’s urogenital system, the allantois also serves as a site of waste elimination for the developing calf (Peter, 2013).

The allantois and chorion layers of the placenta ultimately fuse to form the chorioallantois or fetal placenta. This, combined with the amnion, are considered the extraembryonic fetal membranes. Between gestational day 16 and 27, cotyledons begin to form on the chorioallantois and intermesh with endometrial caruncles to form placentomes. Although placentomes span the entire chorioallantoic surface, the most developed placentomes are located in close vicinity to the fetus. These placentomes grow until day 170 of gestation, when the fetal demand for gas, nutrient, and waste exchange is satisfied by continuous expansion of the villous tree vascular system (Haeger et al., 2016).

d. Functions of the Placenta

The placenta has many critical functions in successful pregnancy establishment and maintenance. It supplies nutrients to the developing fetus, provides a means of waste deposition, serves as a site of respiratory exchange, and mediates communication between the maternal and fetal systems (Nakahara et al., 2020). The fetus is dependent on the placenta to receive nutrients required for growth and survival. Examples of nutrients delivered to the fetus by the placenta include glucose for energy, amino acids for growth and protein synthesis, fatty acids for cellular growth and metabolism, and minerals for skeletal development (Carter, 2012). In cattle, nutrient transfer occurs primarily in placentomes via nutrient-specific transport proteins spanning the polarized microvillous (maternal) and basal (fetal) plasma membranes (Batistel et al., 2017). In a
similar manner to nutrients being transferred to the developing calf, waste products such as urea and bilirubin are taken away from the fetus via the placental vasculature (Carter, 2012).

Another essential function of the placenta is to facilitate the exchange of gases between the fetal and maternal environments. Placenta blood flow is a key factor in the transfer of gases to the fetus, and the proper transfer of gases between the fetal and maternal systems is vital for fetal growth and survival. Fetal blood has a greater affinity for oxygen that maternal blood, which facilitates transfer of oxygen from the maternal to fetal circulation (Comline & Silver, 1975). Gases are delivered to the fetal calf in a similar mechanism as nutrients, via blood vessels in the placentomes. Both oxygen and carbon dioxide are exchanged via simple diffusion following a concentration gradient between the maternal and fetal systems at the placentome villi (Gahlenbeck et al., 1968).

Finally, the placenta also serves as mediator of communication between the maternal and fetal systems and as an endocrine organ. In ruminants, the placenta is responsible for the synthesis and regulation of hormones essential for modulating maternal bodily functions to create conditions that are supportive of pregnancy. Hormones such as prolactin (PRL), growth hormone (GH), P4, and E are either produced directly by the placenta or are distributed in fetal and maternal blood via the placenta (Carter, 2012). Besides hormones, the placenta releases other molecules that mediate fetal-maternal communication. An example of these molecules is EVs, which are released by trophoblast cells of the placenta into maternal circulation to carry a variety of cargo, including proteins, lipids, deoxyribonucleic acid (DNA), and ribonucleic
acid (RNA), between the fetal and maternal systems. As a mediator of cell to cell signaling, EVs play a role in modulating the maternal physiologic system during gestation to create an environment that is supportive of pregnancy establishment and fetal growth.

e. Significance of Fetal-Maternal Crosstalk

The placenta is a key factor affecting reproductive success in cattle. The quality of the pregnancy is determined by the quality of the placenta, as the placenta sets the stage for proper pregnancy establishment and maintenance (Ott, 2019). The placenta has many functions, including nutrient transfer, waste management, and respiratory exchange, in ruminants. Arguably the most important function of the placenta, though, is its role in fetal-maternal crosstalk. Communication between the developing calf and cow is essential for the successful establishment, maintenance, and completion of pregnancy. One key mediator of fetal-maternal crosstalk is EVs, which are released from trophoblast cells of the placenta into the extracellular space. Roles of trophoblast-derived EVs in reproduction include mediating cell to cell communication and maternal immune response (Nair & Salomon, 2018). Failure of fetal-maternal crosstalk and placental dysfunctions during cattle pregnancy can lead to embryonic loss and defects such as hydrallantois, freemartinism, and large placenta syndrome (Peter, 2013).
III. Overview of the Immune System

a. Introduction to Immunity

The immune system is comprised of a complex network of molecules, cells, tissues, and organs that work cooperatively to defend the body against infection. Beyond the mechanical, chemical, and microbial barriers that serve to constantly protect the body from pathogens, the immune system can be divided into two separate but complementary parts: innate immunity and adaptive immunity. The innate immune system is the body’s first line of defense against foreign molecules, or antigens. Innate immunity is antigen-independent (non-specific), has no immunological memory, and acts immediately upon encountering an antigen (Marshall et al., 2018). The innate immune system includes neutrophils, macrophages, dendritic cells (DCs), natural killer (NK) cells, mast cells, eosinophils, basophils, and other non-cellular component parts (Hato & Dagher, 2015). The adaptive immune system is characterized by a delayed, antigen-specific response to the pathogen that results in the development of immunological memory. Key adaptive immune system cells include effector and memory T and B lymphocytes (Bonilla & Oettgen, 2010). Innate and adaptive immunity act jointly to ensure the host animal has both immediate, general protection from all pathogens and delayed, specific protection from previously encountered pathogens.

b. Innate Immunity

Besides the protective mechanisms associated with physical barriers and processes such as sneezing, coughing, and vomiting, the innate immune system is activated when pattern recognition receptors (PRRs), receptors capable of recognizing antigenic
molecules commonly found in pathogens or damaged tissue, on sentinel cells, including macrophages, DCs, and mast cells, recognize an antigen. This antigen can be either a pathogen-associated molecular pattern (PAMP) or a damage-associated molecular pattern (DAMP). PAMPS are small molecular motifs conserved within a class of microbes, such as bacteria and viruses, but are foreign to the host animal. DAMPs are intracellular molecules released by dead or dying cells in damaged tissue. Both PAMPS and DAMPs bind to PRRs on sentinel cells to activate the PRR pathway, in which antigen binding activates transcription factors (NF-κB, IRF-3, and IRF-7) inside the sentinel cell which causes the secretion of pro-inflammatory cytokines and enzymes to initiate an inflammatory response (Vlasova & Saif, 2021).

Macrophages, which arise from circulatory monocytes, are pivotal cytokine-secreting innate immune cells that are long-lived and widely distributed throughout the body. Macrophages, along with DCs and mast cells, are classified as sentinel cells, meaning they constantly monitor the body for the presence of antigens. Macrophages are diverse cells with a wide spectrum of phenotypes and can be broadly divided into pro-inflammatory M1 and anti-inflammatory M2 subpopulations. The classically activated M1 subpopulation is involved in the induction of inflammation and immune cell activation, while the alternatively activated M2 subpopulation is involved in tissue repair and clearance of inflammation. Macrophages are highly plastic and are able to change their polarization from M1 to M2 and vice-versa under evolving tissue microenvironmental conditions and stimuli (C. Zhang et al., 2021). Unless otherwise specified, M1 macrophages are the main effectors of the innate inflammatory responses discussed in
this chapter. Macrophages express PRRs, which recognize PAMPs and DAMPS, and destroy detected pathogens via phagocytosis (Gasteiger et al., 2017).

In contrast to neutrophils, macrophages have a more authoritative role in immune response by coordinating innate immunity and serving as antigen presenting cells (APCs) to trigger adaptive immunity. Cytokines secreted by macrophages after detection of an antigen, including tumor necrosis factor (TNF)-a, interleukin (IL)-1b, and IL-6, stimulate local and systemic inflammatory responses. TNF-a and IL-12 also function to activate NK cells, another component of the innate immune system. Anti-inflammatory M2 macrophage cytokines, namely IL-10 and TGF-b, act later in infection to suppress macrophage activation and pro-inflammatory cytokine production, inhibit antigen presentation to lymphocytes and lymphocyte activity, and maintain the function of T regulatory (Treg) cells (Duque & Descoteaux, 2014).

Macrophages also function at APCs and trigger adaptive immune responses via capture, processing, and presentation of antigens to lymphocytes (C. Zhang et al., 2021). Macrophage-derived cytokines are important in the differentiation of lymphocytes, the principal leukocytes of adaptive immunity. For instance, IL-12 induces naïve T cells to differentiate into T helper (TH)1 cells, which promote cell-mediated adaptive immune responses, and enhances the cytolytic activity of CD8+ T cells (Duque & Descoteaux, 2014).

The pro-inflammatory cytokines, including TNF-a, IL-6, and IL-1b, have local and systemic effects. Locally, these pro-inflammatory cytokines induce vasodilation, decrease vascular permeability, initiate chemokine release to serve as chemo-attractants to recruit leukocytes to the site of infection, and activate lymphocytes.
Systemically, pro-inflammatory cytokines stimulate the release of corticotropic releasing hormone in the hypothalamus to suppress appetite and induce fever, increase the synthesis of C-reactive proteins in the liver for opsonization and complement activation, increase leukocyte production and neutrophil mobilization from the bone marrow, and mobilize protein and energy reserves (Duque & Descoteaux, 2014). After being activated by pro-inflammatory cytokines, resident immune cells, such as DCs and macrophages, produce additional cytokines and chemokines to recruit monocytes and neutrophils to the site of infection, which recruit DCs, NK cells, T lymphocytes, and B lymphocytes to the inflamed area. Mast cells degranulate in response to antigen detection and release cytoplasmic granules, such as histamine and prostaglandins, which up-regulate inflammation and contribute to changes in vascular permeability (Vlasova & Saif, 2021).

Neutrophils are the most abundant white blood cell in circulation and the first leukocyte to respond to antigens in the body. Under normal conditions, short-lived neutrophils are produced in the bone marrow, enter the bloodstream, migrate to infected tissue, complete their functions, and are consumed by phagocytic macrophages (Mayadas et al., 2014). In response to cytokines, neutrophils produced in the bone marrow are released into the bloodstream, where they are recruited into inflamed tissues by a process known as the leukocyte adhesion cascade (Chavakis et al., 2009; Rosales, 2018). During the leukocyte adhesion cascade, endothelial cells lining the blood vessel express adhesion receptors called selectins. Neutrophils in circulation bind to the selectins and begin slowly rolling along the vessel wall. These cells then bind integrin proteins on the endothelial cells and come to a complete stop, adhering to the
vessel wall. Stopped neutrophils transmigrate through the vessel wall and are attracted to sites of infection by following a chemokine concentration gradient, where they eliminate pathogens by phagocytosis and intracellular killing, degranulation of cytotoxic chemicals, and the release of neutrophil extracellular traps (Rosales, 2018). Besides killing pathogens, neutrophils produce cytokines, chemokines, and other inflammatory factors to aid in regulating inflammation (Nauseef & Borregaard, 2014). While the main function of neutrophils is to recognize and destroy microorganisms and tissue debris, accumulating data shows that they also produce molecules such as cytokines, apoptosis inducing factors, and chemokines have some minor roles in coordination of the immune response (Rosales, 2018; F. Yang et al., 2017).

DCs are considered a link between the innate and adaptive immune responses (Zanna et al., 2021). Like macrophages, DCs are part of innate immunity and function as sentinel cells and APCs, but they are more potent activators of the adaptive immune response. Immature DCs are produced in the bone marrow, migrate to peripheral tissues, and search for and capture antigens. The main function of DCs is to survey the body, identify antigenic molecules, and phagocytose, process and present antigens on its surface via major histocompatibility complex (MHC) molecules to activate naïve lymphocytes. DCs then travel to secondary lymphoid tissues, such as the lymph nodes, spleen, tonsils, Peyer’s patches, or bone marrow, and mature through increased expression of MHC molecules, increased expression of cell-surface co-stimulatory molecules (ex. CD80, CD86, and CD40), and increased cytokine production. At secondary lymphoid tissues, mature DCs presenting antigenic peptides can activate naïve T and B lymphocytes to stimulate adaptive immune responses (Cabeza-Cabrerez
et al., 2021). Along with activating lymphocytes, DCs have a pivotal role in promoting the differentiation of CD4+ TH cells into antigen specific TH1, TH2, TH17, and T follicular helper (TFH) cells, depending on which cytokines they secrete. Treg cells, which are created after activation of T helper cells by DCs, are key mediators of peripheral tolerance. TFH cells, induced after the differentiation of T helper cells by DCs, are key effectors in the activation of B lymphocytes, so DCs also play a vital role in B cell development and thus antibody production (Cabeza-Cabrero et al., 2021; Zanna et al., 2021). DCs are the most potent APC in the body and, besides processing antigens and activating the adaptive immune system, have functions in modulating and regulating adaptive immune responses.

As mentioned above, after capturing an antigen, APCs, including DCs and macrophages, process the antigen for presentation to lymphocytes. There are two mechanisms of microbial antigen processing and presentation depending on if the foreign antigen is endogenous or exogenous. Endogenous antigens derived from tumor cells and intracellular microorganisms such as viruses, intracellular bacteria, and protozoa. Endogenous antigens originate within the cell and are marked for destruction by ubiquitin proteins, which are recognized by a proteasome and degraded into small peptide fragments. The peptide fragments are then transported into the ER by a transporter for antigen processing and bound to MHC class I molecules in the ER. The MHC-peptide complex is then transported to the cell surface by vesicles, and endogenous antigen peptide fragments on the MHC class I molecule are presented to CD8+ cytotoxic T lymphocytes (Thery & Amigorena, 2001).
Exogenous protein antigens are derived from endocytosed or phagocytosed extracellular microorganisms such as extracellular bacteria and protozoa and tissue debris. The endosome, which contains the antigen, fuses with a lysosome inside the APC. Proteases within the lysosome degrade the antigen into small peptide fragments. An MHC class II molecule is assembled in the ER, which is then transported to the lysosome containing the antigen peptide fragments. The antigen peptides are bound to the MHC class II molecule, and the MHC-peptide complex is transported to the surface of the APC, where the antigen is presented to CD4+ TH lymphocytes (Thery & Amigorena, 2001).

NK cells are important leukocytes in the innate response towards stressed cells, virally infected cells, and tumors. In humans, they represent 5-20% of the immune cells in circulation (Langers et al., 2012). Upon stimulation, NK cells secrete inflammatory cytokines (interferon (IFN)-γ and TNF-α), and exert cytolytic activity similar to CD8+ lymphocytes. Cytokine production, specifically IFN-γ, activates macrophages and DCs to stimulate further immune response. NK cells attack and kill virally infected or abnormal cells in two mechanisms: cytotoxic granule secretion (perforin and granzymes) and triggering Fas (CD95)/Fas-L (CD95L) (Abel et al., 2018). To kill infected cells via cytotoxic granules, the NK cell first secretes perforin, which creates pores in the target cell membrane. The NK cell then secretes granzymes, which enter the target cell through the pores created by perforin and induce apoptosis in the target cell via a caspase cascade. To kill infected cells using death receptors, NK cells expressing the Fas ligand bind target cells expressing Fas receptor. Like the cytotoxic granule pathway, this induces a caspase cascade in the target cell, resulting in
apoptosis. NK cells can also utilize antibody-dependent cellular cytotoxicity (ADCC) to kill infected cells, bacteria, fungi, and parasites. ADCC involves the binding of antibodies to the surface of target cells or pathogens and to the Fc receptors of the NK cell, as well as macrophages and neutrophils, to induce apoptosis of the infected cell or pathogen (Paul & Lal, 2017). In essence, NK cells are a segment of the innate immune system due to being non-specific and lacking immunological memory, but they share functions with adaptive immune cells (CD8+ lymphocytes) and utilize adaptive immune products (antibodies for ADCC).

Mast cells, basophils, and eosinophils are innate immune cells whose primary protective function is degranulation, or releasing intracellular molecules into the extracellular space to induce an immune response. Like macrophages and DCs, mast cells are sentinel cells that constantly survey the body to recognize and respond to antigenic microbes. Mast cells, eosinophils, and basophils are key leukocytes in protection against parasites and in type I hypersensitivities or allergic reactions (Marshall et al., 2018). Stimuli for degranulation includes antigen recognition by mast cells, cytokine (IL-3, IL-5, granulocyte macrophage colony-stimulating factor (GM-CSF), and histamine-releasing factor) production, and IgE binding to the Fc receptors (FcεRI and FcεRII/CD23) on activated mast cells, basophils, and eosinophils (Stone et al., 2010). After being stimulated, mast cells, basophils, and eosinophils release cytoplasmic granules that have a variety of functions. Mast cells cytoplasmic granules include histamine, serine proteases, carboxypeptidase A, proteoglycans, eicosanoids, TNF-α, IL-3, GM-CSF, IL-5, IL-6, IL-10, IL-13, and IL-8. Basophil cytoplasmic granules include histamine, IL-4, IL-13, and GM-CSF; and eosinophil cytoplasmic granules
include major basic protein, PGE2, TGF-b, IL-3, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, IL-16, IL-18, TNF-a, CCL5, and CCL11 (Marshall et al., 2018; Rigoni et al., 2018; Stone et al., 2010). This diverse group of cytoplasmic granules contains vasoactive mediators, pro-inflammatory mediators, antimicrobial molecules, and chemoattractants that are essential for the body’s immune response against parasites and allergens (Rigoni et al., 2018).

Other components of the innate immune system include the complement system, the coagulation system, antimicrobial molecules, and inflammatory mediators. The complement system comprises plasma proteins that kill microbes in a few ways: by directly forming a membrane attack complex (MAC), by opsonizing and thereby facilitating phagocytosis of pathogens, by inducing inflammation and recruitment of immune cells to the site of infection. The complement system is also responsible for regulating adaptive immune responses and clearing immune complexes (antigen-antibody complexes) from the body. The coagulation system is important in preventing the spread of infection. Antimicrobial molecules kill bacteria and digest bacterial cell walls, and inflammatory mediators initiate inflammation in response to microbial invaders. All of these innate immune system components work collectively during the onset of the body’s immune response to provide rapid, non-antigen-specific defenses against a multitude of microbial invaders by recognizing, responding to, and removing antigens from the body (Gasteiger et al., 2017; Hato & Dagher, 2015; Vlasova & Saif, 2021).

c. Adaptive Immunity
The adaptive immune system involves specific recognition and destruction of antigens while forming immunological memory to enhance the body’s response to future infections. The cellular components of the adaptive immune system include B and T lymphocytes. The latter can be subdivided into gamma-delta (γδ) T lymphocytes or alpha-beta (αβ) T lymphocytes, distinguished by the expression of either a γδ or an αβ T cell receptor (TCR), respectively (Morath & Schamel, 2020).

Unlike αβ T lymphocytes, gd T lymphocytes are a relatively minor lineage of T cells, and their functions are not yet fully characterized. They can bind to many different ligands, and it is speculated that γδ T cells may serve to provide immunological protection to anatomical locations that are not well populated by ab T cells or B cells, considering that they concentrate in peripheral tissues rather than lymphoid organs (Morath & Schamel, 2020; Ribot et al., 2021). While scarce in circulation, γδ T cells are abundant at barrier sites, including the skin, intestines, lungs, and reproductive tract (Mayassi & Jabri, 2018). γδ T cells share some effector mechanisms with ab T cells, such as cytokine production and cytotoxicity. In response to IL-1β, IL-6, and IL-23, they can rapidly produce a burst of pro-inflammatory cytokines, including IFN-γ, IL-17, IL-15, and GM-CSF, in the absence of TCR binding and co-stimulation (Lalor et al., 2011; Woot et al., 2020). As a result of their cytokine secretion, γδ T lymphocytes have the potential to stimulate macrophage activity, enhance antigen presentation by DCs, and initiate further adaptive immunity (Plattner & Hostetter, 2011). Besides enhancing antigen presentation by DCs, γδ T cells have been shown to directly present antigens to CD4+ αβ T cells in cattle to initiate the helper T lymphocyte response (Collins et al., 1998). γδ T lymphocytes can also have immunoregulatory functions. They can regulate
chemokine and cytokine expression through modulating Fas/Fas-L mediated apoptosis of T cells, although this mechanism is not fully described (Ponomarev & Dittel, 2005). In cattle, they have also been shown to suppress antigen-specific αβ T lymphocyte proliferation (Rhodes et al., 2001).

αβ T lymphocytes are the major subset of T cells in mature animals and can be divided into cytotoxic (CD8+) T and helper (CD4+) T effector and memory cells. Cytotoxic T cells are a major portion of circulating T lymphocytes that serve to kill target cells through contact-dependent mechanisms. They are the adaptive immune system's main effector cells for killing pathogens and abnormal host cells (Raskov et al., 2021). CD8+ lymphocytes recognize endogenous antigens presented on MHC class 1 molecules and then destroy the antigenic microbe through either the release of cytotoxic granules or the activation of the Fas/Fas-L apoptosis pathway, similar to the action of NK cells of the innate immune system (Bonilla & Oettgen, 2010).

CD4+ T cells are arguably the most important and influential leukocytes of the adaptive immune system, as they are a required component to activate many adaptive immune responses and modulate several innate leukocyte activities. Depending on the subset of CD4+ cells, they serve to promote the activity of cytotoxic T cells, macrophages, mast cells, basophils, eosinophils, and neutrophils through the secretion of cytokines. They also induce inflammation, activate B lymphocytes, and aid in suppressing immune responses and maintaining tolerance to self-antigens. Subpopulations of CD4+ lymphocytes include TH1, TH2, TH17, TFH, and Treg cells (Bonilla & Oettgen, 2010; Saravia et al., 2019).
Activation of the adaptive immune system to create high-affinity antigen-specific effector and memory cells is a complex process. After lymphocytes are generated in the bone marrow, they mature in the thymus. T cell precursors enter the thymus as double-negative cells that express neither CD4 nor CD8. In the thymus, they proliferate, develop, and undergo rearrangement of TCR genes. Developing T and B cells undergo somatic recombination or random gene rearrangement of the receptor’s variable domain to achieve antigen receptor diversity and increase the quantity and variety of antigens the adaptive immune system can recognize and respond to. γδ T lymphocyte lineages diverge from αβ T lymphocyte lineages during TCR somatic recombination due to the order of γ, λ, and α gene rearrangement. TCR genes rearrange in two consecutive rounds. After the first round of TCR somatic recombination, double-negative T lymphocytes become double-positive, meaning that they express both CD4 and CD8. After the second round of TCR, T cells undergo positive selection in the thymic epithelium. T lymphocytes whose TCRs can bind to MHC complexes with high affinity receive survival signals, while T lymphocytes whose TCRs cannot bind MHC complexes undergo apoptosis. Positive selection establishes single-positive T cells through MHC restriction. Depending on whether the TCR engages with MHC class I or MHC class II, the T cell will express CD8+ or CD4+, respectively. The end result of somatic recombination and positive selection of T lymphocytes is the generation of a diverse set of antigen-specific, high affinity single-positive CD4+ and CD8+ naïve T lymphocytes (Bonilla & Oettgen, 2010; Han & Zuniga-Pflucker, 2021; Kumar et al., 2018).

After completing positive selection to establish MHC restriction, T lymphocytes undergo negative selection in the thymus. Negative selection is a means of establishing
central T cell tolerance and preventing autoimmunity. During negative selection, T cells
with high affinity for self-peptides are destroyed by apoptosis or become T regulatory
cells. This process is responsible for the fact that mature T lymphocytes that leave the
thymus are unresponsive to most cell components (Kumar et al., 2018; Shevyrev &
Tereshchenko, 2020). The self-reactive CD4+ T cells that are not destroyed by
apoptosis differentiate into self-antigen-specific Treg cells, leaving the thymus and
inhibiting responses against these self-antigens in the periphery (Shevyrev &
Tereshchenko, 2020). After undergoing somatic recombination, positive selection, and
negative selection in the thymus, T cells enter circulation as mature but naïve single
positive lymphocytes that circulate through blood and secondary lymphoid tissues
(Bonilla & Oettgen, 2010; Kumar et al., 2018).

Mature, naïve T lymphocytes are activated in secondary lymphoid tissues, such as
the Peyer’s patches, spleen, and lymph nodes. DCs, which have captured and
processed an antigen as previously described, migrate from peripheral tissues to
secondary lymphoid tissues. There, they increase their expression of MHC and co-
stimulatory molecules (CD80 and CD86) and their production of cytokines. While other
APCs can activate memory T lymphocytes, only DCs can activate naïve T lymphocytes
due to their increased MHC and co-stimulatory molecule expression (Hwang et al.,
2020; Iwasaki & Medzhitov, 2015). Activation of naïve T lymphocytes requires direct
contact between both the MHC molecule-bound peptide on the DC with the TCR on the
T cell and the co-stimulatory molecules on the DC and their receptors on the T cell.
Once activated, T lymphocytes can then be differentiated into effector and memory T
cells, and CD4+ T cells further differentiate into various subpopulations including CD4+
TH1, CD4+ TH2, CD4+ TH17, and CD4+ TFH, depending on the cytokines secreted by the DCs (Hwang et al., 2020).

Cytokine production by the DC orchestrates the differentiation of CD4+ T lymphocytes into various lineages. In the presence of IL-12 secreted by DCs and macrophages, T helper cells differentiate into TH1 cells. TH1 cells secrete IFN-γ and IL-2 to stimulate cell-mediated immune responses, including activating macrophages and stimulating cytolytic activity of cytotoxic T cells and NK cells. When activated by DCs secreting IL-4 in the absence of IL-12 and IL-23, CD4+ lymphocytes differentiate into TH2 cells, which mostly promote humoral immunity by stimulating B cell production of antibodies, most notably IgG and IgE. TH2 cells secrete IL-4 and IL-10 to activate mast cells, basophils, and eosinophils, stimulate IgE production by B lymphocytes, and inhibit TH1 cell-mediated immune responses. When stimulated by DCs secreting IL-23, IL-6, and TGF-β, naïve CD4+ cells differentiate into TH17 lymphocytes. TH17 lymphocytes secrete IL-17, IL-21, and IL-22 to promote inflammation and neutrophil production. TH1, TH2, and TH17 lymphocytes can all differentiate into TFH cells in the presence of B lymphocytes in the secondary lymphoid tissues. TFH cells are important in the activation of B cells and promotion of B cell responses (Saravia et al., 2019).

Treg cells are a unique subpopulation of T lymphocytes that are responsible for regulation of the immunological response to maintain specific immunosuppression and self-tolerance. They play a significant role in maintaining the balance between immunological tolerance and reactivity (Shevyrev & Tereshchenko, 2020). During T lymphocyte maturation and negative selection in the thymus, some self-reactive CD4+ T cells differentiate into Treg cells that are specific for these self-antigens. The Treg CD4+
T cells then leave the thymus, enter circulation, and provide peripheral self-tolerance by inhibiting lymphocyte responses to these self-antigens. They can also be induced in the periphery through the production of TGF-β and retinoic acid (Sakaguchi et al., 2020). Treg cells express the transcription factor FOXP3 and mediate immunological suppression in several ways, including cell-contact mediated suppression using CTLA-4, CD25, T cell immunoreceptor with Ig and ITIM domains, CD39, and CD73 cell surface molecules and cytokine-mediated suppression using IL-2, IL-10, TGF-β, and IL-35 (Sakaguchi et al., 2009). Treg cells can suppress T lymphocyte proliferation and prevent T lymphocyte activation to regulate immune responses. They are essential in many biological processes, including preventing autoimmunity and the maternal tolerance of fetal semi-allografts (Sakaguchi et al., 2009).

Besides T lymphocytes, the other cellular component of the adaptive immune system is B lymphocytes. Like T cells, B cells can be long-lived memory cells that maintain immunological memory of previously encountered antigens but may also become secretory plasma cells. Plasma cells secrete antibodies, or soluble Iggs, that serve to protect the protect with numerous functions (Cyster & Allen, 2019). In general, plasma cell-derived antibody effector mechanisms include neutralization of pathogens and toxins, opsonization of antigen to facilitate phagocytosis, activation of the classical complement system, coating antigen to initiate antibody-dependent cellular cytotoxicity, agglutination or clumping of antigens, and activation of NK cells, mast cells, basophils, and eosinophils (Cyster & Allen, 2019; Schroeder & Cavacini, 2010).

B lymphocytes can be divided into thymus-dependent B-2 and thymus-independent B-1 cells. B-2 cells are more complex due to having memory, undergoing somatic
hypermation, and secreting a variety of antibody isotypes. Similar to T lymphocytes, B-2 lymphocytes undergo somatic recombination of their antigen receptors in primary lymphoid organs. Unlike TCRs, however, B cell receptor (BCR) diversity is generated in two steps. After somatic recombination, BCRs experience somatic hypermutation and affinity maturation in secondary lymphoid tissues (Cyster & Allen, 2019; Reed et al., 2016). During somatic hypermutation, point mutations are inserted at high rates into the BCR genome (Reed et al., 2016). Affinity maturation involves selecting B cells whose BCRs bind with high affinity to the antigen. Higher affinity B cells differentiate into plasma and memory cells, while lower affinity B cells die by apoptosis. The end result of this process is a diverse repertoire of antigen-specific mature B cells that produce high-affinity antibodies in response to antigen exposure (Bonilla & Oettgen, 2010; Cyster & Allen, 2019).

To be activated, naïve B cells, along with T cells, need to encounter antigen on the surface of follicular dendritic cells in a lymph node or other secondary lymphoid tissue. Follicular dendritic cells are specialized antigen-presenting cells that bind and retain antigens on their native conformation and present these antigens to naïve B cells. Surface IgG on the B cells binds the antigen, then the B cell endocytoses its BCR with the attached antigen. Meanwhile, activated CD4+ T lymphocytes, which have differentiated into TH1, TH2, and TH17, further differentiate into TFH1, TFH2, and TFH17 in the presence of B cells. The TFH cell searches for a naïve B cell that presents the same antigenic peptide, and the two form an immunological synapse and become a cognate pair (Cyster & Allen, 2019). A germinal center, a secondary follicle, is then
formed, where the B cells undergo somatic hypermutation, isotype switching, and affinity maturation (Tsai et al., 2019).

There are five main isotypes of plasma cell-derived antibodies: IgM, IgD, IgG, IgA, and IgE. Each antibody isotype has unique capabilities giving it specialized functions in fighting infection during the adaptive immune response. IgM is a large pentamer in structure and is the first Ig present during the primary immune response, which is the immune response that occurs during the body’s first exposure to an antigen. IgM is found mainly in circulation, activating the classical complement system, neutralizing pathogens and toxins, and agglutinating antigens. IgD is expressed with IgM on naïve B cells, but its function in circulation or as a membrane-bound molecule has not been fully described (Schroeder & Cavacini, 2010). It is not known to function in any of the main antibody effector mechanisms, but it has been speculated that membrane-bound IgD regulates the fate of B lymphocytes as they develop and become activated (Geisberger et al., 2006).

IgG is the most extensively studied, predominant antibody isotype found in the body and can be divided into several subclasses, including IgG1, IgG2a, IgG2b, IgG3, and IgG4. All IgGs function to neutralize pathogenic microbes and toxins to prevent the binding of these microbes and toxins to and entry into cells. IgG1 and IgG3 also serve to opsonize antigens, activate NK cells, activate the classical complement system, and initiate antibody-dependent cellular cytotoxicity. IgG1 and IgG3 are generally produced by plasma cells in response to peptide antigens, while IgG2 and IgG4 are produced in response to polysaccharide antigens. IgG is small and simple in structure, making it a
good candidate for passing between circulation and tissues, including the placenta and intestinal epithelium (Cavacini et al., 2003; Schroeder & Cavacini, 2010).

Plasma cell-derived secretory IgA is a critical antibody for protecting mucosal surfaces, such as the intestinal, respiratory, and reproductive tracts. IgA is produced by plasma cells in diffuse lymphoid tissues and serves to limit microbial access to the body’s mucosal surfaces by directly neutralizing pathogens or preventing the pathogens from binding to the mucosal epithelium. In non-ruminant species, IgA is elevated in milk and provides intestinal immunity to pathogens. Besides protecting mucosal surfaces, IgA may mediate antibody-dependent cellular cytotoxicity locally (Corthesy, 2007; Schroeder & Cavacini, 2010).

The final antibody isotype, IgE, is present at the lowest serum concentration and has the shortest half-life. IgE is associated with type I hypersensitivities and with immunity to parasites. TH2 lymphocytes stimulate B lymphocytes beneath body surfaces to produce IgE and initiate humoral immune responses. IgE activates mast cells, basophils, and eosinophils through signaling via Fce receptors. Once activated, these granulocytic cells, which contain cytoplasmic granules filled with inflammatory and antimicrobial compounds, degranulate, releasing vasoactive and cytolytic mediators (Chang et al., 2007; Schroeder & Cavacini, 2010).

As previously mentioned, one of the hallmark characteristics of the adaptive immune system is its ability to “remember” pathogens after previous exposure. While short-lived effector T and B lymphocytes serve to subdue current infections, pathogen-specific, long-lived memory T and B cells can also be generated to mirror effector cells and ensure immunological protection against future infections by the same antigenic
molecule. Memory lymphocytes allow for the secondary immune response, or any immune response to subsequent exposures to the same antigen, to respond to the antigenic molecule more effectively (Jameson & Masopust, 2018; Palm & Henry, 2019). During lymphocyte maturation, T and B cells with the highest affinity, or binding strength, for an antigen differentiate into short-lived effector cells, which work to immediately defend the body from the infectious agent. This ensures that the body’s response to the current infection is adequate to subdue the infection. Lower affinity lymphocytes typically differentiate into memory cells and form a reserve of antigen-specific cells in lymphoid tissues to be readily activated in future antigen exposures (Palm & Henry, 2019; Taylor et al., 2012). When re-exposed to a previously encountered antigen, memory lymphocytes initiate a more effective immune response that is both faster and stronger than the primary exposure. When stimulated by an antigen, memory T and B cells rapidly proliferate and amplify the immune response by performing effector functions, such as secreting high-affinity antibodies (Palm & Henry, 2019).

The complement system consists of a sequence of cleavage reactions on the pathogen surface. These reactions occur in response to the presence of pathogen molecules or the binding of antibodies to pathogenic antigens. The three complement pathways are initiated independently from each other and, at a given point, converge into a single pathway. As a result of these reactions, larger fragments stay bound to the pathogen while smaller fragments are released into circulation. Bound components can be recognized by complement receptors on the surface of phagocytic cells, which engulf and ultimately kills these pathogens; or can trigger the assembly of a membrane-attack
complex which results in the osmotic lysis of the pathogen. Small cleavage fragments stimulate inflammation, recruit inflammatory cells, and regulate B cell responses (Java et al., 2020).

During the body’s first exposure to an antigen, a primary adaptive immune response occurs. Because the adaptive immune system has not previously encountered the antigen, it has no memory T and B lymphocytes specific for that antigen. Naïve lymphocytes are activated to create antigen-specific effector cells. Activated lymphocytes undergo clonal expansion, or rapid proliferation, to create a supply of high affinity, antigen-specific cells to fight the immediate infection. A reserve of antigen-specific memory lymphocytes is also generated during the primary adaptive immune response. A secondary adaptive immune response occurs during the body’s subsequent exposures to that same antigen. The secondary adaptive immune response utilizes readily activated memory T and B lymphocytes that have high affinity and are antigen-specific and thus allow the body to respond to the infection in a faster and stronger manner (Riddell, 2022).

d. Mucosal Immunity

The largest organ of the immune system is the mucosa, which includes 4 main component parts: the microbiome, the mucus layer, the mucosal epithelial barrier, and the leukocytes of the mucosa-associated lymphoid tissues (MALT). MALT is located throughout the body in the reproductive tract, mammary glands, gastrointestinal tract, respiratory tract, and urinary tract (Vlasova & Saif, 2021). These thin and easily permeable surfaces make them a common site for pathogen entry. The mucosal immune system is an important portion of the body’s immunological defense, as it
provides protection from microbial invasion at vulnerable mucosal tissues (Chase & Kaushik, 2019).

The microbiome and mucus secretions of the mucosal tissues provide the first layers of defense. Various normal, commensal microorganisms occupy the body’s mucosal surfaces. These harmless occupants of the animal’s mucosal tissues prevent pathogens from establishing themselves through out-competing pathogenic microbes for resources and adhesion sites, as well as directly inhibiting pathogens through the production of antimicrobial molecules (Dadarwal et al., 2017; Vlasova & Saif, 2021). Mucus is secreted by goblet cells and lines mucosal surfaces. The mucus layer not only acts as a barrier between the mucosal epithelium and external microbes, but it also contains mucins, antimicrobial molecules, and secretory IgA (sIgA) which function to destroy pathogenic microbes (Chase & Kaushik, 2019).

At the mucosal epithelium, sIgA is responsible for immune exclusion, neutralizing pathogenic microbes before crossing the epithelial layer into mucosal tissues. When sIgA encounters a pathogen on the mucosal epithelium, it binds to the pathogen through cross-linking. This blocks the pathogen from adhering to the epithelial cell or penetrating and entering the epithelium. The sIgA-pathogen immune complex is then eliminated from the body. In this manner, sIgA on the mucosal epithelium is another barrier that effectively prevents pathogenic microbe entry into the thin, permeable mucosal tissues and protects the animal from infection (Corthesy, 2013).

If a pathogen is able to cross the aforementioned barriers and enter into a mucosal tissue, it then encounters leukocytes, antibodies, and other immune components associated with mucosal immune elimination. Submucosal tissues contain mast cells,
neutrophils, IgE, IgG, and the complement system, which actively target pathogens that have crossed the mucosal epithelium (Abraham & St John, 2010; Chase & Kaushik, 2019). As previously described, mast cells are located in high numbers at mucosal surfaces and degranulate to release inflammatory and antimicrobial mediators.

Neutrophils phagocytose pathogens, release cytotoxic chemicals, and trap pathogens with extracellular nets of bactericidal proteins. IgE is important for activating mast cells, and IgG is important for opsonizing pathogens and activating the complement system, among other roles. The complement system induces inflammation, opsonizes pathogens, lyses pathogens, aids in clearing immune complexes, and regulates the adaptive immune response by activating B-1 cells (Bonilla & Oettgen, 2010; Iwasaki & Medzhitov, 2015; Java et al., 2020). Together, these mucosal immunity components function to attack invading pathogens and clear them from the body’s mucosal tissues (Chase & Kaushik, 2019).

The mucosal immune response is a tightly regulated, intricate process. Antigens are taken up by mucosal DCs at inductive sites, organized lymphoid tissues where mucosal immune responses are initiated. Antigens are processed by DCs and presented to lymphocytes, which then initiate an adaptive immune response. The lymphocytes enter circulation and migrate to mucosal effector sites, diffuse tissues where cell- and antibody-mediated responses are executed. Effector sites contain T and B lymphocytes, IgA-secreting plasma cells, APCs, and mast cells (Chase & Kaushik, 2019; Dadarwal et al., 2017). A mucosal immune response initiated at one mucosal tissue stimulates an immune response in all mucosal tissues as lymphocytes are trafficked from circulation to effector sites throughout the body, a process known as homing (Brandtzaeg, 1997).
In this way, a mucosal immune response triggered at one site induces immunological protection of all mucosal tissues in the body.

The mucosal immune system is an important component of the dynamic immune system of the female reproductive tract. The mucosal epithelium of the uterus is more permeable than other MALTs, and uterine lamina propria contains fewer innate and adaptive leukocytes (Chase & Kaushik, 2019). In contrast to the general mucosal immune system, mucosal immunity in the female reproductive tract is under tight hormonal control. As sex hormones, such as E and P4, rise and wane, they modulate the mucosal immunity of the uterus and other reproductive organs both directly and indirectly via cytokines, chemokines, and growth factors (Rodriguez Garcia et al., 2015).

Physical characteristics of the mucosal immune system, such as the composition and thickness of the mucus layer, change throughout the animal's reproductive cycle and during gestation (Merilan, 1983). Additionally, sex hormones regulate the phenotype and function of mucosa-associated leukocytes in the reproductive tract. During pregnancy, the mucosal immune system of the female reproductive tract, under the influence of sex hormones, is responsible for both allowing the development of a semi-allogeneic calf as well as protecting the uterine mucosal tissue from pathogenic infection (Wira et al., 2014).
IV. **Maternal Immune Response and Changes during Pregnancy**

a. Immune Response During Insemination and Fertilization

A fundamental role of the immune system is to recognize antigens, which are foreign or non-self-molecules that induce an immune response in the host. After detecting an antigen, the immune system responds by initiating inflammation, triggering sickness behavior, and activating immune cells to protect the host animal. Pregnancy in viviparous animals, including cattle, represents an immunological paradox in which the maternal immune system not only tolerates a semi-allogeneic fetus, which contains paternal antigens, but creates a uterine environment supportive of fetal growth. Immune responses in the female reproductive tract are tightly regulated to balance the requirements for protection against pathogens, tolerance of foreign sperm, and support of the semi-allogeneic fetus (Samardžija et al., 2020).

The cow’s first exposure to paternal antigens occurs during insemination. During natural breeding in cattle, semen is deposited in the anterior vagina, and sperm travels from the vagina, through the cervix, into the uterine body, and down the uterine horns before fertilizing the oocyte in the oviduct. During artificial insemination (AI) in cattle, semen is deposited directly into the uterus. Immediately following insemination, paternal antigens in sperm activate the innate immune system and trigger inflammation in the uterine endometrium (Akthar et al., 2022). Sentinel cells in the uterus contain PRRs that identify pathogens via PAMPs to initiate early immune responses. Toll-like receptor (TLR)2 is an important transmembrane PRR in the bovine endometrium that recognizes PAMPs in the seminal fluid, the acellular portion of semen containing molecules like
proteins, buffers, and hormones (Akthar et al., 2022; Mogensen, 2009). After recognizing a PAMP, transcription factors are activated in the sentinel cells that induce changes in messenger RNA (mRNA) expression, resulting in a pro-inflammatory response in the endometrium characterized by increased secretion of inflammatory cytokines $IL-8$, $IL-1\beta$, $GM-CSF$, and $TNF-\alpha$, increased expression of pro-inflammatory prostaglandin E synthase (PGES), and suppression of anti-inflammatory cytokine $TGF-b1$ (Elweza et al., 2018).

Besides upregulating the production of pro-inflammatory factors and suppressing the production of anti-inflammatory factors to cause local inflammation, interactions between sperm and the endometrial epithelium also induce the recruitment of immune cells, namely polymorphonuclear neutrophils (PMNs), macrophages, and DCs, to the uterus. These leukocytes have important functions in innate immunity and acute inflammatory reactions. PMNs are first observed in the uterus 3 hours post-insemination and completely disappear from the uterus 10 hours post-insemination (Marey et al., 2019). They are the first cells recruited in substantial numbers to the site of a new infection, and they serve two main functions in the uterus after insemination: to clear the uterus of excess, abnormal, and dead sperm cells and to clear the uterus of other pathogens introduced into the female reproductive tract during insemination (Akthar et al., 2022). PMNs eliminate sperm and other pathogens from the uterus either by phagocytosis or NETosis (confinement of pathogens into a chromatin extracellular trap containing bactericidal molecules; (Alghamdi et al., 2009; Hong et al., 2017). Macrophages are phagocytic and secrete $TNF-\alpha$, $IL-6$, and $IL-1\beta$ cytokines to promote further inflammation. $IL-6$, secreted from macrophages in the uterus, is required to
induce T lymphocytes to differentiate into TH17 cells, which secrete pro-inflammatory IL-17 and recruit more PMNs to the uterus (Fair, 2015; Schuberth et al., 2008). DCs in the uterus post-insemination function as APCs for the activation of lymphocytes, and these cells ultimately confer and maintain peripheral tolerance of the pregnancy (Dietl et al., 2006; Fair, 2015). Semen-induced immune cell infiltration post-insemination, resulting in inflammation and the clearance of excess sperm and pathogens from the uterus, serves to prepare the uterine environment for implantation of the embryo (Talukder et al., 2020).

After traveling through the uterus, sperm migrate into the oviducts, the site of fertilization in cattle. Although some sperm are consumed by PMNs in the uterus, hundreds to thousands of sperm cells reach the oviducts, where they are capacitated and hyper-activated. In contrast to sperm-uterine crosstalk, which produces a pro-inflammatory immune response, sperm-oviduct crosstalk produces an anti-inflammatory immune response in cattle. An anti-inflammatory response is essential for supporting the survival of sperm cells in the oviduct (Talukder et al., 2020). The presence of sperm in the oviduct up-regulates the secretion of anti-inflammatory cytokines TGF-β1 and IL-10 and down-regulates the secretion of pro-inflammatory cytokines TNF-α and IL-1β in bovine oviduct epithelial cells (Yousef et al., 2016). Bovine oviduct epithelial cells also secrete PGE2, suppressing the phagocytic behavior of PMNs (Marey et al., 2013). Thus, the presence of sperm in the bovine oviduct has anti-inflammatory effects and suppresses neutrophil activity, which promotes the survival of sperm cells in the oviduct to encourage successful fertilization.
After fertilization, the zygote is formed and remains in the oviduct for three to four days, undergoing mitotic cell division and genome activation (Maillo et al., 2015). During this time, the pre-implantation embryo stays in contact with the oviduct epithelial cells and oviductal fluid, which contains carbohydrates, proteins, ions, lipids, metabolites, and EVs (Mazzarella et al., 2021). Although oviduct-embryo interactions in cattle are not fully characterized, it is known that the presence of the embryo in the oviduct affects specific oviductal gene expression, which regulates maternal immune response. The embryo’s ability to alter the cow’s local immune response in the oviduct is important to avoid immunological rejection of the semi-allogeneic embryo (Rodriguez-Alonso et al., 2020). The bovine embryo secretes IFN-τ, a protein that induces an anti-inflammatory response in the oviduct. IFN-τ is immunosuppressive and prohibits clonal expansion of lymphocytes, which protects the embryo from attack from the adaptive immune system (Skopets et al., 1992). The embryo may also decrease inflammation in the oviduct by downregulating pro-inflammatory mediators (NF-κB, CXCL2, and CCL20), decreasing antigen presentation mediators (CD74 and TAP binding protein), and repressing the action of the complement system locally (C3 proteins) (Maillo et al., 2015). Despite only remaining in the oviduct for a short period of time while traveling to the uterus, the embryo’s immunoregulatory interactions with oviduct epithelial cells have critical roles in embryo survival and successful pregnancy establishment in cattle (Talukder et al., 2020).

b. Immune Response during Hatching and Maternal Recognition of Pregnancy
Approximately 4 days post-fertilization, the 16-cell totipotent morula-stage bovine embryo enters the uterus from the oviduct. Interactions between the pre-hatching embryo and uterus in cattle are not thoroughly described, but unhatched bovine embryos likely start secreting significant amounts of IFN-τ, an anti-luteolytic immunosuppressive mediator important for creating an anti-inflammatory environment in the uterus during early pregnancy, as five days post-fertilization (Talukder et al., 2017, 2020). IFN-τ produced by morula-stage embryos acts locally and systemically to create an immune response that supports pregnancy. Locally, IFN-τ generates an anti-inflammatory response by activating IFN-stimulated genes ISG15, OAS1, and MX2 in uterine epithelial cells, which activate the IFN signaling cascade. IFN-τ suppresses the expression of inflammatory transcription factor NF-κB and subsequently, pro-inflammatory cytokines (TNF-α and IL-1β) in uterine epithelial cells. IFN-τ produced by pre-hatching embryos also up-regulates PGES expression and PGE2 secretion in the uterus. Although the exact mechanism is unknown, PGE2 has important roles in immunomodulation and embryo protection from maternal immune attack during pregnancy. Overall, these local IFN-τ actions establish an anti-inflammatory immune response in the uterine epithelium by suppressing innate immune responses of the uterine microenvironment (Passaro et al., 2018; Talukder et al., 2020).

IFN-τ secreted by pre-hatching bovine embryos also has a systemic effect during early pregnancy. PGE2 secretion as a result of IFN-τ production significantly increases the expression of anti-inflammatory cytokine TGF-β1 in peripheral blood mononuclear cells (PBMCs). TGF-β1 works alongside PGE2 to induce Treg development, which promotes immunosuppression and immunological tolerance during early pregnancy.
(Talukder et al., 2017). In PBMCs and PMNs, two IFN-stimulated genes (ISG15 and OAS1) are activated as a result of IFN-τ secretion by the morula and the expression of IL-10, a TH2 cytokine, is upregulated (Shirasuna et al., 2012). In maternal peripheral circulation, anti-inflammatory cytokine (TGF-β1 and IL-10) expression is upregulated in PBMCs and PMNs due to IFN-stimulated gene activation and PGES expression (Talukder et al., 2018). This suggests that peripheral maternal leukocytes acknowledge the presence of the pre-hatching embryo and, as a result, establish an antibody-mediated TH2 immune response during early pregnancy in cattle. Although maternal recognition of pregnancy is classically deemed to occur around day 16 in cattle, “very early” maternal recognition of pregnancy may begin as early as day 8 post-fertilization (Talukder et al., 2017).

By seven days post-fertilization, the morula-stage embryo becomes an unhatched blastocyst, characterized by the beginning of cellular differentiation. Pre-hatching blastocysts continue to secrete IFN-τ and can alter metabolism in the uterine microenvironment to modulate the maternal immune system. Blastocyst-derived signals affect the expression of lipoxygenase enzymes in the endometrium. As a result, the pre-hatching embryo can modulate concentrations of metabolites generated by lipoxygenase enzymes, including products of arachidonic and linoleic acids oxidative metabolism, which have biological functions in vasodilation, regulation of inflammatory response, and immune function (Sponchiado et al., 2019). By day 10 post-fertilization, the bovine embryo hatches from the zona pellucida and begins elongating from a spherical to a filamentous-shaped structure.
During the time between hatching and attachment to the uterine wall, the bovine embryo undergoes a period of rapid elongation and growth and becomes a conceptus with proliferating trophoblast cells. During this phase, which begins around day 13 post-fertilization in cattle, the conceptus secretes IFN-τ in higher quantities than morula and blastocyst stage embryos. Because IFN-τ secretion has a strong positive correlation to conceptus length, embryonic elongation is essential to ensure that adequate amounts of IFN-τ are produced to promote survival of the conceptus and pregnancy maintenance (Hansen et al., 2017). IFN-τ is a type I IFN and the maternal recognition of pregnancy signal in ruminants and has many functions in pregnancy maintenance and immunomodulation (Sanchez et al., 2019). Besides its anti-luteolytic effects that prevent corpus luteum (CL) disintegration by pulses of prostaglandin F2α (PGF2α) by the uterine endometrium, IFN-τ produced by the conceptus is a key regulator of maternal immune responses in cattle and regulates the immune system both in and out of the uterus. IFN-τ stimulates a substantial antiviral, anti-inflammatory response unique to ruminants because IFN-τ emerged in evolution after ruminant species had diverged from other mammals (Roberts et al., 2003).

As in morula and blastocyst stage embryos, conceptus-derived IFN-τ has an anti-inflammatory effect that is important for creating suitable conditions for developing the conceptus and the placenta (Velázquez et al., 2019). IFN-τ upregulates the expression of IFN-stimulated genes, such as TNF-α, peptide TAP1, TAP2, ISG15, MX1, MX2, OAS1, OAS2, IDO1, IL-12b, PTX3, MCP1, and MCP2, which have roles including leukocyte recruitment and activation, inflammatory response, enabling trophoblast adhesion, promoting regulatory T lymphocyte proliferation, and immunoregulation in the
endometrium (Mansouri-Attia et al., 2012; C. G. Walker et al., 2010). Up-regulated adaptive immune system genes likely induce immune tolerance to the semi-allogenic conceptus, while upregulated innate immune system genes likely protect the uterus from infection during local immunosuppression (C. G. Walker et al., 2010). IFN-τ blocks the activation of NF-κB and mitogen-activated protein kinase to suppress inflammation locally and increases MHC class 1 in the embryo, which has functions in embryo-maternal interaction and regulation of the maternal immune response during the pre-implantation period (Zhu et al., 2017). IFN-τ also modulates maternal immune response by activating IFN-stimulated genes in peripheral maternal tissues and immune cells, although the role of functions of peripheral immune cells during preimplantation is not as well characterized in ruminant species as it is in humans and rodents (Hansen et al., 2017; Ott, 2019). When IFN-τ secretion reaches peak levels around day 19 post-fertilization, trophoblast cell attachment to the endometrial wall and the formation of the fetal-maternal interface begin, which marks the end of the preimplantation period (Fair, 2015).

c. Immunotolerance Mechanisms at the Fetal-Maternal Interface

Implantation in cattle is non-invasive and characterized by superficial adherence of the trophectoderm of the conceptus to the endometrium. Successful implantation and maintenance of pregnancy require synchronization of maternal and fetal factors, including hormones, cytokines, and leukocytes. Throughout the implantation process and the remainder of gestation, the maternal immune system is aware of the presence of the semi-allogeneic conceptus, but it is regulated by specific activation, reprogramming, and suppression both locally in the maternal endometrium and
systemically (Ott, 2020). Despite being altered, the immune system at the fetal-maternal interface is not dormant. Rather, it is immunologically active and involves a complex balance between the need for immunological tolerance of paternal antigens in the semi-allogeneic fetus and the need for continuous defense against pathogens (Samardžija et al., 2020). Conceptus-maternal communication at the fetal-maternal interface is essential to induce regulation and balance of the maternal immune system for successful pregnancy maintenance (Ott, 2020).

In contrast to prior immunological events in the establishment of pregnancy, early implantation favors a pro-inflammatory uterine environment. Early implantation is characterized by up-regulation of cell-mediated, pro-inflammatory TH1 responses and pro-inflammatory cytokine (IL-6, IL-8, and TNF-α) production in the uterus (Van Sinderen et al., 2013). Cytokines produced at the fetal-maternal interface during early implantation (IL-6, MIP-1β, CX3CL1, and IP-10) function both to attract and activate leukocytes and to aid in the implantation process by encouraging trophoblast cell migration (Hannan et al., 2006). Leukocytes, including macrophages and DCs, are recruited to the uterus in abundance during the onset of implantation and secrete pro-inflammatory and anti-inflammatory cytokines that function in endometrial remodeling for placentation (Nagamantsu & Schust, 2010). Uterine inflammation, as a result of leukocyte trafficking and pro-inflammatory cytokine secretion by recruited immune cells and endometrial cells, enhances the expression of adhesion molecules necessary for the conceptus to bind to the endometrial epithelium and for implantation to occur (Dekel et al., 2019). Local inflammation during early implantation plays a critical role in uterine receptivity and successful conceptus implantation.
The uterus returns to an anti-inflammatory state following the pro-inflammatory phase of early implantation and placentation. During the first 38 days post-fertilization, \( IFN-\tau \) is secreted by trophoblast cells. As in pre-attachment bovine embryos, \( IFN-\tau \) secretion modulates the expression of genes (IFN-stimulated genes) both in the uterus and the peripheral circulation. Like other Type I IFNs, \( IFN-\tau \) promotes an anti-inflammatory response and induces Treg lymphocytes (Ott, 2019). The effects of \( IFN-\tau \) are exerted through classical type 1 IFN receptors (IFNAR1 and IFNAR2), cell-surface receptors that can stimulate canonical and non-canonical pathways, regardless of tissue type. Conceptus-derived \( IFN-\tau \) produced at the fetal-maternal interface has three modes of action—autocrine, paracrine, and endocrine (Rocha et al., 2021). Bovine trophoblast cells secrete \( IFN-\tau \), which can then act in a dose-dependent, autocrine manner to stimulate trophoblast cell proliferation. This results in the secretion of increased \( IFN-\tau \) levels by the growing trophectoderm (X. L. Wang et al., 2013).

\( IFN-\tau \) acts in a paracrine manner at the fetal-maternal interface by activating IFN-stimulated genes, such as \( IRF1 \) and \( IRF2 \), that promote uterine receptivity, maternal immunomodulation, and conceptus development. In the uterus, \( IFN-\tau \) upregulates the expression of \( MCP1 \) and \( MCP2 \), which recruit monocytes to the endometrium. Monocytes, which become macrophages in tissue, are one of the most important immune cell types during the maternal immune response to the conceptus in bovine pregnancy, as well as being important for placental development and angiogenesis (Rocha et al., 2021). The majority of the macrophages present at the fetal-maternal interface are immunomodulatory, anti-inflammatory M2 macrophages involved in the resolution and clearance of inflammation. M2 cells clear apoptotic cells from the
endometrium to block the production of pro-inflammatory mediators and induce a local immunosuppressive response (Faas et al., 2014; Mansouri-Attia et al., 2012). In ruminant species, IFN-τ stimulates local lymphopenia and neutropenia, which suppress pro-inflammatory responses to pregnancy (Rocha et al., 2021). As described in the Immune Response during Hatching, Implantation, and Maternal Recognition of Pregnancy section, IFN-τ also blocks the activation of NF-κB and MAPK to suppress inflammation in the uterus (Zhu et al., 2017).

An important characteristic of IFN-τ produced by trophoblast cells at the fetal-maternal interface is its ability to leave the uterus and induce endocrine immunomodulatory effects during bovine pregnancy. IFN-τ secretion by the conceptus stimulates the expression of IFN-stimulated genes in systemic tissues, including PBMCs and PMNs. The effects of endocrine stimulation of immune cells by IFN-τ in cattle is not fully characterized. It is possible that IFN-stimulated gene expression in tissues such as the mesenteric lymph nodes may function to traffic peripheral immune cells, that IFN-τ could serve directly as a chemotactic factor to the uterus, or that immune cells could help maintain CL function (Hansen et al., 2017; Rocha et al., 2021).

Type 1 IFNs, such as IFN-τ, also have functions in the PRR pathway during early gestation. The PRR pathway of the innate immune system has roles in pathogen recognition and response and is described in detail previously. Type 1 IFNs can bind to sub-proteins on the PRR complex to activate the PRR signaling cascade (Dai et al., 2016). Besides activating the PRR pathway, type 1 IFNs also regulate the pathway through the production of mRNAs. Nod-like receptors, a intracellular PRR component, are activated by type 1 IFNs and can be divided into three subgroups—inflammasome,
reproductive, and regulatory. Nod-like receptors, once stimulated by type 1 IFNs, modulate important signaling cascades, such as the NF-κB and IFN pathways (Coutermarsh-Ott et al., 2016). Stimulation of PRR and nod-like receptor pathways by type 1 IFNs, such as IFN-τ, have the capacity to regulate inflammation and immune response through IL and caspase 1 maturation, IFN-stimulated gene expression, and IRF production (Rocha et al., 2021).

IFN-τ is not the only immunoregulatory molecule present during early gestation in cattle. Besides its direct immunomodulatory functions, a key role of IFN-τ is to block pulsatile secretion of luteolytic PGF2α by the uterine endometrium to preserve the life of the CL. This is vital for maintenance of the pregnancy because the CL secretes P4, an important regulator of the maternal immune system during early gestation (Vlasova & Saif, 2021). Luteal P4 is anti-inflammatory and down-regulates uterine immune functions. P4 suppresses lymphocyte proliferation in the endometrium, even in the presence of antigen. (Lewis, 2003). Decreased lymphocyte proliferation subdues the cow’s adaptive immune response to antigens, such as the semi-allogeneic conceptus. P4 also has a role in down-regulating eicosanoid synthesis in the uterus. Eicosanoids, including PGF2α, up-regulate immune responses by enhancing pro-inflammatory cytokine production, phagocytosis, and lymphocyte function. By inhibiting eicosanoid production, P4 decreases local inflammation and adaptive immune response in the uterus (Seals et al., 2002). Although down-regulation of immune responses by luteal P4 decreases the ability of the uterus to resist infection, an anti-inflammatory uterine environment is essential to protect the bovine conceptus from maternal immune attack. In humans, P4 has been shown to inhibit the production of IL-17 by peripheral TH17
lymphocytes. IL-17 is a pro-inflammatory cytokine with roles in inflammation and neutrophil recruitment, and increased levels of IL-17 is associated with loss of pregnancy. P4 in humans also suppresses production of TH1 cytokines (IFN-γ, TNF-α, and IL-2) that function to promote cell-mediated immunity. Thus, P4 has a role in the shift from a TH1 and TH17 cytokine bias to TH2 cytokine bias, an important event for maternal tolerance of the semi-allogeneic conceptus (AbdulHussain et al., 2020). P4 has also been shown to promote proliferation of immunosuppressive Treg cells and enhance their activity in humans, which aids in establishing immunological tolerance to paternal antigens present in the conceptus (Tai et al., 2008).

Prostaglandins, namely PGE2, are secreted by both the bovine conceptus and the uterine endometrium during early gestation. Prostaglandins secreted by the conceptus act in a paracrine manner to enhance ISG expression and function in the uterus. PGE2 has anti-luteolytic properties, thus prolonging the lifespan on the CL for continued P4 production (Bridi, Perecin, & da Silveira, 2020). PGE2 is also immunosuppressive and down-regulates the activity of T lymphocytes, NK cells, DCs, and macrophages via EP2 and EP4 receptors. The binding of PGE2 ligands to EP2 and EP4 receptors initiates a cyclic adenosine monophosphate (cAMP), protein kinase A, and cAMP response element binding protein pathway that up-regulates the expression of anti-inflammatory genes. Similar to P4, PGE2 inhibits cell-mediated responses by down-regulating TH1 cytokine production. It also promotes the differentiation of immunosuppressive cells, such as M2 macrophages, myeloid derived suppressor cells, and Treg lymphocytes. In cattle, PGE2 regulates PD-1 and lymphocyte activation gene-3 activity, which are immune system inhibitory mediators that function in a negative-feedback manner to
prevent excessive immune responses. PGE2 also inhibits the proliferation of CD4+ and CD8+ T lymphocytes and up-regulates the production of *IL-10* and *STAT3* mRNAs in PBMCs, thus shifting the animal to a TH2 humoral-mediated immune bias (Sajiki et al., 2018).

Indoleamine 2,3 dioxygenase 1 (IDO1) is another important immune regulator present at the fetal-maternal interface in cattle. IDO1 is an immunosuppressive enzyme activated by *IFN*-τ that causes an anti-inflammatory immune response by down-regulating T lymphocyte function. By consuming and sequestering tryptophan from the uterine microenvironment, IDO1 inhibits T lymphocyte function by starving endometrial T lymphocytes of this essential amino acid. Tryptophan deficiency blocks progression of the cell cycle and causes apoptosis of T lymphocytes due to arrest at the mid-G1 phase of the cell cycle. Ultimately, T lymphocytes are unable to proliferate in the presence of IDO1 (G. K. Lee et al., 2002). Besides its role in T lymphocyte suppression, IDO1 has been observed to significantly increase anti-inflammatory cytokine (*IL-4*, *IL-10*, and *TGF-β1*) production under the influence of *IFN*-τ. *IL-4* is a key cytokine causing the differentiation of naïve T helper lymphocytes into TH2 cells, which promote further TH2 differentiation and regulate humoral and adaptive immunity (Sokol et al., 2008). *IL-10* is an anti-inflammatory mediator produced by TH2 cells, as well as macrophages and monocytes, and *TGF-β1* has immunoregulatory and anti-inflammatory functions (Ayatollahi et al., 2007). Up-regulation of TH1 cell differentiation in the absence of *IL-4* results in pro-inflammatory cytokine (*IFN-γ* and *TNF-α*) production, which is associated with pregnancy complications and loss of pregnancy in mammalian species. Thus, IDO1 may have a role in suppressing pro-inflammatory cytokine producing TH1 cells and...
promoting anti-inflammatory cytokine producing TH2 cells, an important event for immunological tolerance of the conceptus and pregnancy success (Mohapatra et al., 2020).

Pregnancy associated glycoproteins (PAGs) are a family of aspartic proteinases expressed by the ruminant placenta and are found in high concentrates both at the fetal-maternal interface and in maternal circulation in cattle (Ott et al., 2014). PAGs are secreted by TGCs of the placenta. TGCs begin secreting PAGs on day 15 post-fertilization, but PAGs do not reach significant levels until day 24 post-fertilization. The specific roles of PAGs are unclear, but they are hypothesized to function in immune modulation and maternal immune tolerance of the conceptus during gestation in cattle (Oliveira Filho et al., 2020). Treatment of bovine immune cells with PAGs in vitro decreased granulocyte, monocyte, macrophage, and DC proliferation, indicating a potential anti-inflammatory role of PAGs (Hoeben et al., 1999). PAGs induce the release of GCP-2 (also known as CXCL6), a chemotactic factor that attracts neutrophils through binding CXCR1 and CXCR2 receptors on neutrophils, from day 24 post-fertilization until the end of gestation (Austin et al., 1999). While evidence of PAGs both being anti-inflammatory by suppressing immune cell proliferation and pro-inflammatory by initiating the release of a neutrophil chemoattractant seems contradictory, alpha chemokines, like GCP-2, also have non-immunomodulatory functions in the formation of new blood vessels or angiogenesis, which important for placental development during early pregnancy (Balestrieri et al., 2008). It is possible that GCP-2 released due to PAG secretion serves to promote placenta formation and development rather than as a pro-inflammatory mediator in bovine pregnancy. The functions and relevance of PAGs at
the fetal-maternal interface have not been completely described, but it is apparent that PAGs have a role in regulating the immune response and inflammation at the fetal-maternal interface in ruminants.

The bovine fetal-maternal interface plays host to a variety of immune cell types, including monocytes, macrophages, DCs, and T and B lymphocytes. Monocytes are circulating leukocytes, and in tissue, such as the endometrium, they differentiate into either macrophages or DCs. Both macrophages and DCs are major subsets of the immune cell population at the fetal-maternal interface. It is likely that IFN-τ produced by trophoblast cells at the fetal-maternal interface has a role in trafficking monocytes to the uterus from circulation and promoting the differentiation of monocytes into macrophages or DCs in uterine tissue (Mansouri-Attia et al., 2012). MCP1 and MCP2, monocyte chemoattract chemokines stimulated by IFN-τ, are potent recruiters of monocytes in vivo. Although MCP1 and MCP2 are characterized as pro-inflammatory mediators, they has been suggested to induce antibody-mediated TH2 responses in mice models (Gu et al., 1997). Thus, up-regulation of MCP1 and MCP2 chemokines during early bovine pregnancy could be a result of monocyte recruitment from circulation to enhance the TH2-type response characteristic of pregnancy (Mansouri-Attia et al., 2012).

Macrophages are rare in the uterus of open cows, but intrauterine macrophage populations begin accumulating significantly as early as day 13 post-fertilization. Endometrial macrophages have roles in immunomodulation, fetal growth, and parturition. Their functions may vary depending on anatomical location, as caruncular macrophages and interplacentomal macrophages vary in morphology and gene expression (Oliveira et al., 2010). Macrophages can be generally divided into two
subclasses—M1 and M2. M1 macrophages are pro-inflammatory and characterized by high expression of IL-12 and IL-23 and low expression of IL-10. M2 macrophages are anti-inflammatory and characterized by high expression of IL-10 and low expression of IL-12 and IL-23 (Hooshmandabbasi et al., 2021; Schebesch et al., 1997). M2 macrophages are pivotal regulators of immune responses. They serve to suppress inflammatory immune response, heal damaged tissue, and stimulate antibody production (Mills & Ley, 2014). Regulation of correct intrauterine macrophage subpopulation proportions is vital for successful pregnancy maintenance. After the placenta has developed, M2 macrophages are the dominant population at the fetal-maternal interface and are essential to sustain maternal immune tolerance of the conceptus (Hooshmandabbasi et al., 2021). M2 macrophages phagocytose apoptotic cells and trophoblast debris, resulting in the down-regulation of pro-inflammatory cytokines (IL-12, P70, IL-1β, and IL-8) and the up-regulation of anti-inflammatory cytokines (IL-10, IL-6, IL-1Ra, and IDO). M2 macrophages also function as APCs and clear antigen-antibody immune complexes to regulate immune function locally. M1 macrophage bias at the fetal-maternal interface during early and mid-gestation is associated with pregnancy complications and loss (Yao et al., 2019). In essence, macrophages are recruited to the uterus in significant numbers during early bovine pregnancy, and an intrauterine M2 macrophage bias is essential for maternal immune tolerance of the semi-allogeneic conceptus and pregnancy maintenance.

DCs are another important cell type at the fetal-maternal interface. DCs have. DCs can be considered the “gatekeepers” of the intrauterine environment during pregnancy due to their unique ability to induce both antigen-specific activation of the adaptive
immune system and immunosuppression. Under normal conditions, DCs establish an anti-inflammatory environment but, when stimulated by cytokines, allow protective immunity and sufficient immune activation (Blois et al., 2007; Mansouri-Attia et al., 2012). DCs are a significant source of PX3, an important molecule for female fertility. PTX3 is involved in the innate immune system and has functions in selective pathogen recognition, opsonization, enhancing phagocytosis, modulation of inflammation, complement-mediated functions, and regulation of autoimmunity (Fair, 2015; Mansouri-Attia et al., 2012). As the most potent APC and activator of the adaptive immune system, DCs are required for activating naïve T lymphocytes and differentiating T lymphocytes into several subpopulations, including TH1, TH2, TH17, and Treg. As previously mentioned, cell-mediated TH1 responses and highly inflammatory TH17 responses are detrimental to pregnancy viability, and antibody-mediated TH2 responses and tolerance-inducing Treg responses are supportive of pregnancy. DCs, though the activation of naïve T lymphocytes and regulation of cytokine secretion, are the key mediator behind the polarization from a TH1 bias to a TH2 bias at the fetal-maternal interface (Saito et al., 2010; Wei et al., 2021). Besides up-regulating TH2 differentiation, DCs also aid in establishing maternal immunological tolerance by promoting immunosuppressive Treg cell differentiation through the secretion of $TGF-\beta$ (Wei et al., 2021).

Treg cells are a specialized subset of immunosuppressive T lymphocytes with an important role in establishing immunological tolerance. Treg cells are essential for pregnancy maintenance, as pregnancy is a physiological condition that is highly dependent on maternal tolerance of the semi-allogeneic conceptus, which contains
many paternal antigens (Huang et al., 2020). During pregnancy, the number of Treg cells in maternal circulation significantly increases, and Tregs are specifically recruited to the fetal-maternal interface. Tregs, primed by paternal antigens present in sperm and seminal fluid, induce immunological tolerance of the semi-allogeneic conceptus by blocking the action of effector CD4+ and CD8+ T lymphocytes via cell contact dependent and cell contact independent mechanisms. Exposure to paternal antigens during insemination results in the expansion of antigen-specific Treg lymphocytes that work to create an immune-privileged environment to protect the conceptus from immunological rejection at the fetal-maternal interface (Clark et al., 2008; Huang et al., 2020). Intrauterine Treg populations peak shortly following implantation and continue to maintain immunological tolerance of the conceptus throughout gestation until declining in numbers near parturition (Huang et al., 2020).

As mentioned previously, there is a shift from a cell-mediated TH1 bias at implantation to an antibody-mediated TH2 bias in the post-implantation period. TH2 lymphocytes at the fetal-maternal interface, which are activated by uterine DCs presenting paternal antigens, release TH2-type cytokines, including IL-4, IL-5, IL-10, and IL-13 (Wang et al., 2020). Because TH1 and TH17 responses induce cytotoxic and pro-inflammatory responses, suppressing their activity at the fetal-maternal interface is essential for maintenance of pregnancy. Cytokines produced by TH1 (IFN-γ, TNF-α, and IL-2) and TH17 (IL-17) cells have been shown to stimulate trophoblast cell death, inhibit fetal development, and induce spontaneous abortions in murine models (Chaouat et al., 1990). By secreting cytokines that actively suppress the differentiation of TH1 and TH17 lymphocytes, TH2 cells at the fetal-maternal interface modulate the maternal
immune system in a way that supports pregnancy and conceptus viability (R. E. Mitchell et al., 2017). *IL-10*, which is secreted by TH2 lymphocytes, also aids in activating M2 macrophages, a pivotal intrauterine leukocyte population for maternal immune response during early bovine pregnancy (Fair, 2015; Wang et al., 2020). TH2 cells stimulate antibody-mediated immunity, as opposed to the cell-mediated immunity associated with TH1 cells. TH2-type cytokines could promote the development of B lymphocytes into antibody-producing plasma cells, and the production of protective antibodies directed against paternal antigens is important for pregnancy maintenance and maternal immunological tolerance (Maddur & Bayry, 2015).

At the fetal-maternal interface, a variety of immune cell types, including monocytes, DCs, effector T lymphocytes, Treg cells, and B cells, express PD-1, a transmembrane inhibitory co-stimulatory molecule (Meggyes et al., 2019). During pregnancy, PD-1 receptor and its ligand, PD-L1, regulate the balance of T lymphocyte activation, T lymphocyte homeostasis, peripheral tolerance of fetal antigens, and immune-mediated tissue damage (Sharpe et al., 2007). Naïve T cell activation requires two signals from APCs, mainly DCs: presentation of the antigen via an MHC molecule and co-stimulation by co-stimulatory molecules on the DC. The co-stimulatory pathway of T lymphocyte activation involving PD-1 (CD274) delivers a negative signal that contributes to T cell exhaustion by down-regulating T cell-specific cytokine production and inhibiting T cell proliferation and survival (Gianchecchi et al., 2013). PD-1/PD-L1 interactions at the fetal-maternal interface also promote the differentiation of naïve T lymphocytes into Treg cells and enhance the immunosuppressive activity of Tregs (Francisco et al., 2010). Immune cells present at the fetal-maternal interface have significantly increased
PD-1 receptor expression compared to peripheral leukocytes, likely due to the need for increased regulation of the immune system at the fetal-maternal interface to avoid excessive inflammation (Meggyes et al., 2019). The PD-1/PD-L1 pathway is critical for immunomodulation at the fetal-maternal interface in order to regulate leukocyte activity and induce immunological tolerance of the semi-allogeneic conceptus (Y.-H. Zhang et al., 2015).

Other cell types present in smaller numbers at the bovine fetal-maternal interface include NK cells and B lymphocytes. NK cells are recruited abundantly to the uterus and have important roles during pregnancy in humans but are found only scarcely at the bovine fetal-maternal interface. Uterine NK cells likely have a function during vascular remodeling of discrete placentomal regions during peri-implantation period in cattle, but their significance during bovine pregnancy wane off after the placenta has developed (Oliveira et al., 2013). B lymphocytes are present at the fetal-maternal interface throughout pregnancy, but little is known about their involvement in bovine gestation compared to human and mice. In humans, intrauterine B cells are physiologically distinct from peripheral B cells. B lymphocytes at the fetal-maternal interface have increased expression of CD27, a memory marker, when compared to peripheral populations, suggesting that intrauterine B lymphocytes enhances B cell immunological memory. Intrauterine B cells also have the capacity to produce IL-10, a key cytokine for stimulating humoral immunity and TH2 differentiation (Benner et al., 2020).
V. Overview of Extracellular Vesicles

a. Description of Extracellular Vesicles

EVs are a heterogeneous group of lipid bilayer membrane-enclosed molecules released from cells into the extracellular space and biological fluids. These vesicles play a critical role in cell-cell communication by carrying cargo from donor cells to target cells. This cargo includes micro RNAs (miRNAs), mRNAs, proteins, cytokines, metabolites, lipids, and other small biological substances (Das & Kale, 2020). EVs and their cargo are internalized into target cells by various mechanisms, including micropinocytosis, phagocytosis, and lipid raft mediated interactions. After being taken into a recipient cell, the EV’s cargo molecules are released into the target cell's cytoplasm to cause changes in the function of the target cell (Godakumara et al., 2021).

In mammals, EVs target and alter the function of a diverse range of cell types and body systems. For example, EVs in synovial fluid deliver autoantigens and initiate joint inflammation (Mor-Vaknin et al., 2011). EVs in blood affect coagulation and delivery of pro-angiogenic factors to initiate the formation of new blood vessels (Castaman et al., 1996; van Balkom et al., 2013). EVs in saliva contain peptide-cleaving proteins and coagulation factors, which aid in the healing process when animals lick wounds (Berckmans et al., 2011; Ogawa et al., 2011). EVs released from the T lymphocytes can activate and suppress the immune system (Nolte-'t Hoen & Wauben, 2012). In the nervous system, EVs play a role in neural cell communication and mediate synaptic plasticity (Lachenal et al., 2011). In the female reproductive tract, EV cargo affects sperm capacitation, fertilization, immune system modulation, embryo implantation and
development, and endometrial-embryo crosstalk (Al-Dossary et al., 2013; Oreshkova et al., 2012). In summary, EVs are found in many biological fluids and tissues and can produce a wide range of effects on target cell function.

b. Extracellular Vesicle Synthesis

Based on size and biogenesis, EVs can be categorized into exosomes, microvesicles (MVs), and apoptotic bodies. Exosomes have a spherical shape in solution and typically range from 30 to 200 nanometers in diameter. They are synthesized via an endocytic pathway and are released via exocytosis (Gurung et al., 2021). Inside the cell, structures known as multivesicular bodies are filled with intraluminal vesicles, which are formed via the inward budding of the endosomal membrane. These intraluminal vesicles are loaded with proteins, lipids, nucleic acids, and other cellular components sequestered from the cytosol. Multivesicular bodies travel along the cell’s cytoskeletal network to the plasma membrane and fuse with the plasma membrane to release the intraluminal vesicles, now known as exosomes, into the extracellular space via exocytosis (Colombe et al., 2014). Exosome biogenesis is a tightly regulated pathway important for cellular communication, and exosomal cargo reflects the physiological state of the donor cell (Zamani et al., 2019).

MVs are heterogenous in shape and range from 100 to 1000 nanometers in diameter. They are released from the donor cell into the extracellular space directly from the outward budding of the donor cell’s plasma membrane (Gurung et al., 2021). During MV biogenesis, molecular cargo like proteins, DNA, and miRNA are trafficked to the donor cell’s plasma membrane. MV cargo components are selectively trafficked to the membrane and are not random amalgamations of cellular components (D’Souza-
Schorey & Clancy, 2012). The plasma membrane then blebs outward and pinches off using adenosine triphosphate (ATP)-dependent contractile machinery to form membrane-enclosed, irregularly shaped MVs, which carry cargo to target cells (Tricarico et al., 2017).

Apoptotic bodies, the largest EVs, are variably shaped and over 1000 nanometers in diameter (Gurung et al., 2021). Apoptotic bodies are released from cells undergoing apoptosis or programmed cell death. During the final phase of apoptosis, the cell fractures into subcellular apoptotic body fragments, which are irregular and inconsistent in size and cargo. Apoptotic body cargo includes DNA fragments, miRNAs, histones, chromatin remnants, degraded proteins, and intact organelles. Once released into the extracellular space, they are rapidly phagocytosed by surrounding cells and digested in phagolysosomes. Apoptotic bodies pass useful materials and information from dying cells to target cells (Battistelli & Falcieri, 2020).

c. Extracellular Vesicle Uptake and Cell-Cell Signaling Mechanisms

EVs are secreted by most cell types and mediate intercellular communication both locally and systemically. Non-specific uptake is common to all EV types, but specific targeting to recipient cells is characteristic of exosomes. This is accomplished by the surface structure of exosomes, which contains molecules such as glycoproteins, lipids, and recognition motifs that facilitate exosome cargo delivery to specific target cells (Gurung et al., 2021). After coming into contact with their target cell, exosomes can trigger intercellular signaling via internalization by the target cell, direct binding with the target cell, or fusion with the target cell plasma membrane. The primary mechanism by which exosomes mediate intercellular communication is via rapid internalization by
target cells. Exosome internalization can be accomplished by Clathrin-mediated endocytosis, lipid-raft mediated endocytosis, caveolin-mediated endocytosis, phagocytosis, macropinocytosis, or a co-existing combination of these. Regardless of the mode of internalization, this route of EV signaling is tightly regulated and involves the uptake of the entire exosome and its cargo into the recipient cell, where the exosome then fuses with an intracellular compartment of the target cell to release its cargo (Gurung et al., 2021).

When exosomes interact with the target cell via direct binding, transmembrane ligands on the exosome bind to extracellular receptors on the target cell plasma membrane. This binding initiates a downstream signaling cascade that alters the function of the target cell (Gurung et al., 2021). An example of this route of EV signaling is dendritic cell-derived exosomes expressing MHC peptide complexes binding to Toll-like receptor ligands on the extracellular surface of target cells to enhance immune responses (Tkach et al., 2017).

Exosomes can also mediate cell-cell signaling by releasing their cargo directly into their target cell’s cytosol via fusion with the target cell’s plasma membrane. Specialized proteins pull the exosome into close vicinity to the target cell so that the membrane of the exosome and the membrane of the target cell fuse to create one continuous membrane, causing the internal release of the exosome’s cargo into the target cell (Gurung et al., 2021). This mechanism of EV signaling has been observed in DCs and tumor cells (Montecalvo et al., 2011; Y. Zheng et al., 2019).
VI. Overview of MicroRNAs

a. Description of miRNAs

miRNAs are non-coding RNA segments with an average length of 22 nucleotides that are not translated directly into proteins but are components of the epigenome, heritable phenotypic alterations that are not linked to nucleic acid sequence changes (Holtzman & Gersbach, 2018). miRNAs are present both intracellularly and extracellularly. Many biological fluids, including plasma, serum, cerebrospinal fluid, saliva, breast milk, urine, tears, colostrum, peritoneal fluid, bronchial lavage, seminal fluid, and ovarian follicular fluid contain circulating extracellular miRNAs either in EVs or associated with proteins (O’Brien et al., 2018). miRNAs can interact with mRNA, proteins, and other non-coding RNAs to elicit phenotypic changes important for the function and survival of an animal (P. Zhang et al., 2019).

miRNAs can regulate gene by silencing, activating, and stabilizing gene expression. To silence genes, miRNA molecules can bind to the 3’ untranslated region, 5’ untranslated region, or coding regions of a specific target mRNA, leading to deadenylation and decapping of the mRNA strand. This ultimately causes translational repression of the target mRNA. In contrast, miRNAs can initiate translational activation by interacting with promoter regions of DNA sequences. This leads to up-regulation of gene expression. Besides altering gene expression, dynamic miRNA binding can promote stable gene expression by buffering out stochastic fluctuations in transcription (O’Brien et al., 2018). Gene expression regulation by miRNAs has roles in many
biological processes including reproduction, which affects pregnancy establishment, embryonic development, and fetal-maternal communication (Bredi et al., 2021).

b. miRNA Synthesis and Uptake

Synthesis of a miRNA begins with the processing of RNA polymerase transcripts and can be classified into either canonical or non-canonical pathways. Canonical miRNA synthesis, the predominant pathway, involves forming primary miRNA hairpins from independent genes and then processing the primary miRNA segments by a microprocessor protein complex. The pri-miRNA strand is then transported to the cytoplasm for further enzymatic processing to form a mature miRNA duplex. Non-canonical miRNA synthesis is less common and uses different combinations of canonical protein complexes to create functional miRNAs (O'Brien et al., 2018). The biogenesis of miRNA is regulated both pre-transcriptionally by transcription factors and enhancers and post-transcriptionally by modulation of protein complexes involved in miRNA synthesis and interactions with RNA binding proteins (Dexheimer & Cochella, 2020).

While some extracellular miRNAs are released due to cell death or injury, the release of miRNAs into the extracellular space can also be a regulated process for targeted cell-cell communication. Once in the extracellular environment, free miRNAs may interact with cell-surface receptors, and vesicle-enclosed miRNAs may be taken into target cells via endocytosis, micropinocytosis, phagocytosis, or fusion with the target cell’s plasma membrane. miRNAs can also be exchanged directly between cells via cell-cell contact and gap junctions. Once inside recipient cells, miRNAs localize in
the rough ER, trans-Golgi network, stress granules, processing bodies, multivesicular bodies, lysosomes, mitochondria, and nucleus (O’Brien et al., 2018).
VII. Immunomodulatory Roles of Extracellular Vesicles in Pregnancy

Establishment and Maintenance

a. Extracellular Vesicle Interactions with the Maternal Immune System

As previously discussed, EVs play an important role in mediating intercellular communication through physical interaction with target cells and cargo transfer, such as proteins, lipids, and nucleic acids, between cells. As mediators of cell-to-cell communication, EVs are involved in a wide variety of biological functions, including regulation of the immune system (Chen et al., 2019; Gurung et al., 2021). Depending on the state of the parent cell, EVs can have either pro- or anti-inflammatory effects through the trafficking of mediators like miRNAs which stimulate cytokine production, leukocyte development and recruitment, and other immune system functions and activities (Buzas, 2022).

Pregnancy establishment is an intricate process that requires a dynamic balance between pro- and anti-inflammatory processes (PrabhuDas et al., 2015; Z. Wang et al., 2021). During early pregnancy, inflammation is required for biological processes such as implantation of the embryo to the endometrial epithelium and the formation of the placenta (Mor et al., 2011). While some inflammation is necessary to establish pregnancy, excessive inflammation can be detrimental to the viability of the embryo. An excessive inflammatory response at the fetal-maternal interface can result in complications including implantation failure, abnormal placental development, embryo loss, and pathological pregnancies (Sieg et al., 2022). The maternal immune system must be tightly regulated so that the uterine microenvironment remains tolerant and supportive of the semi-allogeneic embryo while simultaneously allowing sufficient
inflammation for pregnancy establishment and defense against pathogens (Olmos-Ortiz et al., 2019). The immunological balance required for establishment and maintenance of a healthy pregnancy is organized by carefully coordinated intercellular communication between the fetal and maternal systems (Talukder et al., 2020).

Accumulating research shows that EVs released from the trophoblast cells of the placenta at the fetal-maternal interface are key mediators of the fetal-maternal crosstalk necessary for regulation of the immunological processes during establishment and maintenance of pregnancy (Bai et al., 2021; Godakumara et al., 2021). Trophoblast-derived EVs regulate crosstalk between the fetal membranes and maternal leukocytes to create a hospitable uterine environment for embryo implantation and development (Giacomini et al., 2019; Godakumara et al., 2021). When cell-to-cell dialog at the fetal-maternal interface is insufficient or abnormal, the biochemical processes necessary for pregnancy establishment and maintenance are disrupted, resulting in reproductive failure (Bashiri et al., 2018).

b. Roles of Extracellular Vesicles in Healthy Pregnancy Establishment and Early Pregnancy

EVs play essential roles in many aspects of healthy female reproduction and pregnancy, including sustaining reproductive organ homeostasis, regulating ovulation, supporting embryo implantation, and mediating crosstalk between the fetal and maternal systems at the fetal-maternal interface (Smith & Russell, 2022). Prior to fertilization, ovarian EVs function to promote folliculogenesis, and miRNAs isolated from follicular fluid-derived EVs target genes associated with meiosis, cell proliferation, metabolic pathways, transcriptional regulation, immune system development, and
creating a favorable uterine environment for fertilization (Hasan et al., 2020; Noferesti et al., 2015; Sang et al., 2013; Smith & Russell, 2022). Bovine ovary- and oviduct-derived EV molecular cargo content has been shown to vary as the animal progresses through the different phases of the estrous cycle, indicating that EVs likely play a role in regulating dynamic reproductive changes in cyclic females (Almiñana et al., 2018; de Ávila et al., 2020; Navakanitworakul et al., 2016).

Oviductal and uterine fluid-derived EVs have been shown to have multiple functions during the process of fertilization in normal reproduction. In humans and mice, EVs aid in sperm capacitation in the female reproductive tract by transferring tyrosine phosphorylated proteins and miRNAs to sperm (Bathala et al., 2018; Fereshteh et al., 2018; Franchi et al., 2016). Besides being taken up by sperm, oviduct-derived EVs are also taken up by oocytes and deliver proteins responsible for the prevention of polyspermy (Alcântara-Neto, Fernandez-Rufete, et al., 2020; Coy et al., 2008). In vitro, incubation of sperm with healthy oviduct EVs has been shown to positively affect sperm survival and motility in a dose and time-dependent manner (Alcântara-Neto, Schmaltz, et al., 2020). Following fertilization, embryo-, seminal fluid-, and endometrium-derived EVs function in promoting processes necessary for implantation of the embryo, such as increasing PRL secretion, promoting angiogenesis, and activating focal adhesion kinase signaling (Desrochers et al., 2016; Rodríguez-Caro et al., 2019; Q. Tan et al., 2020).

After implantation, placental-derived EVs are critical mediators of the fetal-maternal communication necessary for creating a uterine environment that is supportive of pregnancy maintenance. As previously reviewed, communication at the fetal-maternal interface is necessary to modulate the maternal immune system to prevent
immunological rejection of the semi-allogeneic embryo. The complex regulatory mechanisms that protect the fetal allograft from immunological attack while maintaining maternal immunocompetence against pathogens are largely unknown, especially in cattle. Most information on the immunomodulatory roles of EVs in pregnancy comes from in vitro and ex vivo studies using human and mouse models (Morelli & Sadovsky, 2022). Increasing evidence, however, shows that EVs released from placental cells are important components of the fetal-maternal crosstalk that establishes immunotolerance to the embryo in healthy bovine pregnancies (Smith & Russell, 2022).

In healthy human pregnancies, first-trimester placenta-derived EVs have been documented to express immunomodulatory proteins, including human leukocyte antigen-G5, B7-H1 (PD-L1), and B7-H3, to suppress the maternal immune system and modulate the effector functions of T lymphocytes to establish immunotolerance (Kshirsagar et al., 2012). Additionally, EVs isolated from human trophoblast cells during early gestation have been shown to carry bioactive FasL and TRAIL, proteins that induce apoptosis in maternal T lymphocytes (Stenqvist et al., 2013). Syncitin-1, expressed in human trophoblast EVs, suppresses pro-inflammatory TNF-α, IFN-γ, and CXCL10 cytokines to mediate the pregnancy-dependent TH1 to TH2 shift (Tolosa et al., 2012). Beyond their immunosuppressive functions, human placenta-derived EVs have also been reported to recruit monocytes to the fetal-maternal interface and to express pro-inflammatory cytokines IL1-β, IL-6, CSF, and TNF-α, which aids in initiating the mild inflammation necessary for processes such as implantation during early gestation (Atay et al., 2011). miRNAs isolated from human trophoblast EVs, such as miR-519c, miR-517-3p, miR-516-5p, and miR-512-3p, demonstrated immunoregulatory capabilities that
can be transferred to non-trophoblast cells via EV trafficking (Delorme-Axford et al., 2013; Tiozzo et al., 2021). Evidence suggests that, rather than localizing to the uterus, placental EVs distribute throughout the body during pregnancy. In mouse models, placenta-derived exosomes have been located in the maternal lungs, kidneys, and liver, where they interact with leukocytes through surface integrins to establish maternal immunotolerance (Nguyen et al., 2021). As demonstrated in a mouse model, placental-derived EVs can also be trafficked into the fetal system to induce functional changes in embryonic tissues (Sheller-Miller et al., 2019).

Though research is more limited in cattle, it is speculated that bovine EVs have similar immunomodulatory roles during pregnancy establishment and maintenance. In high-fertility dairy cows, plasma-derived EVs have been shown to act on endometrial epithelial and stroma cells to downregulate prostaglandin synthases (PTGS1 and PTGS2), downregulate pro-inflammatory cytokines, and upregulate anti-inflammatory cytokines in the uterus during pregnancy establishment to create an intrauterine environment that is favorable for fertility, receptivity, and embryo viability (Abeysinghe et al., 2023). Bovine trophoblast-derived EVs have been found to upregulate the expression of integrin αn, β3, and Wnt7a, proteins associated with embryo-maternal interactions and endometrial embryo receptivity, and downregulate MUC1, an anti-adhesive factor that represses embryo attachment, in endometrial epithelial cells in cattle (Su et al., 2022). Cattle trophoblast EVs upregulate the expression of vascular cell adhesion molecule-1, a cell adhesion molecule necessary for lymphocyte homing in endometrial epithelial cells. Because vascular cell adhesion molecule-1 is reportedly under-expressed in infertile females its compared to fertile controls at the peri-
implantation stage, the upregulation of the molecule by EVs supports embryonic implantation (Kusama et al., 2018). Pregnancy-associated miRNAs isolated from bovine EVs, including bta-miR-450b, bta-miR-146b, bta-miR-26b and bta-miR-27b, have exhibited significant variation in expression between early pregnant and non-pregnant cattle and may be involved in maternal immune response and fetal health (Markkandan et al., 2018). Besides regulating fetal-maternal communication and pregnancy receptivity, proteins isolated from bovine trophoblast EVs regulate the fusion of trophoblast cells and uterine epithelial cells during placentation (Kusama et al., 2018). In healthy pregnancies, trophoblast-derived cattle EVs have been found to target the maternal immune and endocrine systems to create a uterine environment that is supportive of pregnancy through improving the extracellular environment, controlling cellular redox homeostasis, and regulating cell-to-cell signal transduction (Su et al., 2022).

c. Roles of Extracellular Vesicles in Infertility and Pregnancy Complications

Early pregnancy failure can occur in as many as 50% of high-producing dairy cows, generally due to insufficient communication between the maternal and fetal systems in utero (Lonergan et al., 2019; Su et al., 2022). During the reproductive process, trophoblast cells can alter and regulate maternal physiological function through the packing and release of specific bioactive molecules into the maternal system via EVs (Pegtel et al., 2010; Sarker et al., 2014; Su et al., 2022). Appropriate release and expression of trophoblast-derived EVs during the female reproductive process is paramount for establishing and completing a healthy pregnancy (Kusama et al., 2018; Su et al., 2022). Because EVs have a variety of functions in supporting female fertility
and pregnancy via mediating fetal-maternal communication, when EV secretion or cargo content at the fetal-maternal interface is abnormal, infertility and pathological pregnancies may occur. While other factors, such as metabolic pressure and animal management, play a role in the observed decline in dairy cattle fertility, research demonstrates that atypical EV biology is correlated with reproductive failure and pregnancy complications (Roche et al., 2018; Turner et al., 2021). EVs released by the placenta are of central interest to current reproductive research, as pregnancy success in mammalian species is highly dependent on normal placenta formation, development, and function (Gurunathan et al., 2022). Accumulating data documents variation between placenta-derived EVs from healthy and pathological pregnancies in many species, although most current studies are performed using EVs derived from human and mouse fluid samples and in vitro trophoblast cell culture supernatant. Abnormalities that have been associated with infertility and reproductive complications can be broadly grouped into two categories: atypical EV production and atypical EV cargo content.

Abnormal EV production during pregnancy establishment and maintenance has been identified in many pathological reproductive conditions; however, the conclusions that can be drawn from this data are limited due to the wide variation and no standardization in the techniques used to isolate, quantify, and analyze EVs from biological samples, which impedes precise interstudy comparisons (Block et al., 2021). Regardless of this, irregularities in placental EV production, most commonly increased EV secretion and abnormally high EV concentration in maternal circulation, has been associated with multiple reproductive complications and may be reflective of pathologies
such as irregular placental development, maternal immune dysregulation, and inadequate cell functionality (Block et al., 2021; Smith & Russell, 2022).

Concentrations of circulating EVs are significantly elevated in pregnant females when compared to nonpregnant females, and the quantity of placenta-derived EVs in maternal circulation naturally increases as gestation progresses in healthy pregnancies (Salomon et al., 2014; Sarker et al., 2014). Pathological reproductive conditions, however, can cause abnormal increase in EV production and subsequent elevated EV concentration in maternal circulation, and increased quantities of placental-derived EVs in maternal blood may be indicative of pregnancy complications (Block et al., 2021).

Preeclampsia, a human pregnancy disorder characterized by hypertension, inadequate placentation, endothelial dysfunction, and proteinuria, is well documented by multiple groups to be associated with elevated concentrations of placental EVs in maternal blood (Germain et al., 2007; Gilani et al., 2017; Goswami et al., 2006; Guller et al., 2011; Pillay et al., 2016; Salomon et al., 2017). Excessive placental EV production has been demonstrated in cases of gestational diabetes mellitus compared to healthy controls both in vivo and in explanted gestational diabetes-affected placental tissue (Nair et al., 2018; Salomon et al., 2015). In vitro research has demonstrated that hyperglycemic conditions, reflective of maternal insulin resistance during gestation, significantly increases the release of EVs from human trophoblast cells, which subsequently increases the production of GM-CSF, IL-4, IL-6, IL-8, IFN-γ, and TNF-α cytokines from the umbilical vein blood and impacts maternal immune response at the fetal-maternal interface (Rice et al., 2015). While increased concentration of placental EVs has been more prominently associated with pathological pregnancies, decreased quantities of
EVs during gestation have also been associated with adverse reproductive conditions. Research has demonstrated reduced amounts of placental EVs in both maternal and fetal circulation in cases of fetal growth restriction and in small for gestational age fetuses, implying that decreased EV production at the fetal-maternal interface is negatively linked to fetal growth and may be indicative of poor placental function (Miranda et al., 2018). In instances of unexplained infertility in women, expression of EV protein CD63 is upregulated and EV protein CD9 is downregulated in the endometrium during the proliferative phase of the menstrual cycle when compared to fertile controls, suggesting that EV secretion in the uterine environment is abnormal in infertile women (Uyar et al., 2020).

Compared to numerous human studies, current data on EV secretion and resulting EV concentrations in pathological reproductive conditions in cattle is more limited. A 2016 study demonstrated that the concentration of circulating exosome-sized plasma-derived EVs is 50% lower in dairy cows characterized as phenotypically subfertile compared to cows characterized as phenotypically fertile on the basis of genetic ancestry (M. D. Mitchell et al., 2016). While the variation in EV concentration between fertile and subfertile cattle is interesting, the reason and biological significance of this is unknown. In this study, it was hypothesized that the difference in EV concentration is likely due to differences in fertility between the groups, but other causes such as milk yield, metabolic activity, or health status may also influence EV production (M. D. Mitchell et al., 2016; Skog et al., 2008). Similarly, a 2018 comparison of high-fertility dairy heifers and low-fertility dairy heifers (based on substantial diversity in fertility estimated breeding value but similar genetic character in all other traits) found that the
total exosome-sized plasma EV concentration was 23% greater in the high-fertility group than in the low-fertility group, although the EVs isolated from low-fertility heifers were more saturated with cargoes (Koh et al., 2018). As EVs have been shown to function in promoting maternal-fetal tolerance during the establishment of pregnancy through the trafficking of immunomodulatory molecules in other species, it can be speculated that the noted reductions of circulating EVs in cows with reduced fertility may be a contributing factor in their poor fertility and reproductive outcomes, although further research is necessary to determine the functional significance of EV concentration on bovine reproductive health (M. D. Mitchell et al., 2016; Théry et al., 2009).

As previously described, EVs mediate intercellular communication via the transfer of various cargoes, such as proteins, lipids, nucleic acids, metabolites, and signaling molecules, between cells (Gurung et al., 2021). When EV cargo is aberrant, normal cell-to-cell communication is disrupted, and diseases can occur. Amassing research has demonstrated deviations between the cargo content isolated from placental-derived EVs in healthy and pathological pregnancies in a variety of species (Gurunathan et al., 2022). For example, in humans, proteomic analysis of placental-derived EVs in preeclampsia patients have been demonstrated to have increased expression of serpin peptidase inhibitor-1, porphyria cutanea tarda, S100 calcium-binding protein B, TGF-β, vascular endothelial growth factor receptor-1, natriuretic peptide B, and placental growth factor, as well as significantly enriched expression of miRNAs including miR-486-1-5p, miR-486-2-5p, miR-210, miR-517-5p, miR-518b, miR-155, and miR-520h (Bai et al., 2021; Hian Tan et al., 2014; H. Li et al., 2020; K. H. Tan et al., 2017). This abhorrent EV
cargo is associated with inhibition of trophoblast cell invasion, poor endothelial function, and abnormal T lymphocyte activation (Bai et al., 2021). EVs derived from human preeclampsia placentas have also been found to have substantially reduced expression of PD-L1, a key molecule involved in macrophage polarization and Treg cell differentiation at the fetal-maternal interface (Levine et al., 2020). Interestingly, EVs with abnormal cargoes isolated from placental tissues of preeclampsia patients injected into the tail vein of healthy mice evoke preeclampsia symptoms of hypertension and proteinuria, which suggests an important causative role in, rather than just an association with, the pathology of preeclampsia (Cha Han et al., 2020).

Preterm labor, defined as childbirth occurring prior to 37 weeks of gestation, is a significant cause of fetal morbidity and mortality in humans and is often associated with dysregulated inflammation in the placental and uterine tissues (Buca et al., 2020). Research has demonstrated that many cargoes, including 164 miRNAs that interact with over 11,000 genes, differ between circulating EVs isolated from women who experienced preterm labor and women who maintained their pregnancy to full term (Fallen et al., 2018). This suggests that circulating EVs have the potential to be used as non-invasive diagnostic biomarkers for preterm labor, and because nearly half of the identified divergent miRNAs belong to the placental-expressed C19MC and C14MC clusters, abnormal EV cargo may be reflective of pathological conditions at the fetal-maternal interface (Fallen et al., 2018).

Human EV cargo content has also been shown to be divergent in cases of abnormal fetal growth. For example, EVs isolated from maternal blood in instances of small-for-gestational age pregnancies have been demonstrated to be enriched in specific
miRNAs including miR-20b-5p, miR-942-5p, miR-324-3p, miR-223-5p, and miR-127-3p, while circulating EVs isolated from large-for-gestational age pregnancies have elevated levels of miR-661, miR-212-3p, and miR-197-3p (Rodosthenous et al., 2017). Further, research has identified a correlation between the concentration of certain miRNA EV cargoes, including miR-483-5p, miR-10a-5p, miR-204-5p, miR-202-3p, miR-345-5p, miR-885-5p, miR-127-3p, miR-148b-3p, miR-324-3p, miR-1290, miR-597-5p, miR-139-5p, miR-215-5p, and miR-99b-5p, isolated from maternal serum and the birthweight-for-gestational-age z-score, a standardized measurement for comparison of fetal and neonate growth and size (Rodosthenous et al., 2017). This data suggests that circulating EV cargo content has the potential to be used as a non-invasive biomarker to surveil fetal size and to predict abnormal fetal growth throughout gestation (Saadeldin et al., 2022).

Though cattle-specific data are limited, several studies have demonstrated that atypical bovine EV cargo can stimulate dysregulation of the maternal immune system and subsequent aberrant intrauterine inflammatory responses in cattle to negatively affect fertility and reproductive success. A 2020 study found that when compared to circulating EVs from high fertility dairy heifers, EVs from low fertility heifers enhance the expression of pro-inflammatory cytokines, including IL-1α and IL-8 (CXCL8), and decrease the expression of anti-inflammatory IL-4 in endometrial epithelial cells, triggering irregular intrauterine inflammation that contributes to the observed reduced fertility phenotype (Koh et al., 2020). Further, when incubated with EVs derived from low fertility heifers, bovine uterine epithelial cells exhibit a strong TH1 immune response (Koh et al., 2020). Predominant TH1 immune responses at the fetal-maternal interface
are associated with pathological pregnancy conditions, such as uncontrolled inflammation and preterm labor in humans (Sykes et al., 2012). A 2023 study demonstrated that plasma-derived small EVs isolated from low-fertility dairy cows contain divergent cargos that cause a dysregulated production of inflammatory mediators by uterine epithelial and stromal cells when compared to high-fertility dairy cows. EVs from low-fertility cows contain cargoes that upregulate prostaglandin synthases and pro-inflammatory cytokines and downregulate anti-inflammatory cytokines (Abeysinghe et al., 2023). Taken together, these data overall suggest that EV cargo abnormalities contribute to recurrent implantation failure and recurrent early embryo loss in cattle by creating dysfunction in the intrauterine immune environment.

Amassing research has demonstrated that small non-coding miRNA EV cargo content notably varies between open cows, cows carrying healthy pregnancies, and cows carrying pathological pregnancies. This observations has prompted investigation into the use of miRNAs isolated from circulating EVs as novel biomarkers for the viability of the pregnancy and physiological status of the embryo (Ealy & Seekford, 2019; Gilad et al., 2008). EV-derived miRNAs have been linked to endometrial fertility status in cattle and exhibit detectable variation between healthy and unhealthy pregnancies in cattle as early as eight days post-insemination (Ealy & Seekford, 2019; Ioannidis & Donadeu, 2016; Ponsuksili et al., 2014). A 2017 study to identify specific differentially expressed circulating EV miRNA biomarkers of pregnancy condition in cattle found 27 mature miRNA EV cargos that were significantly enriched in EVs isolated in instances of embryonic mortality when compared to EVs isolated from healthy bovine pregnancies as early as day 17 of gestation (Pohler et al., 2017). These
identified divergent miRNAs, including miR-339-5p, miR-100-5p, miR-143-3p, miR-409-3p, and miR-16-5p, are associated with inflammation, cell proliferation and viability, cell cycle progression, endometriosis, apoptosis, and infection (Pohler et al., 2017). While the exact function of these miRNAs is unclear, it is speculated that they aid in sequence-specific epigenetic regulation of gene expression during pregnancy establishment, and, in the case of embryo mortality, cause dysregulation of maternal immune response or maternal recognition of pregnancy (Pohler et al., 2017).

Research has also demonstrated that the protein cargo content of EVs isolated from cattle may be reflective of their physiological condition, which includes their reproductive health, fertility, and pregnancy viability (M. D. Mitchell et al., 2016; Muroya et al., 2015). In postpartum cows, circulating exosomes are enriched in cargo proteins associated with immune response and cell signaling pathways, which eludes to their immunomodulatory functions (M. D. Mitchell et al., 2016; Théry et al., 2009). As postpartum dairy cows frequently experience inflammation of the uterine and mammary glands in the weeks after calving, resulting in reduced fertility and poor conception rates during the initial rebreeding period, it can be theorized that the EV-derived proteins notably elevated in postpartum dairy cows with endometritis and mastitis may orchestrate inflammatory responses and subsequent adverse reproductive outcomes (Bertoni et al., 2008; M. D. Mitchell et al., 2016). Further, a 2016 study seeking to compare EV-derived proteins isolated from dairy cows categorized as phenotypically fertile or subfertile by genetic ancestry revealed that the circulating EVs of both groups were enriched in protein cargoes associated with immune response gene ontology terms; however, the EV-derived proteins found within these terms varied between the
fertile and subfertile groups (M. D. Mitchell et al., 2016). Although the immune function of the cows in the aforementioned 2016 study was not intensively monitored, a 2012 study on the same animals highlighted differences between the fertile and subfertile groups in the endometrial gene expression of immunoregulatory pathways in response to pregnancy in the cows at day 17 of gestation (C. G. Walker et al., 2012). When considered together, this data provides indirect evidence of differences in immune health and function between fertile and subfertile cows and suggests that the divergence in EV-associated protein cargo content between the groups may be a source of this immunomodulatory variation (M. D. Mitchell et al., 2016; C. G. Walker et al., 2012).

Similarly, a 2018 comparison of the protein content of EVs derived from high-fertility and low-fertility dairy heifers, classified by fertility estimated breeding value but genetically similar in other biology characteristics, found that plasma-derived EVs from the low-fertility group both had greater overall abundance of protein content and contained 31 unique proteins, including tetratricopeptide repeat protein 41-related, glycodelin, and Kelch-like protein 8, relative to the high-fertility group (Koh et al., 2018). While further work is still necessary to reveal the complete biological significance of the divergent EV-bound protein cargoes in bovine fertility, the proteins found to be enriched in the low-fertility bovine EVs in the present study have functions associated with protein-protein interaction, cellular and metabolic processes, and immunologic and hormonal regulation (Alok & Karande, 2009; Koh et al., 2018). Considering this, it can be inferred that abnormal EV protein cargo could create adverse reproductive
conditions by disrupting biological processes, cell-cell interactions, and regulation of the maternal immune and endocrine systems at the fetal-maternal interface.

Additionally, exosome-sized plasma-derived EVs in dairy cattle with endometrial infections have been documented to be enriched in protein cargos, such as Talin-2, that are associated with physiological stress, cellular signaling, angiogenesis, inflammation, and immune system processes by PANTHER analysis, a database providing information about the evolutionary pathways affecting functions of protein-coding genes, when compared to EVs from uninfected cows (Almughlliq et al., 2018). These data show that EV protein cargo may be both capable of modulating the immune system in response to infection and reflective of the inflammatory state of the uterus. Because activation of the immune system and promotion of inflammation is associated with impaired fertility and reproductive performance in cattle, EVs containing proteins associated with bovine uterine infection may have roles in establishing poor reproductive conditions in pathological pregnancies.
VIII. Applications, Further Directions, and Challenges of Research

a. Use of Extracellular Vesicles as Diagnostic Biomarkers of Fertility and Pathological Pregnancy Conditions

As previously mentioned, declining cow reproductive performance is a significant issue facing the dairy industry, and herd infertility and subfertility is detrimental to the economic viability and success of dairy production (Ott, 2019). Currently, there are tools such as fertility estimated breeding value and expected progeny difference data to accurately predict fertility in cattle aside from imperfect methods relying on outward phenotypical attributes such as body condition scoring and genetic lineage estimated breeding value expected progeny difference (Turner et al., 2021). A reliable, easily attainable early biomarker of fertility would allow dairy producers to select for high-fertility replacement heifers when developing their herds and subsequently reduce the burden of poor cow reproductive performance on the industry. EVs are promising novel candidates for evaluating fertility and for diagnosing reproductive complications (Block et al., 2021; Levine et al., 2020; Turner et al., 2021).

EVs are gaining significant interest as quantitative, qualitative biomarkers of reproductive performance because, as discussed previously, they contain cargoes that are representative of the state of their parent cell, as well as the tissue and organ of origin (Vagner et al., 2019). Additionally, EVs are highly stable, have low immunogenicity, and are able to be isolated noninvasively from all body fluids (Ciferri et al., 2021; Zhou et al., 2021). Because diseased states affect EV concentration, cargo content, and overall function, isolation of EVs and analysis of these characteristics from
affected individuals can potentially be used to successfully predict fertility as well as
diagnose a variety of reproductive conditions involving dysfunction of the maternal
immune system, such as endometritis, preeclampsia, and recurrent implantation failure
(Gilani et al., 2016; Gurunathan et al., 2022; C. Liu et al., 2021).

For EVs to be practically used in a clinical veterinary or medical setting to
noninvasively gauge an individual’s fertility status or diagnose pathological reproductive
conditions, further research is warranted to identify specific EV-bound cargo molecules
associated with reproductive performance and health. As of present, research has
identified detectable cargo variation between plasma-derived EVs in healthy and
unhealthy cattle pregnancies as early as eight days post-insemination (Ealy & Seekford,
2019; Ioannidis & Donadeu, 2016; Ponsuksili et al., 2014). Many specific cargoes,
including various miRNAs and proteins, have been identified to be differentially
contained in EVs isolated from high-fertility versus low-fertility dairy cattle (Bridi et al.,
2021; Markkandan et al., 2018; M. D. Mitchell et al., 2016; Vagner et al., 2019).
Research in cattle is limited, and more extensive work has been done on human
pathological pregnancy conditions. Currently, specific EV cargoes have been identified
as potential indicators of conditions such as preeclampsia and gestational diabetes
mellitus (Cha Han et al., 2020; Hian Tan et al., 2014; Nair et al., 2018).

Major hurdles in biomarker research include that current methods of EV isolation
lack standardization and vary greatly in regard to purity and concentration, and that,
once EVs have been isolated, cargo characterization can be complex and time-
consuming (McAlarnen et al., 2022; Zhou et al., 2021). As a consequence of
complicated, non-standardized isolation and characterization, reproducibility and
consistency of results between different affected individuals and studies are currently insufficient to definitively pinpoint specific, unique identifiers of fertility status and reproductive conditions. Continuous work and development of a standardized procedure for EV isolation and characterization will make provision for the clinical use of EVs as predictors of fertility and as diagnostic biomarkers of pathological pregnancy conditions in all species, including cattle (McAlamen et al., 2022).

b. Use of Extracellular Vesicles as Treatment for Infertility and Pathological Pregnancy Conditions

Beyond being used as a diagnostic tool, administration of EVs with beneficial cargoes to affected patients may be a suitable therapy for many reproductive and immunological diseases. Due to their involvement in the establishment and maintenance of pregnancy, EVs have potential clinical applications in preventing, diagnosing, and treating pregnancy complications in cattle and other species, including humans. EVs serve as mediators of communication between cells, and by administering EVs with specific therapeutic cargoes, this communicative pathway can be controlled and manipulated (Man et al., 2020). EVs make favorable candidates for novel therapy delivery systems for the treatment of disease due to their non-toxicity, safety, compatibility with living tissues, organ-specific distribution, and ability to target specified locations in vivo (Ailuno et al., 2020; Lu & Huang, 2020; Mecocci et al., 2022). Along with administration of naturally-derived EVs for treatment of reproductive disease, therapeutic EVs can be engineered through manipulation of their internal or external cargoes and manipulation of their scale of secretion (Kim et al., 2020). While further research is necessary, the clinical use of EVs in treating infertility and other adverse
reproductive conditions is gaining considerable interest and being studied \textit{in vitro} and in animal models (Goss et al., 2022; Zhao et al., 2019).

While the use of EV therapies to treat reproductive conditions is novel, the research and limited clinical studies are promising. A 2020 case study documented that intrauterine infusion of amnion-derived EVs treated chronic degenerative endometritis and enabled pregnancy establishment in mare with a history of numerous failed insemination attempts over two breeding seasons. The administration of EVs as therapy for the mare’s chronic uterine inflammation had a regenerative, anti-inflammatory effect on the injured endometrium and, after only two infusions, resulted in successful pregnancy establishment in the previously infertile mare (Lange-Consiglio et al., 2020). Further equine studies have demonstrated that amnion-derived EV treatment reduced pro-inflammatory \textit{TNF-\alpha, IL-6, IL-1B}, and metalloproteinase 1 and 13 and initiated the release of some anti-inflammatory mediators to reduce inflammation in endometriosis-affected uterine cells \textit{in vitro} (Perrini et al., 2016). Intraovarian therapeutic injection of human amnion epithelial cell-derived EVs to infertile mouse models with premature ovarian insufficiency initiated changes in the expression of 109 cytokines and 34 proteins associated with regulation of immune response, such as \textit{TGF-b} (Q. Zhang et al., 2017). In an \textit{in vitro} mouse model, human umbilical cord mesenchymal stem cell-derived EVs had therapeutic effects on injured endometrial epithelial cells by significantly inhibiting \textit{IL-6, IL-1\beta, TLR4,} and v-rel reticuloendotheliosis viral oncogene homolog A and promoting \textit{TNF-\alpha} production by injured cells, thus exhibiting anti-inflammatory properties, improving cell viability, and reducing apoptosis in injured endometrial cells (Liang et al., 2020). Administration of EVs to improve the outcomes of
assisted reproductive technologies, such as AI, in vitro fertilization, and intracytoplasmic sperm injection, is also being explored (Gervasi et al., 2020). Co-incubation of in vitro embryos and EVs has been shown to enhance embryo competence and quality, cryo-survival rate, fertilization rate, and cell proliferation in bovine, canine, and mouse models (Bai et al., 2021; Lange-Consiglio et al., 2017; Qu et al., 2019).

Similar to the obstacles facing the use of EVs as diagnostic biomarkers of fertility and pathological pregnancy conditions discussed previously, the clinical use of EVs as therapies for reproductive disease is complicated by the difficulty of complete EV cargo characterization, the labor intensiveness and lack of standardization of EV isolation protocols, and the logistical challenges associated with handling and storing EVs (Williams & Ehrhart, 2022). Current research and limited clinical studies have undoubtedly demonstrated that EVs have the capacity to cure or mitigate a wide variety of adverse reproductive conditions through the trafficking of curative and regenerative cargo molecules to affected tissues (Ciferri et al., 2021; Ghafourian et al., 2022; Lange-Consiglio et al., 2020; Zhou et al., 2021). While it has been shown that EVs have the potential to serve as novel treatments for infertility and pathological pregnancies, further work is warranted to identify specific beneficial EV cargoes to remediate various diseases, as well as optimize EV isolation and cargo characterization methods, to be used practically in a veterinary or medical setting.

c. Other Uses of Extracellular Vesicles in Nonreproductive Inflammatory Disease

As accumulating research continues to demonstrate that EVs and EV-associated cargo molecules are qualitatively and quantitatively different when compared between
healthy individuals and individuals affected by adverse health conditions, the potential of utilizing EVs as a noninvasive diagnostic tool for a wide variety of pathologies outside of reproductive complications is steadily gaining interest, particularly for inflammatory conditions or diseases resulting in dysregulation of the immune response. Quantification and cargo qualification of plasma-derived EVs isolated from individuals with systemic lupus erythematosus, a serious autoimmune disorder affecting multiple organs simultaneously, shows that EVs exhibit increased concentration and unique cargo molecules when compared to unaffected individuals (Duval et al., 2010; López et al., 2017; Mobarrez et al., 2016; Paul R. Fortin et al., 2016; Xu et al., 2020). These data, suggesting that EVs isolated from a blood may be used to identify lupus-affected individuals, is particularly exciting because currently there is no one single diagnostic test for lupus. Vasculitis, inflammation of the blood vessels, can be diagnosed through the assessment of concentration and enzymatic cargo content of EVs isolated from blood samples, and the level of EVs in patients correlates proportionately with the Birmingham Vasculitis Activity Score, demonstrating that EVs from affected individuals can be used both to diagnose vasculitis as well as quantify disease activity (Brogan et al., 2004; Erdbruegger et al., 2008; Karpman & Tontanahal, 2021). EVs and their associated cargoes are being investigated as a simple tool to screen for, diagnose, determine the severity of, and monitor progression and treatment efficacy for many cancers, which is significant because early detection and close monitoring of cancer is key to patient survival (Beck et al., 2022; D'Souza-Schory & Clancy, 2012; Johnsen et al., 2019; Melo et al., 2015). Presently, EV-bound cargo molecule markers have been identified for a variety of cancer types, including CD151 for lung cancer, CD147 for
ovarian cancer, and glypican-1 for pancreatic cancer (Y. Li et al., 2015; Madhavan et al., 2015; Thakur et al., 2014; H. Zheng et al., 2018). The list of inflammatory conditions that EVs may be used as diagnostic biomarkers for, including sepsis, type 1 diabetes mellitus, and neurodegenerative diseases, continues to grow with further research and clinical trials (Casella et al., 2018; Noren Hooten et al., 2022; Raeven et al., 2018; Sun et al., 2021).

Through the trafficking of beneficial, curative cargo molecules to sites of inflammation, EVs may also be used as therapies for many inflammatory diseases in cattle and other species. In a 2022 study, local injection of EVs isolated from bovine umbilical cord blood following delivery of a healthy calf effectively treated subclinical mastitis and significantly reduced milk somatic cell count in a group of dairy cows by increasing the expression of anti-inflammatory IL-10, cathelicidin, cystatin, lipocalin 2, and angiopoietin while decreasing pro-inflammatory IL-6; all treated cows were permanently cured of mastitis within 15 days post-treatment (Ghai et al., 2022; Merlo et al., 2022). In an in vitro model of inflammatory bowel disease-affected intestinal tissue, bovine milk-derived EV treatment had regenerative, anti-inflammatory effects on inflamed tissue through decreasing expression of pro-inflammatory CXCL8, TNF-α, IL-1β, and IL-17 and increasing expression of anti-inflammatory IL-10 and TGF-β1, while also downregulating matrix metallopeptidase-9 and upregulating mucin-2 and tight junction protein-1 to restore homeostasis and mucosal function (Mecocci et al., 2022). Human mesenchymal stem cell-derived EVs have been shown to generate skeletal muscle tissue repair in injured mouse models through having significant anti-inflammatory effects by downregulating inflammatory cytokine IL-6,
upregulating anti-inflammatory cytokine IL-10, and switching macrophages from an
M1 to an M2 phenotype polarization (Lo Sicco et al., 2017). Further, administration
of EVs has also been demonstrated to be an effective therapeutic method of
modulating inflammation and restoring balanced immune function in clinical trials for
many other inflammatory conditions, including multiple sclerosis, some cancers,
SARS-Cov-2 infections, and cardiovascular disease (Casella et al., 2018; Ciferri et
al., 2021; B.-C. Lee et al., 2021; Scott et al., 2022; Zamani et al., 2019). Overall,
these studies demonstrate the therapeutic potential of EVs as treatments for a
variety of inflammatory conditions and pathologies, even outside of reproductive
conditions. To summarize, growing research shows the promising clinical relevance
and usefulness of EVs as both diagnostic biomarkers of and therapies for an
expansive range of reproductive and non-reproductive conditions in medical and
veterinary settings. While EVs may be valuable tools for diagnosing and treating
pathologies in the future, the use of EVs in a clinical setting is currently complicated
by logistical challenges associated with their isolation, characterization, and storage.
Further research is thus warranted improve the practicality and functionality of EVs
as viable diagnostic and treatment options in medical and veterinary settings.
CHAPTER II
THE ROLE OF EXTRACELLULAR VESICLES IN IMMUNOMODULATION DURING BOVINE PREGNANCY

Abstract

During pregnancy, the maternal immune system must be altered to allow the tolerance of a semi-allogeneic fetus. Our previous studies show a strong inflammatory response in the uteri of cows carrying somatic cell nuclear transfer (SCNT) pregnancies due to dysregulation in the proteins expressed by trophoblast cells. Between 30 and 90 days the rate of pregnancy loss is 50-100% for SCNT compared to 2-10% for artificial insemination (AI) pregnancies. Abnormal immunological crosstalk during placentation is a major cause of this loss in SCNT pregnancies. The trafficking of extracellular vesicles (EVs), membrane-bound cargo carriers, potentially represents a key form of crosstalk between the conceptus and endometrium at the fetal-maternal interface. The aims of this study were to determine the role of trophoblast-derived EVs in healthy bovine pregnancies established by AI and abortion-prone pregnancies established by SCNT. We hypothesized that EVs from SCNT pregnancies would stimulate maternal immune cells to express more pro-inflammatory mediators when stimulated compared to cells treated with EVs from AI pregnancies.

Pregnancies were established by AI or SCNT (n = 6/group) and placental tissue was collected at 42±3 days, a time of major embryonic loss in cattle. The tissue was digested and cultured in EV-depleted medium at a density of 500 cells/well for 21 days. EVs were isolated from trophoblast supernatant by size exclusion chromatography.
Peripheral blood mononuclear cells (PBMCs) were collected from day 35-70 AI pregnant cows and isolated by density gradient centrifugation. Isolated cells were sorted for CD4+, CD8+, and CD14+ using flow cytometry, plated at an average density of 5,000 cells/well (44-96 wells/cow), and treated with $10^7$ to $10^5$ EVs/well. Cells were cultured for 24 hours then frozen at -80°C. Reverse transcription quantitative polymerase chain reactions using primers for pro- and anti-inflammatory genes were performed on the collected cells using the high-throughput Fluidigm BioMark system. Relative expression was evaluated for 30 genes. Experimental data were analyzed as a randomized block design using SAS® University Edition Version 3.8, where block was cow and experimental unit was the cell culture well. The interaction between treatment and block was investigated.

Our data reveal changes in the relative gene expression level of several genes in maternal immune cell populations between AI EV-treated cells and SCNT EV-treated cells. Rather than establishing either a solely anti-inflammatory or pro-inflammatory phenotype, our findings suggests that EVs from both healthy and abortion-prone pregnancies may orchestrate a complex interplay between the expression of anti- and pro-inflammatory genes to establish immunological balance at the fetal-maternal interface during early gestation.
1. Introduction

Reproductive success is a critical measure of profitability in all sectors of the livestock industry but is especially important in the dairy cattle industry, where sufficient reproductive performance is essential for milk production and the generation of a constant stream of replacement heifers. Dairy cow fertility, however, has been waning for several decades (Berry et al., 2016; Garnsworthy et al., 2008; Koh et al., 2018). This presents a challenge for dairy farmers, whose profit margins are heavily reliant on the ability of their cows to conceive, maintain their pregnancy, and produce a healthy calf. Although poor reproductive performance in dairy cattle can be attributed to many factors, loss of established pregnancies is arguably the most deleterious contributor (Sigdel et al., 2021). In high producing dairy cows, fertilization rates reach 70-75%, yet calving rates drop to 30-35% as a result of pregnancy loss (Diskin et al., 2011; Santos et al., 2004). The majority of this pregnancy loss (25-40%) is embryonic loss occurring during the first 42 days of gestation (Wiltbank et al., 2016). Dysregulation of the maternal immune system during early pregnancy has been identified as a paramount source of embryonic death, and thus reproductive failure, in dairy cattle (Fair, 2015).

For pregnancy to be maintained, it must be tolerated by the maternal immune system. Pregnancy represents an immunological paradox in which the maternal immune system not only accepts the presence of the semi-allogeneic embryo but is reconditioned to establish a uterine environment that is promotive of the survival of the semi-allograft (Bridi et al., 2021; Ott, 2020; Talukder et al., 2020). Despite being reprogrammed during pregnancy, the maternal immune system is not dormant. Rather, it is active and maintaining the complex balance between tolerating embryonic antigens.
while also defending the body from pathogenic antigens (Samardžija et al., 2020). The immunological balance essential to preserve a viable pregnancy is maintained through continuous dialogue between the embryo and uterine environment at the fetal-maternal interface of the placenta (Nakahara et al., 2020).

Extracellular vesicles (EVs) are catalysts of intercellular communication that serve as vital mediators of the fetal-maternal crosstalk during conception and throughout the duration of gestation (Bridi, Perecin, & da Silveira, 2020; Bridi et al., 2021). Extracellular vesicles, lipid membrane-bound structures secreted by all cell types, direct cell-cell communication through the trafficking of biological cargoes, including proteins, nucleic acids, and lipids, between cells (Gurung et al., 2021). The cargo content of EVs is representative of the physiological state of the donor cell and, when taken up by a recipient cell, can initiate changes in its function (Buca et al., 2020). Depending on the cargo content of the EVs, different transcription factors are activated, genes are transcribed, and proteins are synthesized in the recipient cell to stimulate a variety of responses, including pro- or anti-inflammatory reactions to the embryo (Bridi, Perecin, & Coelho da Silveira, 2020; Buca et al., 2020; Giacomini et al., 2019).

Many recent reviews and studies have extensively highlighted the immunomodulatory roles of placental-derived EVs at the fetal-maternal interface and demonstrated the significance of EV-mediated intercellular communication during early gestation in human and mouse models (Bridi, Perecin, & Coelho da Silveira, 2020; Buca et al., 2020; Das & Kale, 2020; Hadley et al., 2018; Nakahara et al., 2020; J. Zhang et al., 2020). Extracellular vesicles orchestrate the crosstalk between the fetal and maternal systems through the trafficking of various biological cargoes, such as
cytokines and miRNAs, from donor to recipient cells. As mediators of fetal-maternal crosstalk, EVs are responsible for facilitating a broad spectrum of physiological processes during normal gestation, including immunomodulation, to establish an intrauterine environment that is tolerant of the semi-allogeneic conceptus (Almiñana et al., 2017; Buca et al., 2020; Das & Kale, 2020). When EV concentration or cargo content is abnormal during the pregnancy, however, EVs may contribute to the development of pathological conditions and adverse reproductive outcomes (Zhang et al., 2020). The immunomodulatory function of EVs at the fetal-maternal interface is well characterized in humans and mice, but much less is known about their roles in bovine reproduction.

Our group has demonstrated that somatic cell nuclear transfer (SCNT)-established pregnancies exhibit immune dysregulation at the fetal-maternal interface (Koroghli et al., 2018; Rutigliano et al., 2016, 2017, 2022). The objective of the current study was to determine the role of trophoblast-derived EVs in regulating the maternal immune response against the embryo in pregnancies established by artificial insemination (AI) and SCNT. It is hypothesized that EVs from SCNT pregnancies will stimulate maternal immune cells to express more pro-inflammatory mediators when compared to immune cells treated with EVs from AI pregnancies.

2. Materials and Methods

2.1 Establishment of pregnancies

In this study SCNT pregnancies were used as a model for abortion-prone pregnancies. These pregnancies were established as previously described (Aston et al.,
2006). Briefly, oocytes were isolated from ovaries collected at a local abattoir, and fibroblasts from an Angus beef cow were used as nuclear donor cells. A day 7 embryo was transferred to each of the 38 estrus synchronized recipient cows. Control pregnancies were established by AI. All pregnancies were diagnosed by transrectal ultrasound at day 28 of gestation. Cows were considered pregnant if a conceptus with a heartbeat was present.

2.2 Placental tissue collection, isolation, and culture

For collection of 42±3-day placental tissue, pregnant cows carrying SCNT and AI-established pregnancies were humanely euthanized at Utah State University. Reproductive tracts were immediately collected, placed on ice, and transported to the laboratory. Placentomes were cut from the placental tissue using sterile scissors and placed in a Dulbecco’s Modified Eagle Medium (DMEM) with 4X Penicillin-Streptomycin and 1X Amphotericin B. Uterine caruncular tissue was separated from placenta cotyledonary tissue using sterile forceps. Approximately 120 g of cotyledonary tissue was collected per placenta and washed twice in PBS with 4X Penicillin-Streptomycin.

Cotyledonary tissue was then digested in warm trypsin with DNase (1 mL of DNase per 100 mL trypsin) and incubated on a shaker in 37°C for 30 minutes. After digestion, the supernatant was filtered through a cheesecloth, the remaining tissue was discarded, and the supernatant was pipetted into 50 mL tubes with 5 mL exosome-free fetal bovine serum (FBS). The supernatant was centrifuged for 10 minutes at 100 x g at room temperature, pellets were resuspended in 10 mL of DMEM solution, and layered over 10 mL of 40% Percoll in 50 mL tubes. The tubes were then centrifuged at room
temperature for 10 minutes at 800 x g with the brake off. After centrifugation, the interface was harvested and diluted in 45 mL of DMEM solution. The tubes were centrifuged at room temperature for 10 minutes at 100 x g. Following centrifugation, the pellet was washed once again as described above. The pellet was then resuspended in 5-10 mL of DMEM/F-12 medium with 10% exosome-free FBS, 4X Penicillin-Streptomycin, and 1X Amphotericin B. The cells were counted using a hemocytometer and cultured in tissue culture flasks at 37°C in 5% CO2. After 48 hours of culture, cell culture supernatant was collected and frozen at -80°C.

2.3 Extracellular vesicle isolation

Frozen trophoblast cell supernatant was thawed at 4°C overnight. Thawed supernatant was concentrated by ultrafiltration using Centricon Plus-70 centrifugal filter units (Millipore Sigma), as described by Burns et al., (2018). Briefly, 10-15 mL of supernatant per sample was centrifuged in the Centricon Plus-70 filter unit at 2,093 x g for 15 minutes, then the filter unit was inverted, and concentrated filtrate was collected by centrifugation at 1,000 x g for 2 minutes. The filtrate was then resuspended in 500 µL of sterile PBS (Burns et al., 2018). Supernatant filtrate was further sieved by using a 0.2 µm sterile syringe filter. Concentrated supernatant was then centrifuged at 500 x g for 10 minutes. The pellet was discarded, and supernatant was centrifuged again at 10,000 x g for 10 minutes. Supernatant was separated from the pellet, and the pellet was discarded.

Extracellular vesicles were then isolated from the filtered supernatant using size exclusion chromatography (SEC). An IZON qEV single 35 nm size exclusion
chromatography column was secured upright using an adjustable clamp stand and one column volume (approximately 3.5 mL) of sterile PBS was run through the column to equilibrate it. When the column stopped flowing, 150 µL of the prepared supernatant was pipetted into the loading frit followed by additional PBS, and immediately 0.5 mL fractions started being collected in microcentrifuge tubes. When the column stopped flowing again, the loading frit was topped with sterile PBS. Ten 0.5 mL fractions were collected per trophoblast supernatant sample.

Collected fractions were pipetted into individual Amicon Ultra-15 devices (Millipore Sigma) then centrifuged at 2093 x g for 15 minutes. To recover the concentrated fraction, a pipette was inserted into the bottom of the Amicon device and the sample was withdrawn using a sweeping, side-to-side motion. Concentrated EV fractions were pipetted into labeled microcentrifuge tubes and stored at -80°C.

2.4 Quantification and analysis of EVs

Protein quantification of trophoblast supernatant fractions following size exclusion chromatography was done by a bicinchoninic acid (BCA) assay and NanoDrop spectrophotometry. The Pierce BCA Protein Assay kit (ThermoFisher) standard microplate procedure was used, and absorbance was read at 562 nm using a BioTek Synergy H1 Hybrid Multi-Mode Microplate Reader. Protein concentration was also measured using the A280 direct protein measurement on a NanoDrop Microvolume Spectrophotometer (Thermo Scientific). Protein concentration of the EV fractions was standardized to PBS, which is what the supernatant was diluted in during SEC. Extracellular vesicle content in the trophoblast supernatant SEC fractions were
characterized by nanoparticle tracking analysis (NTA) using the Nanosight NS300 system (Malvern Panalytical). A total of eight 500 µL fractions were collected per sample of trophoblast supernatant, and the total concentration of particles in each fraction and the size distribution of particles were measured.

2.5 Peripheral blood mononuclear cell isolation and culture

Peripheral blood mononuclear cells (PBMCs) were collected from day 35-70 Al-established pregnant Holstein cows via puncture of the coccygeal vein into glass vacutainer tubes containing anticoagulant tri-sodium citrate acid and dextrose (Becton Dickinson). Blood samples were centrifuged at 800 x g for 10 minutes to isolate buffy coats, and 2-4 buffy coats per cow were combined in 50 mL conical tubes. Buffy coat samples were then diluted with an equal volume of PBS + 2% FBS. Peripheral blood mononuclear cells were then isolated from the buffy coats via density gradient centrifugation using SepMate PBMC Isolation 50 IVD tubes (Stemcell Technologies). Fifteen mL of Lymphoprep density gradient medium (Stemcell Technologies) were pipetted through the central hole of the SepMate insert. Keeping the SepMate tube vertical, the diluted blood sample was slowly pipetted down the side of the SepMate tube so that it layered above the insert and Lymphoprep medium. The SepMate tube was then centrifuged at 1,200 x G for 20 minutes.

Following centrifugation, the top layer of the SepMate tube, containing enriched mononuclear cells, was poured into a new 50 mL conical tube. The cells were pelleted by centrifugation at 300 x g for 8 minutes, and supernatant was discarded. Two mL of erythrocyte shock lysis solution was added to the 50 mL tube to remove contaminating
red blood cells, and the pellet was vortexed and resuspended. Immediately, 8 mL of PBS + 2% FBS was added to the tube. The tube was again centrifuged at 300 x g for 8 minutes to pellet cells. The enriched PBMCs were then washed twice in 3 mL of PBS + 2% FBS. The final pellet was resuspended in 3 mL of RPMI 1640 + 5% exosome-free FBS + 100 µg/mL penicillin/streptomycin with glutamine.

Isolated PBMCs were stained with trypan blue and counted using a hemocytometer, and viability data and cell concentration were recorded. Two million cells were transferred into labeled 12x75 Falcon 5 mL round bottom polystyrene tubes (ThermoFisher) and centrifuged at 800 x g and 4°C for 3 minutes to pellet the cells. Supernatant was discarded. PBMCs (2x10^6 cells/tube) were then incubated on ice in the dark for 15 minutes with 100 µL of the following primary goat anti-mouse antibodies at 15 µg/mL: CD4 (clone ILA11A1), CD8 (clone BAT8LA), CD14 (clone MM61A). One Falcon tube was incubated with E. Coli antibody (clone COLIS69A). All monoclonal antibodies were purchased from the Washington State University Monoclonal Antibody Center unless otherwise stated (Table 1).

Peripheral blood mononuclear cells were then centrifuged twice with RPMI 1640 + 5% exosome-free FBS + 100 µg/mL penicillin/streptomycin with glutamine for 3 minutes at 800 x g and 4°C. Supernatant was discarded. Cells were then suspended in a secondary goat anti-mouse antibody solution with Alexa Fluor 488 (ThermoFisher), Alexa Fluor 647 (ThermoFisher), and Phycoerythrin (PE, Jackson ImmunoResearch). One microliter of live/dead blue stain (Fisher Invitrogen) was added to each tube, and the tubes were incubated in the dark on ice for 15 minutes. Cells were then washed twice with RPMI 1640 + 5% exosome-free FBS + 100 µg/mL penicillin/streptomycin with
glutamine for 3 minutes at 800 x g and 4°C. The supernatant was discarded, and the pellet was resuspended in 1 mL of RPMI 1640 + 5% exosome-free FBS + 100 µg/mL penicillin/streptomycin with glutamine and transferred to labeled 5 mL Falcon tubes with strainer caps (ThermoFisher) for flow cytometry. Peripheral blood mononuclear cell populations were sorted via flow cytometry (FACS Aria II equipped with FACS Diva software, Beckton, Dickinson and Company, Franklin Lakes, NJ). Sorted cell populations included CD4+, CD14+, and CD8+ based on their forward and side scatter qualities and the fluorescence intensity of secondary antibodies following staining as described above.

Following flow cytometry, walls of the Falcon tubes containing the sorted PBMC populations were rinsed with RPMI 1640 + 5% exosome-free FBS + 100 µg/mL penicillin/streptomycin with glutamine then centrifuged for 3 minutes at 800 x g and 20°C. Supernatant was discarded, and the pellets were resuspended in 110 µL of RPMI 1640 + 5% exosome-free FBS + 100 µg/mL penicillin/streptomycin with glutamine. Cells were counted using a hemocytometer using Trypan blue stain (ThermoFisher), and their viability was assessed and recorded. Cells were seeded at a density of 5,000 to 10,000 cells per well in 96-well flat bottom culture plates (ThermoFisher), and 200 µL of RPMI 1640 + 5% exosome-free FBS + 100 µg/mL penicillin/streptomycin with glutamine were added to each well.

For each PBMC population, representing a pooled sample from multiple cows, cells were cultured with one of six treatments: $10^5$-$10^7$ AI-derived EVs (AI EV), $10^5$-$10^7$ SCNT-derived EVs (SCNT EV), $10^5$-$10^7$ AI-derived heat inactivated EVs (AI HI), EVs placed in a 65°C water bath for 30 minutes), $10^5$-$10^7$ SCNT-derived heat inactivated
EVs (SCNT HI), whole trophoblast culture supernatant (WSP), and PBS. Cells were then cultured for 24 hours at 37°C and 5% CO2. Following the 24 hour incubation, the 96-well plates were centrifuged at 800 x g for 6 minutes. Supernatant was collected and stored in -80°C. Pelleted cells were resuspended in CellsDirect One-Step q-RT-PCR 2X reaction mix (Invitrogen) and stored at -80°C until gene expression was assessed.

2.6 Gene expression profiling of leukocytes

The CellsDirect One-Step q-RT-PCR kit (Invitrogen) was used to isolate and purify RNA from the isolated PBMC samples without the need for an RNA purification step. Relative gene expression of the treated PBMC populations was assessed using the Fluidigm BioMark high throughput RT-qPCR system (Standard BioTools) according to (Yang et al., 2016). All primers were designed and produced by Fluidigm/Standard BioTools. Pro- and anti-inflammatory gene primers assessed include CD28, CSF2, CTLA4, CXCL8, EIF4A1, FOXP3, GATA3, IFNA, IFNG, IFNT, IL1B, IL2, IL2Ra, IL4, IL5, IL6, IL10, IL12b, IL13, IL15, IL17, IL18, IL23A, IL27, TGFBI, TNFA, and TXB21. Housekeeping gene references included ACTB, GAPDH, and YWHAZ (Table 2). Manufacturer instructions were followed for priming, loading, and measuring relative gene expression abundance in the Fluidigm 48.48 chip. Relative gene expression was quantified using the $2^{-\Delta\Delta Ct}$ method compared to the average CT values of the housekeeping genes.

2.7 Statistical analysis
The effects of EVs derived from AI and SCNT pregnancies on gene expression of specific T cell subpopulations (CD4+, CD8+, and CD14+ T cells) were analyzed using a factorial design using JMP (version 16.2.0, SAS Institute Inc.). The interactions between EV origin (PREG) and EV treatment (EVTRT) were included in the model. PREG and EVTRT were considered fixed factors while a random factor was the cow (donor of immune cells) in the model. Pairwise comparisons with Tukey’s correction were used to determine which factors held significance. Adjusted p values were calculated using a False Discovery Rate test (Benjamini & Hochberg, 1995). Statistical difference was considered when p value was equal to or less than 0.05. For the gene expression data, statistical differences were considered when p value was less than 0.05 and the fold-change was greater than two.

3. Results

3.1 Flow cytometry analysis and sorting of bovine peripheral immune cells

The proportions of CD4+, CD8+, and CD14+ cells among mononuclear cells in peripheral blood in cows at 35-70 days of gestation by flow cytometry was 18.2%, 7.2%, and 36.2%, respectively. The lymphocyte population was defined as a parent population composed of small cells with low granularity based on forward and side scatter; while the monocyte/macrophage population was composed of larger, more granular cells. Immune cells in the lymphocyte parent population were then characterized based on their fluorescence intensity representing the surface marker expression of CD4 and CD8, and cells in the monocyte/macrophage population were characterized by CD14 marker expression.
3.2 Characterization of trophoblast cells in culture

Expression of trophoblast-specific genes such as PAG10, PAG11 and PLAC1 before and after culture was comparable, and the expression levels of these genes was significantly greater than those of fibroblast cells (data not shown).

3.3 Characterization of trophoblast-derived extracellular vesicles

Nanoparticle tracking analysis results consistently detected a large spike of total particles per fraction in fraction 3. Further, results showed that fraction 3 consistently had the highest concentration of particles between 30 and 150 nm in size, the size range for exosome-sized EVs. Other fractions had lower concentrations of particles per fraction, as well as lower concentrations of exosome-sized particles (Figure 1). For these reasons, only fraction 3 was used for EV treatments.

Results from BCA assays of isolated EV fraction found an inversely proportional relationship between particles per fraction (as detected by NTA) and total protein quantity per fraction. As the concentration of exosome EV-sized particles per fraction increased, the total protein concentration per fraction as detected by the BCA assay decreased in this study. As a consequence, low protein concentration per fraction, relative to the entire set of fractions per sample, could be used as another means of verifying the EV-rich SEC fractions.

3.4 Trophoblast extracellular vesicle-induced gene expression in immune cells
Of the genes investigated, trophoblast-derived EV treatments affected relative expression of *CTLA4, CD28, IL6, IL2, IL1B, IL15*, and *IFNA* in CD4+ cells. As shown in Figure 2, there was an interaction between PREG and EVTRT on the expression levels of *CTLA4* in CD4+ cells. The gene was upregulated (*p* < 0.001) in AI HI compared with AI EV, AI WSP, SCNT EV, and SCNT HI; and it was downregulated in AI EV and AI WSP compared with SCNT EV, SCNT HI, and SCNT WSP. There was also an interaction between PREG and EVTRT on *CD28* expression levels in CD4+ cells; AI EV *CD28* expression was decreased compared to AI HI, AI WSP, and SCNT EV (*p* < 0.05).

Both EVTRT, and EVTRT and PREG interaction were significant on relative *IL6* expression in CD4+ cells. AI HI upregulated *IL6* in CD4+ cells when compared to AI EV, AI WSP, SCNT EV (*p* < 0.0001), and SCNT HI (*p* = 0.0002). AI WSP caused lower *IL6* expression in CD4+ cells when compared to SCNT EV (*p* = 0.001) and SCNT HI (*p* = 0.0036), and SCNT WSP downregulated the gene when compared to SCNT EV (*p* < 0.001) and SCNT HI (*p* = 0.004). In CD4+ cells, EVTRT impacted the expression of *IL2* (*p* = 0.0048). SCNT WSP upregulated *IL2* expression compared to AI EV (*p* = 0.002), AI HI (*p* = 0.003), AI WSP (*p* = 0.003), SCNT EV (*p* = 0.002), and SCNT HI (*p* = 0.003). Further, AI EV decreased *IL2* expression compared to AI WSP (*p* = 0.0018), and SCNT EV downregulated the gene when compared to AI HI (*p* = 0.0066). Similarly, *IL1B* expression was affected by EVTRT. *IL1B* expression was downregulated by AI EV treatments in CD4+ cells compared to AI HI (*p* < 0.0001), AI WSP (*p* = 0.0017), and SCNT EV (*p* = 0.0013). AI HI caused *IL1B* expression to be increased when compared to AI WSP (*p* = 0.002), SCNT EV (*p* < 0.0001), SCNT HI (*p* < 0.0001), and SCNT WSP.
IL1B expression was downregulated in CD4+ cells following SCNT WSP treatment when compared to SCNT EV (p < 0.0001) and SCNT HI (p < 0.0001).

In CD4+ cells, both EVTRT and EVTRT and PREG interactions influenced IL15 expression. AI EV downregulated IL15 relative expression when compared to AI HI (p = 0.0023), SCNT EV (p = 0.0005), and SCNT HI (p < 0.05). Contrastingly, AI HI increased IL15 expression in CD4+ cells compared to SCNT EV (p = 0.0006), SCNT HI (p = 0.0096), and SCNT WSP (p = 0.0065). SCNT EV treatment upregulated IL15 when compared to AI WSP (p = 0.007), SCNT HI (p = 0.04), and SCNT WSP (p = 0.0026); SCNT HI increased IL15 expression when compared to SCNT WSP (p = 0.021). Finally, EVTRT impacted IFNA expression in CD4+ cells; AI HI downregulated IFNA when compared to AI WSP (p = 0.0346), SCNT HI (p = 0.0063), and SCNT WSP (p = 0.039); and SCNT EV downregulated IFNA expression compared to all other treatments (p < 0.01).

Of the genes examined, IFNG, IFNT, and IFNA were found to be significantly affected by trophoblast-derived EV treatments in CD14+ leukocytes. As illustrated in Figure 3, PREG influenced the expression of IFNG in this cell population (p = 0.0102). While all treatments exhibited decreased IFNG expression relative to the PBS control, AI EV and AI WSP upregulated the gene’s expression compared to SCNT EV and SCNT WSP, respectively, in the CD14+ population. AI EV treatment significantly increased IFNG expression in these cells relative to SCNT EV (p = 0.03), SCNT HI (p = 0.03), and SCNT WSP (p = 0.006). AI WSP upregulated the gene in CD14+ cells when compared to AI HI (p = 0.04), SCNT EV (p = 0.04), and SCNT WSP (p = 0.01). Following this trend, PREG also impacted IFNT expression in CD14+ cells (p = 0.09),
with AI EV, AI HI, and AI WSP treatments increasing the expression of *IFNT* in these cells when compared to SCNT EV, SCNT HI, and SCNT WSP treatments, respectively. AI EV significantly upregulated *IFNT* relative to SCNT WSP (*p* = 0.006), AI WSP upregulated the gene compared to SCNT WSP (*p* = 0.007), and SCNT EV upregulated the gene compared to SCNT WSP (*p* = 0.008). Both PREG (*p* = 0.045) and EVTRT (*p* = 0.002) caused differential expression of *IFNA* in CD14+ cells. Compared to SCNT WSP, *IFNA* was expressed at a higher level in the cells treated with AI EV (*p* = 0.004), AI WSP (*p* = 0.02), SCNT EV (*p* = 0.005), and SCNT HI (*p* = 0.003).

Out of the genes studied in the CD8+ population, only *IL4* expression was significantly influenced by PREG following trophoblast-derived EV treatments (*p* = 0.0132); however, no pairwise comparisons between treatments showed significant variation.

4. Discussion

This is the first study to investigate gene expression and proliferation profiles in bovine immune cells in response to EV treatment. The paramount finding of the present study are that trophoblast-derived EVs likely do play some role in modulating cytokine production by immune cells to modulate the inflammatory response to the conceptus in early bovine pregnancy, as demonstrated by EV treatments initiating differential gene expression relative to the control. Additionally, trophoblast EVs derived from AI and abortion-prone SCNT pregnancies influence gene expression of maternal immune cells differently.
This study utilized a primary culture of trophoblast cells collected at 42 ± 3 days of gestation. Cells were cultured for three to four weeks, and supernatant was collected for EV isolation, characterization, and culture with immune cells. Gene expression data obtained from cells used in this study and cell used in our previous study show that this culture is composed of predominantly trophoblast cells; and that these cells maintain the expression of trophoblast specific genes in culture (Leppo et al., 2021).

In this study, EVs were isolated by SEC, and the particle size and concentration were assessed by NTA. Isolation of EVs through SEC yields fractions containing specific particle sizes. In the present study, NTA detected the highest concentration of both total particles and exosome EV-sized particles per fraction in SEC fraction 3, which provided the justification for using SEC fraction 3 as the source of EVs for treating the PBMCs. Compared to other popular methods of EV isolation by particle size differentiation, such as ultrafiltration, sequential filtration, field-flow fractionation, and hydrostatic filtration dialysis, SEC has several advantages. It requires no extensive sample pretreatment, is highly consistent, and conserves the structure, integrity, and functionality of the EVs (Doyle & Wang, 2019; D. Yang et al., 2020). However, SEC is more time consuming and lower yield than some of the aforementioned methods (Akbar et al., 2022).

The expression of both CTLA4 and CD28 were significantly affected by trophoblast-derived EV treatments in CD4+ T lymphocytes. CTLA4 and CD28 are homologous costimulatory receptors with shared ligands that modulate the body’s inflammatory response (Chambers, 2001; Y. Liu, 1997). They serve opposite functions in T cell activation; pro-inflammatory CD28 promotes the activation of T cells while
immunosuppressive CTLA4 blocks T cell activation (Rowshanravan et al., 2018). Previous research has demonstrated that, in instances of first trimester human miscarriages, *CTLA4* is downregulated and *CD28* is upregulated in decidual tissue (Jin, Fan, & Li, 2011; Jin, Fan, Zhang, et al., 2011). Beyond their individual expression, data shows that the *CTLA4*/*CD28* ratio is important in pregnancy viability, as this ratio is notably reduced in cases of miscarriage relative to normal human pregnancies (Jin et al., 2009). In the current study, both immunosuppressive *CTLA4* and cell-mediated response-promoting *CD28* expression were lower in CD4+ cells treated with AI EVs relative to cells treated with SCNT EVs. During normal pregnancy, the maternal inflammatory response must be tightly regulated to prevent immunological overreaction and rejection of the semi-allogeneic conceptus. As *CD28* is pro-inflammatory with major roles in naïve T cell activation, it is expected that AI EVs, which model normal pregnancy, would downregulate *CD28* expression in CD4+ cells.

Interestingly, however, AI EVs also downregulated *CTLA4*, an anti-inflammatory co-stimulatory molecule that inhibits T cell function, when compared to the abortion-prone pregnancy model SCNT EV treatment. A possible explanation for this is that the decreased *CTLA4* expression noted in AI EV treated CD4+ cells is associated with regulation of the *CTLA4*/*CD28* ratio during early pregnancy. As previously discussed, it has been observed that low *CTLA4*/*CD28* ratios are associated with cases of first trimester miscarriages in humans (Jin et al., 2009; Jin, Fan, & Li, 2011; Jin, Fan, Zhang, et al., 2011). Potentially, there is an ideal range of *CTLA4*/*CD28* ratios for proper maternal immune response to the embryo, and the noted decrease in *CTLA4* expression by AI EVs functions to establish a *CTLA4*/*CD28* ratio within this range.
(Kieffer et al., 2019; S. Liu et al., 2014; L. S. K. Walker, 2013). *CTLA4* decreases the function of T cells, but certain T cell subsets such as T regulatory (Treg) cells, have roles in healthy pregnancy establishment and maintenance, and completely inhibiting them would negatively affect the health of the pregnancy. Research has demonstrated that reduction of Treg cell activity and clonal expansion at the fetal-maternal interface is associated with pathological conditions including miscarriage and preeclampsia (Tsuda et al., 2019). While some immunosuppression is necessary to prevent an excessive inflammatory response, some essential events during early pregnancy, such as embryo implantation and development of the placenta, require inflammation, even in normal healthy pregnancies; therefore, completely abolishing T cell activity and inflammation would likely not be beneficial to the viability of the pregnancy (Miko et al., 2019; Mor et al., 2011). It is possible that AI EVs downregulate immunosuppressive *CTLA4* expression in CD4+ cells to allow T cells to perform their necessary functions in pregnancy establishment and maintenance while preventing severe inflammatory response to the conceptus, an example of establishing immunological balance at the fetal-maternal interface.

Further, this study showed that the expression of the pro-inflammatory cytokine *IL6* was affected by the trophoblast-derived EV treatments in CD4+ cells. *IL6* has many functions, including promoting the synthesis of acute phase proteins and C reactive protein by the liver, B cell differentiation, promotion of inflammatory TH17 differentiation, inhibition of Treg differentiation, and regulation of serum iron and zinc levels (Tanaka et al., 2014). *IL6* expression was downregulated in CD4+ cells relative to the PBS control in nearly every treatment. It appears that *IL6* is downregulated in response to
trophoblast EVs in general, in both the AI and abortion-prone SCNT pregnancies. As *IL6* has roles in chronic inflammation, the noted decrease in expression in the current study is likely to prevent a chronic maternal inflammatory response to the semi-allogenic conceptus (Neurath & Finotto, 2011). Interestingly, both AI WSP and SCNT WSP treatments downregulated the expression of *IL6* in CD4+ cells relative to the EV and HI treatments, suggesting that the inhibition on this cytokine is not mediated by EVs. Rather, other soluble factors released into the supernatant by trophoblast cells besides EVs may be serving to inhibit chronic inflammation during early pregnancy.

*IL2* is a cytokine that exhibits both pro-inflammatory and anti-inflammatory functions. It stimulates T cell, NK cell, monocyte, macrophage, and neutrophil activation, which promotes an inflammatory response. Contrastingly, it is important for the development, regulation, proliferation, and maintenance of immunosuppressive Treg cells (Shachar & Karin, 2013). In the present study, both AI EV and SCNT EV treatments downregulated the expression of *IL2* in CD4+ cells compared to the HI and WSP treatments, suggesting that control of T cell proliferation may be EV-mediated. Neither AI EV nor SCNT EV caused the gene to be expressed vastly differently than the PBS control, though, potentially suggesting that EVs may only play a minor role in controlling *IL2* gene expression or that the regulation of *IL2* transcription is mostly independent of EVs during early bovine pregnancy. Another potential explanation for this result is that, because *IL2* has both anti-inflammatory and pro-inflammatory roles, both normal and abortion-prone pregnancy models would affect the expression of this gene similarly and neither would cause drastic shifts in its expression level relative to the control. This could be an example of how trophoblast-derived EVs serve to establish
a tightly regulated immunological balance at the fetal-maternal interface. Trophoblast soluble factors likely promote a certain level of \textit{IL2} expression in CD4+ cells for its anti-inflammatory Treg functions but also limit its overexpression to prevent excessive pro-inflammatory immune cell activation, and vice versa for the SCNT EVs.

Our study found that the expression of \textit{IL1B}, a key mediator of the inflammatory response, is affected by trophoblast-derived EVs in CD4+ cells. As a pro-inflammatory cytokine, \textit{IL1B} has roles in increasing vascular permeability, enhancing cytokine production, and activating lymphocytes (Kaneko et al., 2019). \textit{IL1B} expression was downregulated in these cells by the AI EVs relative to SCNT EVs. Further, both SCNT EV and SCNT HI increased \textit{IL1B} expression relative to the SCNT WSP. This suggests that EVs produced by SCNT placentas, regardless if they are unaltered or heat inactivated, carry cargoes that stimulate a pro-inflammatory response by \textit{IL1B} in CD4+ cells. Interestingly, the AI HI treatment increased \textit{IL1B} expression in CD4+ cells compared to many treatments, including SCNT EV and SCNT HI. A potential explanation for this observation is that AI EVs actively control their cargo delivery to prevent the release of pro-inflammatory mediators, such as \textit{IL1B}. However, once the EV lipid membrane is disrupted, as it is in heat inactivation, this control is lost, and cargo delivery becomes passive. Other studies have shown that exposing EVs to high temperatures affects the physio-chemical properties of the EVs and induces release of their encapsulated proteins but does not affect the degree of which they are taken up by target cells (Schulz et al., 2020). Therefore, it is possible that \textit{IL1B} expression may have been upregulated in CD4+ cells by AI HI treatments due to temperature-induced
changes in the vesicles’ physio-chemical characteristics which inhibited their ability to control cargo transfer.

The expression of *IL15* was also significantly affected by trophoblast-derived EV treatments in CD4+ lymphocytes. As a pro-inflammatory cytokine, *IL15* regulates natural killer and T cell activation and proliferation. Previous studies in humans and mice have found that *IL15* is richly expressed in the uterus, but poorly expressed in placental tissue, during early pregnancy (Gordon, 2021). Though expressed, it must be tightly regulated for pregnancy maintenance, as dysregulation of *IL15* is associated with adverse conditions including preeclampsia, fetal growth restriction, and abortion (Gordon, 2021). Our study found that SCNT EV treatment upregulates *IL15* expression in the CD4+ cells relative to the AI EV treatment. Interestingly AI HI treatments increased the expression of *IL15* in the CD4+ population relative to the other treatments and PBS control. Similar to *IL1B*, a possible explanation of this result is that EVs from healthy pregnancies tightly regulate delivery of cargoes to target cells, but once the EV lipid membrane is disturbed by heat inactivation, the vesicles’ ability to control cargo delivery is lost.

The final gene our study found to be significantly affected by trophoblast-derived EV treatments in CD4+ population is *IFNA*. As a type I interferon, *IFNA* induces interferon-stimulated genes with non-specific anti-viral effects, enhances natural killer cell and T cell responses, and contributes to regulate the body’s innate and adaptive immune response through the activation of leukocytes including lymphocytes, monocytes, and macrophages. (Cha et al., 2014; M. Zhang et al., 2021). Besides these pro-inflammatory functions, however, *IFNA* also has limited anti-inflammatory roles,
such as blocking the production of some antigen-specific pro-inflammatory cytokines (Chalise et al., 2013). The present study found that IFNA expression was upregulated by most treatments relative to the PBS control, with the exception of SCNT EV which downregulated the gene. Interestingly, of all the treatments, IFNA was expressed at the highest level in CD4+ cells receiving SCNT HI treatment and the least following SCNT EV treatment. This potentially suggests that EVs from the SCNT pregnancies may excessively limit the delivery of cargoes that would stimulate IFNA expression in target cells but that, following membrane disruption by heat inactivation, the vesicles' mechanism of cargo trafficking control is lost, a phenomenon similar to what was described for IL1B and IL15.

Our results show that trophoblast-derived EV treatments affected the relative gene expression of CD28, CTLA4, IL6, IL2, IL1B, IL15, and IFNA in the CD4+ population in this study. The CD4+ cell expression profiles of IL6, IL1B, and IL15 were very similar. In most of the aforementioned genes, variation was observed in the relative expression induced by treatments from the AI-established and SCNT-established pregnancies. While, in this study, the AI EVs generally appeared to stimulate a more anti-inflammatory phenotype while SCNT EVs promoted a pro-inflammatory phenotype of CD4+ cells overall, results were more ambiguous than anticipated. Both AI and SCNT treatments had anti- and pro-inflammatory effects on the CD4+ population. This suggests that it is potentially more accurate to view EV-mediated maternal immunological response in normal pregnancies as possessing appropriate balance between the expression of anti- and pro-inflammatory genes, rather than arguing that the maternal immune system is simply suppressed during gestation.
Our study found that, in the CD14+ population, three genes, *IFNG, IFNT,* and *IFNA,* were significantly affected by trophoblast-derived EV treatments. *IFNG* has functions in modulating both innate and adaptive immune responses, including activating macrophages, stimulating natural killer cells and neutrophils, and promoting cell-mediated immune responses by encouraging differentiation of naïve T helper cells into TH1 cells and inhibiting TH2 differentiation. In normal pregnancies, its expression is a source of the necessary inflammation required for implantation and development of the placenta, and irregularities in *IFNG* expression are associated with pathological conditions, such as abortion and preeclampsia, in humans and livestock (Murphy et al., 2009). In the current study, both AI EV and AI WSP treatments upregulated the expression of *IFNG* in CD14+ cells in comparison to the SCNT treatments. With the exception of AI EV and AI WSP, all treatments downregulated the gene relative to the PBS control. This is likely an example of how some inflammation is necessary during the establishment and maintenance of a healthy, normal pregnancy. Higher expression of *IFNG* in CD14+ cells treated with AI EVs and AI WSP than in cells treated with the SCNT treatments demonstrates how, in healthy pregnancies, inflammatory cytokines are still expressed but in a controlled, and tightly regulated manner. Further, in other species such as humans, uterine natural killer cells are a significant source of the *IFNG* necessary for the inflammatory processes of early pregnancy; however, natural killer cells are scarce in the uterus in cattle (Murphy et al., 2009). CD14+ cells which include macrophages, monocytes and neutrophils (Paape et al., 1996) on the other hand, have been observed in significant amounts in the pregnant bovine uterus (Oliveira & Hansen, 2009; H. Rutigliano et al., 2022). The observed elevated *IFNG* production by CD14+
cells in the current study following AI EV treatment may reflect a mechanism by which trophoblast-derived EVs in healthy gestations ensure adequate IFNG is produced for the inflammatory processes of pregnancy establishment to occur, and that this process is not restricted to intrauterine natural killer cells.

IFNT, the signal for maternal recognition of pregnancy in ruminants that also has some mild anti-inflammatory properties (Bazer et al., 2015; Choi et al., 2003), was also significantly affected by trophoblast-derived EV treatments in the CD14+ population. Our work found that the AI-established pregnancies upregulated IFNT in these cells in comparison to the SCNT-established pregnancies, which reflects that the AI pregnancies would promote an anti-inflammatory response in the maternal immune cells. The AI-established pregnancies are our normal ruminant pregnancy model, so it is expected that they would increase the relative expression of anti-inflammatory IFNT compared to the pro-inflammatory SCNT pregnancies. Another explanation for the observed relative decrease of IFNT in the SCNT treatments is that, in SCNT pregnancies, the embryo is cultured for a significant amount of time in vitro before being transferred to a recipient female. IFNT is produced transiently by the conceptus beginning around the time of blastocoel formation (Hernandez-Ledezma et al., 1992). Compared to AI-established pregnancies, in which the entire process of fertilization and embryo development is in vivo, SCNT pregnancy establishment exposes the developing embryo to multiple factors that have been documented to negatively affect IFNT secretion, including cell culture media composition, cell culture density, and overall embryo manipulation (Sanchez et al., 2019).
The final gene we found to be significantly influenced by trophoblast-derived EV treatments in CD14+ cells was *IFNA*. As previously described, *IFNA* is type I interferon with non-specific antiviral effects that serve to modulate both innate and adaptive immune responses through the activation of many types of immune cells. While it is considered mostly pro-inflammatory, it also has some immunosuppressive properties, as it blocks the production of some antigen-specific pro-inflammatory cytokines (Cha et al., 2014; Chalise et al., 2013; M. Zhang et al., 2021). The present study found that, in the CD14+ population, all treatments, with the exception of SCNT WSP, increased *IFNA* expression relative to the PBS control. In human and mouse models, *IFNA* expression has been shown to be essential for blastocyst implantation and remodeling of maternal tissues at the implantation site for placental development (Casazza et al., 2020). Though cattle and other ruminants have a less invasive placental attachment than humans and mice, our results indicate that *IFNA* is likely still important during implantation and placental development during early gestation in cattle in both AI and SCNT pregnancies. Increased relative *IFNA* expression in the AI pregnancies compared to the SCNT pregnancies was an unexpected result as elevated type I interferon expression has been linked to poor reproductive outcomes, including preeclampsia and pregnancy loss, in humans (Crow & Manel, 2015). A potential explanation is that the reproductive complications associated with excessive *IFNA* production are generally correlated with increased gestational age. While studies in cattle are limited, knockout mouse models that lack type I interferon signaling receptors demonstrate incomplete placenta development and consequent poor fetal-maternal nutrient and waste
exchange, further suggesting that *IFNA* secretion to stimulate controlled inflammation is important during early gestation, even in normal pregnancies (Yockey & Iwasaki, 2018).

Intriguingly, the only genes found to be significantly affected by the EV treatments in the CD14+ population in the present study were interferons, which implies that there is potentially a link between EV secretion by the trophoblast cells of the conceptus and the macrophage interferon production. CD14+ cells are essential immunomodulatory cells with a multitude of functions during pregnancy establishment and maintenance of early pregnancy (Faas et al., 2014). Because they are pivotal in creating intrauterine conditions that support embryonic growth, infection of this cell population would be detrimental to the viability of the pregnancy, especially considering their critical roles in placentation (Haese et al., 2021). As type I and III interferons are key antiviral cytokines, it is a possibility that trophoblast-derived EVs specifically target interferon transcription in CD14+ cells during early gestation, a time in which the maternal immune system is being generally suppressed by other cytokines to shield the semi-allogenic conceptus from a maternal immunological attack, in order to specifically protect the macrophage population from infection. Research in women has also demonstrated that decreased CD14+ activation and functionality paired with low interferon levels during early gestation is associated with adverse outcomes, namely preterm delivery, likely due to reduced immune responsiveness in both reproductive and peripheral tissues (Mdletshe et al., 2021). Considering this limited data with the results of our study, it can be argued that there is evidence that trophoblast-derived EVs target interferons specifically in CD14+ cells to influence the outcome of pregnancy.
In the CD8+ population, there appeared to be very poor EV-mediated immunomodulation, as EV treatments affected only one gene, IL4, yet no pairwise comparisons between the treatments showed significant variation. This is possibly explained by the fact that CD8+ cells are more terminally differentiated cells. Once fully developed, they develop specialized functions and acquire a stable phenotype (Joshi & Kaech, 2008). They are less plastic and more terminally specialized than CD4+ or CD14+ cells, and for this reason may be less apt to be responsive to the EV treatments.

This study utilized immune cells obtained from circulating blood, and not from the endometrium, to model the trophoblast-derived EV and immune cell interactions at the fetal-maternal interface. Though PBMCs are likely still a reliable model, it is possible that endometrium-derived maternal immune cells would behave differently when treated with the trophoblast-derived EVs. The endometrial immune cell population is exposed to paracrine signaling factors in the uterus, is in close proximity to the conceptus, is composed of different relative proportions of cell types, and expresses genes at different levels when compared to peripheral immune cells (Rutigliano et al., 2022). This was also an in vitro study, which does not reflect the interactions between cells in the endometrium and the indirect effects of EVs. Numerous factors, such as exposure to culture media components and simplified experimental conditions, may affect the ability of an in vitro study to accurately replicate the way cells would respond in a complex, living organism. Additionally, the present study solely investigated the isolated direct effects of trophoblast-derived EVs on isolated maternal immune cells but did not consider the indirect EV-mediated effects or cell-cell interactions. Finally, while the study was organized in a way to reduce the effect of individual animals on the results, it
is still possible that individual animal variation, including immunological variation, gestational age, genetic differences, energy balance, and pathogen exposure, had some influence on the outcomes of the study. Likewise, individual differences in the limited number of pregnancies from which placenta samples were collected, such as genetic influence, placentation quality, and embryo health, may have affected the contents of EVs released by the trophoblast cells and thus impacted our results.

Future studies are warranted to fully understand the processes by which trophoblast-derived EVs stimulate modulation of the maternal immune system during pregnancy establishment and maintenance. Studies utilizing uterine-derived maternal immune cells, as opposed to PBMCs, may create a more accurate model of EV-leukocyte interactions at the fetal-maternal interface; and ultimately, an in vivo study would shed light on the complex interactions between the conceptus and the endometrium. Because the present study investigated changes in relative gene expression in leukocyte populations and found some proliferative cytokines to be upregulated, it would be interesting to quantify the proliferation of maternal immune cells in response to EV exposure. Gene ontology and KEGG analysis may also be implemented to further understand the effects of trophoblast-derived EVs in maternal immune cell populations.

The current study serves to expand our knowledge base of immunomodulation during pregnancy establishment and maintenance, and this increased understanding of how the maternal immune system is regulated during early pregnancy has many downstream applications. New knowledge gained through this study, as well as future research, on what molecules mediate the communication between the conceptus and the maternal immune system could ultimately result in novel approaches to reduce
embryonic loss in cattle and may be at least partially transferable to prevent pregnancy complications and loss in other livestock species and in humans. Increased understanding of EV contents associated with pregnancy complications may eventually lead to EVs being used as diagnostic biomarkers based on mRNA profiles or to the creation of engineered EVs loaded with therapeutic cargoes for prevention and treatment of pathological reproductive conditions.

These findings highlight the unique ability of trophoblast-derived EVs to affect the relative gene expression profiles of maternal immune cells during early pregnancy in cattle. We identified several genes that were affected by EV treatments in CD4+, CD14+, and CD8+ populations and identified variation between the effects of EVs isolated from a normal pregnancy model, AI, and an inflammatory pregnancy model, SCNT. Our study demonstrates that trophoblast EVs and soluble factors contribute to promoting an immunological balance at the fetal-maternal interface. Our study serves as a basis for future experiments to further understand the role of trophoblast-derived EVs in immunomodulation at the fetal-maternal interface during pregnancy establishment and maintenance.
5. Tables

Table 1. Antibodies used for lymphocyte identification and sorting by fluorescence-activated cell sorting.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Antibody</th>
<th>Supplier</th>
<th>Isotype</th>
<th>Primary or Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8</td>
<td>BAT82LA</td>
<td>WSU(^a)</td>
<td>IgG1</td>
<td>Primary</td>
</tr>
<tr>
<td>CD4</td>
<td>ILA11A1</td>
<td>WSU</td>
<td>IgG2a</td>
<td>Primary</td>
</tr>
<tr>
<td>CD14</td>
<td>MM61A</td>
<td>WSU</td>
<td>IgG1</td>
<td>Primary</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>A21131</td>
<td>Thermo Fisher(^b)</td>
<td>Goat anti-mouse IgG2a</td>
<td>Secondary</td>
</tr>
<tr>
<td>Alexa Fluor 647</td>
<td>A28181</td>
<td>Thermo Fisher</td>
<td>Goat anti-mouse IgG1</td>
<td>Secondary</td>
</tr>
<tr>
<td>Control</td>
<td>S69A</td>
<td>WSU</td>
<td></td>
<td>Primary</td>
</tr>
</tbody>
</table>

\(^a\) Washington State University Monoclonal Antibody Center (Pullman, WA)

\(^b\) Thermo Fisher Scientific (Waltham, MA)
Table 2. Genes, gene definitions and primer sequences used for real time reverse transcription-polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Definition</th>
<th>Primer sequence (forward, reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>β-actin</td>
<td>FP: GGCCGAGCGGAAATCG&lt;br&gt;RP: GCCATCTCCTGCTCGGAAGTC</td>
</tr>
<tr>
<td>CD28</td>
<td>Cluster of differentiation 28</td>
<td>FP: GGAGGTCTGTGCTGTGAATGG&lt;br&gt;RP: CGGTGCAAGTCTCCTTATTTTTTTTTTTTTTATTTT</td>
</tr>
<tr>
<td>CSF2</td>
<td>Colony stimulating factor 2</td>
<td>FP: CAGAAGTGAGCTTTACCTCAGACAAGA&lt;br&gt;RP: CCTCCAGTGTAAGATCCTGAGTT</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-lymphocyte-associated protein 4</td>
<td>FP: GCAGCCAGTGACGAAGA&lt;br&gt;RP: TCATCCAGGAGGTAGCTCATC</td>
</tr>
<tr>
<td>CXCL8</td>
<td>C-X-C motif chemokine ligand 8</td>
<td>FP: GGAAAAGTGAGCTGTGAAGG&lt;br&gt;RP: GGTGGTCTTTCTTTTTCTTTTCATGGA</td>
</tr>
<tr>
<td>EIF4A1</td>
<td>Eukaryotic translation initiation factor 4A1</td>
<td>FP: GGATTGCACCAGGAAACTTTC&lt;br&gt;RP: GGGTCAAGGAGGAAGGAAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>
| **IL4** | Interleukin 4 | FP: GCCGTATCTACAGGAGCCAC  
           RP: CAAGAGGTCTTTTCAGCGTACTTG |
| **IL5** | Interleukin 5 | FP: TGGTGGCAGAGACCTTGACA  
           RP: GAATCATCAAGTTCCCATACCTA |
| **IL6** | Interleukin 6 | FP: GGCTCCCATGATTGTTGCTAGTT  
           RP: GCCCAGTGACAGGTTCCTT |
| **IL10** | Interleukin 10 | FP: GAGCAAGGCGGTGGAGAAGG  
           RP: GATGAAGAGTGCAAATCTCAGTATGG |
| **IL12B** | Interleukin 12β | FP: GCTGGGAGTACCCTGACACG  
           RP: GGCTGAGGTTTGGTCCATGAAG |
| **IL13** | Interleukin 13 | FP: CAGTGTCATCCAAAGGACCAAG  
           RP: CGGACGTACTCACTGGAAAC |
| **IL15** | Interleukin 15 | FP: GGGCTGTATCAGTGCAAGTCTTC  
           RP: ATTGGGATGAGCATCCTTCAG |
| **IL17** | Interleukin 17 | FP: CATCATCCACAGAGTCCAGG  
           RP: CACTTGCCCTCCAGATCAC |
| **IL18** | Interleukin 18 | FP: ACTGTTCAGATAATGCACCCCAG  
           RP: GAAACAATTGTGCTCACAGGAG |
| **IL23A** | Interleukin 23A | FP: CCTCCTTTCTCGTCTCAAGATC  
           RP: CGGAGGTCTGGGTGTCATCCT |
| **IL27** | Interleukin 27 | FP: ATGGTACTGGGATGATGGTGACAA  
           RP: CAGAAGCAAGAAGACAGCAAAAG |
| **TBX21** | T-box transcription factor 21 | FP: GCACGACTGAAAGCCAGTTTATAAC  
           RP: CCAACCTAAGCAGATTTCCTGT |
| **TGFβ1** | Transforming growth factor β1 | FP: CTGAGCGGCGGGCATAC  
           RP: TGGCGTATCCACCATTAGCA |
| **TNFA** | Tumor necrosis factor-α | FP: TCTACCAGGGAGAGTCTTCCA  
           RP: GTCCGGCAAGTTGATTCCTCA |
| **YWHAZ** | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein Z | FP: GCATCCCACAGACTTTTCC  
           RP: GCAAAGACAATGACAGACCA |
6. Figures

Figure 1. Characterization of EVs by nanoparticle tracking analysis (NTA). (A) Total particle concentration of size exclusion chromatography (SEC) fractions; (B) Particle size distribution (nm) in SEC fractions.

(A)

(B)
Figure 2. CD4+ relative gene expression for significant genes CTLA4 (A), CD28 (B), IL6 (C), IL2 (D), IL1B (E), IL15 (F), and IFNA (G) in response to incubation with artificial insemination (AI) or somatic cell nuclear transfer (SCNT) pregnancy established trophoblast derived extracellular vesicle treatment (EV), heat inactivated extracellular vesicles control (HI), or whole supernatant control (WSP). Bars with different superscript letters indicate $p < 0.05$. 2A: CTLA4 was upregulated in AI HI compared with AI EV, AI WSP, SCNT EV, and SCNT HI, and downregulated in AI EV and AI WSP compared with SCNT EV, SCNT HI, and SCNT WSP. 2B: CD28 expression was decreased in AI EV compared to AI HI, AI WSP, and SCNT EV. 2C: IL6 was upregulated by AI HI compared to AI EV, AI WSP, SCNT EV, and SCNT HI. AI WSP decreased IL6 compared to SCNT EV and SCNT HI, SCNT WSP downregulated IL6 compared to SCNT EV and SCNT HI. 2D: SCNT WSP upregulated IL2 expression compared to AI EV, AI HI, AI WSP, SCNT EV, and SCNT HI. AI EV decreased IL2 compared to AI WSP. SCNT EV downregulated IL2 compared to AI HI. 2E: IL1B was downregulated by AI EV compared to AI HI, AI WSP, and SCNT EV. AI HI increased IL1B compared to AI WSP, SCNT EV, SCNT HI, and SCNT WSP. SCNT WSP decreased IL1B compared to SCNT EV and SCNT HI. 2F: AI EV downregulated IL15 compared to AI HI, SCNT EV, and SCNT HI. AI HI increased IL15 compared to SCNT EV, SCNT HI, and SCNT WSP. SCNT EV upregulated IL15 compared to AI WSP, SCNT HI, and SCNT WSP. SCNT HI increased IL15 expression compared to SCNT WSP. 2G: AI HI downregulated IFNA compared to AI WSP, SCNT HI, and SCNT WSP; and SCNT EV downregulated IFNA expression compared to all other treatments. AI = artificial insemination; SCNT = somatic cell nuclear transfer; EV = trophoblast derived extracellular vesicles; HI = heat inactivated trophoblast derived extracellular vesicles; WSP = whole supernatant.
Figure 3. CD14+ relative gene expression for significant genes \textit{IFNG} (A), \textit{IFNA} (B), and \textit{IFNT} (C) in response to incubation with artificial insemination (AI) or somatic cell nuclear transfer (SCNT) pregnancy established trophoblast derived extracellular vesicle treatment (EV), heat inactivated extracellular vesicles control (HI), or whole supernatant control (WSP). Bars with different superscript letters indicate $p < 0.05$. 

\textbf{3A}: AI EV and AI WSP upregulated \textit{IFNG} expression compared to SCNT EV and SCNT WSP, respectively. AI EV increased \textit{IFNG} expression in these cells relative to SCNT EV, SCNT HI, and SCNT WSP. AI WSP upregulated \textit{IFNG} compared to AI HI, SCNT EV, and SCNT WSP. 

\textbf{3B}: \textit{IFNA} was expressed at a higher level in the cells treated with AI EV, AI WSP, SCNT EV, and SCNT HI compared to SCNT WSP. 

\textbf{3C}: AI EV, AI HI, and AI WSP increased \textit{IFNT} expression compared to SCNT EV, SCNT HI, and SCNT WSP, respectively. AI EV upregulated \textit{IFNT} relative to SCNT WSP, AI WSP upregulated it compared to SCNT WSP, and SCNT EV upregulated it compared to SCNT WSP.
REFERENCES


Akthar, I., Marey, M. A., Kim, Y., Shimada, M., Suarez, S., & Miyamoto, A. (2022). Sperm interaction with the uterine innate immune system: Toll-like receptor 2 (TLR2) is a main
https://doi.org/10.1071/RD21265


https://doi.org/10.1016/j.theriogenology.2020.05.043


https://doi.org/10.1016/j.anireprosci.2008.10.015


Proteins Are Altered by the Methionine Supply during Late Gestation in Dairy Cows and Are Associated with Newborn Birth Weight. *The Journal of Nutrition.*


https://doi.org/10.1007/s00726-014-1905-x


https://doi.org/10.1016/j.celrep.2020.108204


https://doi.org/10.1146/annurev-animal-021815-111406


https://doi.org/10.3390/ijms21031163

https://doi.org/10.3390/ijms21031163


https://doi.org/10.1146/annurev-immunol-061020-053707


Imakawa, K., Zaghloul, A. H., & Miyamoto, A. (2018). A proinflammatory response of
bovine endometrial epithelial cells to active sperm in vitro. *Molecular Reproduction and

*Rheumatology, 47*(12), 1820–1825. https://doi.org/10.1093/rheumatology/ken373

Faas, M. M., Spaans, F., & De Vos, P. (2014). Monocytes and macrophages in pregnancy and
pre-eclampsia. *Frontiers in Immunology, 5*(298).
https://doi.org/10.3389/fimmu.2014.00298

Fair, T. (2015). The contribution of the maternal immune system to the establishment of
pregnancy in cattle. *Frontiers in Immunology, 6.*
https://doi.org/10.1016/j.theriogenology.2008.07.020

Wang, K. (2018). Extracellular vesicle RNAs reflect placenta dysfunction and are a
biomarker source for preterm labour. *Journal of Cellular and Molecular Medicine, 22*(5),

Fereshteh, Z., Schmidt, S. A., Al-Dossary, A. A., Accerbi, M., Arighi, C., Cowart, J., Song, J. L.,
(OVS) microRNA profiling during the estrous cycle: Delivery of OVS-borne microRNAs to
sperm where miR-34c-5p localizes at the centrosome. *Scientific Reports, 8*(1), 16094–
16094. PubMed. https://doi.org/10.1038/s41598-018-34409-4


early-onset pre-eclampsia, but not normotensive intrauterine growth restriction.


https://doi.org/10.4049/jimmunol.2000889


https://doi.org/10.3390/ijms21155365


https://doi.org/10.1002/ijc.29324


https://doi.org/10.1095/biolreprod.115.127969


https://doi.org/10.3390/nano10091838


https://doi.org/10.1095/biolreprod.112.101121


insemination. *In: The 112th Meeting of the Society for Reproduction and Development*. 
https://doi.org/10.14882/jrds.112.0.

https://doi.org/10.1007/s13258-018-0668-2

https://doi.org/10.1186/s13223-018-0278-1


https://doi.org/10.1016/j.omto.2022.08.005
Mdletshe, N., Thobakgale, C., Malaba, T. R., Madlala, H., Myer, L., Muema, D. M., Mogeni, P.,
Early Pregnancy Is Associated With Preterm But Not Small-for-gestational-age Delivery
in Women Infected With Human Immunodeficiency Virus Initiating Antiretroviral
Therapy in Pregnancy: A Prematurity Immunology in HIV-infected Mothers and their
Infants Study (PIMS) Case-control Study in Cape Town, South Africa. *Clinical Infectious
Diseases, 73*(12), 2205–2216. https://doi.org/10.1093/cid/ciab151

Mecocci, S., Ottaviani, A., Razzuoli, E., Fiorani, P., Pietrucci, D., De Ciucis, C. G., Dei Giudici, S.,
an In Vitro Model of Intestinal Inflammation. *Biomedicines, 10*(3).
https://doi.org/10.3390/biomedicines10030570

Meggyes, M., Miko, E., Szigeti, B., Farkas, N., & Szereday, L. (2019). The importance of the PD-
https://doi.org/10.1186/s12884-019-2218-6

Mittendorf, E. A., Weitz, J., Rahbari, N., Reissfelder, C., Pilarsky, C., Fraga, M. F., Piwnica-


Merlo, B., Gugole, P. M., & Iacono, E. (2022). An Update on Applications of Cattle Mesenchymal


systemic lupus erythematosus (SLE): Phenotypic characterization and clinical associations. *Scientific Reports, 6*(1), 36025. https://doi.org/10.1038/srep36025


https://doi.org/10.1111/imr.13074


https://doi.org/10.1371/journal.pone.0075571


https://doi.org/10.1371/journal.pone.0013213.t003


https://doi.org/10.3168/jds.2018-15668


https://doi.org/10.1016/j.theriogenology.2020.04.010


https://doi.org/10.3389/fimmu.2019.01787


https://doi.org/10.3389/fimmu.2017.01124


Polei, M., Gunther, J., Koczan, D., & Furbass, R. (2020). Trophoblast cell differentiation in the bovine placenta: Differentially expressed genes between uninucleate trophoblast cells and trophoblast giant cells are involved in the composition and remodeling of the
extracellular matrix and O-glycan biosynthesis. *BMC Molecular and Cell Biology, 21*(1).
https://doi.org/10.1186/s12860-020-0246-8


https://doi.org/10.1038/ni.3131


https://doi.org/10.1016/j.jaci.2009.09.046


https://doi.org/10.1038/s41598-021-92525-0


https://doi.org/10.1038/ncb1800


https://doi.org/10.1016/0165-2427(92)90153-H


https://doi.org/10.20517/evcna.2022.27


https://doi.org/10.1038/s41598-019-44590-9


https://doi.org/10.4049/jimmunol.1301885


*Journal of Allergy and Clinical Immunology*, 125, S73-80.

https://doi.org/10.1016/j.jaci.2009.11.017


https://doi.org/10.5603/GP.2020.0048


https://doi.org/10.1002/pmic.201800167


https://doi.org/10.3389/fimmu.2019.00792


