THESIS

MATERNAL IMMUNOTOLERANCE: DEFINING THE DEVELOPMENT OF T CELLS DURING EARLY PREGNANCY IN THE HORSE

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ABSTRACT

MATERNAL IMMUNO-TOLERANCE: DEFINING THE DEVELOPMENT OF T CELLS DURING EARLY PREGNANCY IN THE HORSE

Pregnancy acceptance requires immune tolerance of the embryo, which is initiated upon maternal exposure to the paternal antigen into the uterus. Stimulation is believed to be initiated by paternal antigen presentation within the seminal plasma (SP) fraction to developing regulatory T cell (Treg) populations within the maternal immune system. Tregs then suppress effector T cells, such as Th1. The mechanisms in which this is modulated have yet to be fully elucidated, but the equine model may offer unique insights, as SP is frequently reduced during assisted reproductive techniques (ART) such as insemination with cryopreserved semen. Therefore, we aimed to assess developing Treg and Th1 populations following insemination, in addition to evaluating the impact of SP exposure on these populations. To do so, peripheral blood mononuclear cells (PBMCs) were collected from mares prior to insemination in addition to weekly following ovulation for 28 days. Following insemination, mares were categorized into groups based on breeding protocol; mares were bred with fresh semen (n=10), cooled semen (n=8), and frozen semen (n=6). RNA was isolated from PBMC for qRT-PCR of the transcription factors associated with Treg (*FoxP3, TGF-β, CD25*) and Th1 (*TBX21, IFNγ)* development. Statistics were performed utilizing SAS 9.4®, where the effect of time, pregnancy outcome, and SP exposure was assessed. *FoxP3* expression was found to increase in expression over time (p=0.04) in pregnant animals, while no changes were noted for *TGF-β* (p=0.38), *CD25* (p=0.67), *TBX21* (p=0.39) or *IFNγ* (p=0.14). While a significant effect of pregnancy status was noted for IFNg (p=0.05), noted as increased *IFNγ* expression in the nonpregnant animals, no effect of

pregnancy status was noted when assessing *FoxP3* (p=0.61), *TGF-β* (p=0.66), CD25 (p=0.29), or TBX21 (p=0.93). A significant effect of SP exposure at time of breeding was noted for *CD25* (p=0.04) with a trend noted for expression of *TGF-β* (p=0.06). In both Treg-related transcripts, expression levels were found to be highest in mares bred with fresh semen (full SP exposure) in comparison to cooled or frozen semen, where SP was reduced. In conclusion, these findings suggest that exposure to seminal plasma at the time of breeding may influence the development of maternal immune tolerance by promoting Treg-associated signaling pathways. Mares bred with fresh semen, and therefore exposed to greater concentrations of seminal plasma, demonstrated increased expression of Treg-associated transcripts compared to mares bred with cooled or frozen semen. Collectively, these data support a role for seminal plasma in shaping early maternal immune responses following insemination and highlight a potential immunological consequence of reduced seminal plasma exposure during assisted reproductive techniques in the horse.

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DEDICATION

I first want to dedicate this work to my late grandfather, Robert Ney Crook Jr., who I know would be proud to see that his granddaughter turned her crazy horse obsession into a dedicated career as a research scientist.

I come from a long line of overachievers, with family members and great-grandparents who were engineers, chemists, musicians, journalists, teachers, ranchers, and pastors. Yet, many of the women who came before me were not afforded the same opportunities to pursue their own careers or academic ambitions, setting aside their own aspirations to support their families. I am deeply grateful for the privilege to access continued education and the ability to follow my own path. This work is dedicated to the women in my family whose perseverance made my journey possible, even if their own wasn't fully recognized.

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1.1 INTRODUCTION

The successful establishment of pregnancy is dependent on the maternal immune system undergoing a series of specific changes throughout gestation to support the developing embryo. The immune system of the female must recognize the foreign paternal antigen, thus accepting the conceptus and preventing the maternal immune system from destroying the developing fetus that is 'non-self' (Munoz-Suano et al., 2011). This process is stimulated upon maternal exposure to paternal antigens within the ejaculate into the uterine lumen, initiating immuno-tolerance to the pregnancy. The mechanisms in which this is modulated have yet to be fully elucidated, but data from various species have demonstrated that regulatory T cells (Tregs) are imperative for successful pregnancy outcomes (Robertson et al., 2018). The primary function of Tregs lies in the suppression of immune responses through the cytokine transforming growth factor-beta (TGF-β) (Chen et al., 2003). Tregs produce immunosuppressive TGF-β to inhibit populations of other T effector cells, including T helper cells (Th). In contrast to Tregs, T helper 1 (Th1) cells promote pro-inflammatory responses through cytokines such as interferon- γ (IFN-γ) and interleukin-12 (IL-12) (Robinson & O'Garra, 2002). The overstimulation of potential pro-inflammatory responses during early pregnancy may impact gestational success, as the dominance of Th1 type immunity during this time is associated to abortion and pre-term labor in both mouse and human models (Saito, 2010). Another study demonstrated that Tregs were significantly downregulated in women with miscarriages in comparison to those with successful pregnancy outcomes, suggesting that the establishment of this lymphocyte population is crucial to maintaining early pregnancy (Winger & Reed, 2011). This phenomenon of Treg dominance

during early pregnancy has not been extensively studied in the horse, but one study showed a relationship between decreased circulating Treg populations and early embryonic loss in the mare (Aurich et al., 2014).

Another component potentially impacting the resulting T cell phenotype in response to mating is the exposure to the seminal plasma (SP) portion of the ejaculate. Cytokines, hormones, and proteins that originate from SP contribute to the programming the immunological response to the spermatozoa and developing pregnancy in both murine and human models (Schjenken & Robertson, 2015). Initial studies have investigated the role of reduced SP (and thereby reduced paternal antigen) exposure and pregnancy loss in women (du Fosse et al., 2022), suggesting that the reduction of SP that is associated with assisted reproductive technologies (ART)s may be impeding the successful immunotolerance of embryonic development. Thus, research in a variety of species has focused on potential ramifications of ART-derived pregnancies. Specifically in human and mouse research, decreased Treg concentrations have been associated with reduced SP exposure at the time of breeding, potentially highlighting a relationship between these two factors and successful pregnancy outcomes (Kanannejad et al., 2020). Investigating this theory in the equine model may offer unique insights into applications in the human model as SP is frequently reduced in breeding doses prepared in commercial breeding operations (Noronha & Antczak, 2010). In the horse, the extent to which SP reduction impacts early pregnancy tolerance through immune cell expansion has not been fully described; thus, a study dedicated to elucidating maternal lymphocyte expression prior to breeding and during early pregnancy relative to SP exposure is warranted.

1.2 IMMUNE RESPONSE TO SEMEN DEPOSITION

1.2.1 Overview of Innate and Adaptive Immunity

Across all mammalian species, the immune system as a whole operates to defend an organism from pathogens and/or foreign material and sustain a long-term immune response to assist in future antigen exposures. This is regulated by a system that recognizes antigens as self or not, referred to as the non-specific innate immune response (Murphy, 2017). Anything that is foreign, or non-self, is quickly recognized by pathogen-associated molecular patterns (PAMPs) localized on cell membranes which signal for the recruitment of innate immune cells (Medzhitov & Janeway Jr, 1998). These cells, such as neutrophils, macrophages, and monocytes, act as the first line of defense by phagocytosing antigens and releasing cytokines that recruit other adaptive immune cells to the site of inflammation (Medzhitov & Janeway, 2000). Within days, the adaptive branch of the immune system is activated by said antigen, inducing a specific, long-term immune response (Mempel et al., 2004).

Immune cells can be broadly categorized into three groups by function: granulocytes, monocytes, and lymphocytes (Murphy, 2017). Granulocytes include neutrophils, basophils, eosinophils, and mast cells, all of which carry out the innate immune response against pathogens by way of phagocytosis, degranulation, and histamine release (Dale et al., 2008). In the context of reproduction, the most predominant granulocyte identified within the reproductive tract are populations of neutrophils, acting as ‘first responders’ in the female tract providing a broad-spectrum, non-specific inflammatory response to anything foreign detected (Nathan, 2006).

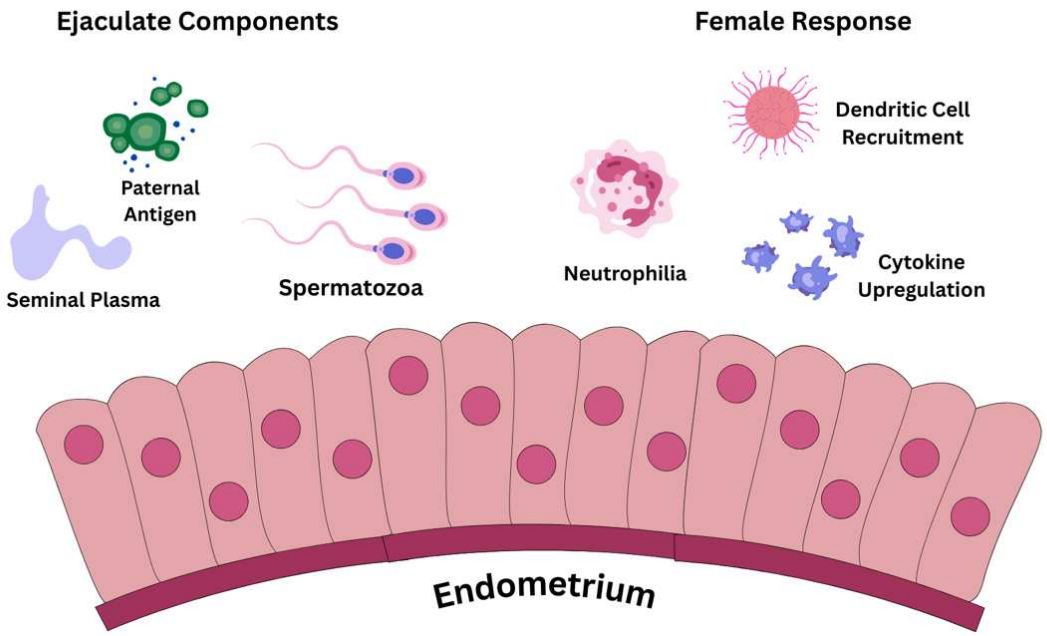
The second category of leukocytes are monocytes, which are the precursor to dendritic cells and macrophages (Heikkinen et al., 2003). Both of these immune cells play a role in antigen

presentation and prompting the adaptive immune response to respond following the initial influx of neutrophilia and cytokine production (Laskarin et al., 2007). The adaptive immune response is highly specific in terms antigen reactivity, in the manner that naïve lymphocytes are programmed only to serve a single function against a specific antigen (Bretscher, 1999). These lymphocytes, once stimulated by an antigen, costimulatory molecules, and cytokines, are able to carry out adaptive immune responses of various phenotypes. B cells, T cells, and natural killer cells (NK cells), initiate these processes associated with immune memory, antibody production, and antigen tolerance (Meng et al., 2023).

1.2.2 Innate Immune Response to Semen

In the equine model, stallions produce a relatively large volume of semen that is deposited into the uterus, directly at the site of the endometrium (Bowen, 1969). Billions of spermatozoa, amongst SP, are released into the uterine lumen where spermatozoa navigate epithelial folds towards both uterine horns. Motile spermatozoa quickly migrate to the oviduct from the uterus, creating a sperm reservoir that is identifiable in the oviduct within 30 minutes to four hours of insemination (Troedsson et al., 1998). Only sperm that make it to the oviduct within this four hour timeframe survive the inflammatory response that floods the uterus (Katila et al., 2000). Once the ejaculate is introduced to the uterine environment, an acute inflammatory response is stimulated from the mare's innate immune system within 30 minutes of exposure (Katila, 2012). A schematic representation of the innate immune response to mating at the maternal endometrium is proposed below in **Figure *1***.

Figure 1. Representation of the female response to components within the male ejaculate at the site of the endometrium.



The immune response to mating begins at the site of the endometrial epithelium, which serves as the first line of defense against foreign invaders. The innate immune response is immediately triggered once foreign antigens are recognized by receptors, recruiting immune cells such as neutrophils and dendritic cells to the site by upregulating the endometrial production of pro-inflammatory cytokines. Excess spermatozoa and fluids are eliminated by these neutrophils throughout the endometrium as part of the innate immune response. Within days, the adaptive branch of the immune system is activated by the dendritic cells that first captured the paternal antigen.

The innate response is stimulated by proteins and antigens found within the ejaculate, specifically introduced by both the sperm and plasma fractions (Troedsson et al., 2005). Endometrial epithelial cells synthesize pro-inflammatory cytokines and chemokines initiating chemotaxis, the recruitment of peripheral immune cells to the site of inflammation (Skarzynski et al., 2020). Neutrophils, or more specifically, polymorphonuclear neutrophils (PMNs), act as the first line of defense against any foreign antigens introduced to the endometrial environment. Following recruitment by the pro-inflammatory chemokine ligand-8 (CXCL-8), PMNs migrate through the uterine lumen from the stroma through chemotaxis across membranes (Katila, 2018).

These cells serve to bind to excess spermatozoa and debris, engulfing anything deemed foreign through phagocytosis (Troedsson et al., 2001). In response to increased neutrophilia, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) secretion is upregulated, initiating uterine contractions to begin to clear debris, immune cells, spermatozoa, and the resulting fluid production (Troedsson et al., 1998).

Dendritic cells (DCs) also bind foreign antigens during the innate immune response, but they also capture the paternal antigen from the ejaculate and travel to the lymph node to stimulate the formal adaptive immune response within days (Laskarin et al., 2007). This phenomenon has been described extensively in human models, but the elucidation of DCs specifically carrying out this antigen presentation process in the equine model during early pregnancy has yet to be reported. Based on consistent findings in other species, equine researchers hypothesize that DCs similarly play a role in stimulating the adaptive immune response, but additional studies are needed to further validate this. Overall, the pro-inflammatory response to semen deposition that is generated by the maternal immune system is crucial to clearing the uterine environment of debris and potential pathogens, but an anti-inflammatory response is required to resolve this initial pro-inflammatory wave at the site of the uterus.

In the horse model, the acute inflammatory response is transient and within 6 hours the recruitment of anti-inflammatory cytokines IL-10 and IL-1 receptor antagonist are initiated, serving to control the previous inflammatory recruitment of cytokines and neutrophils (Woodward et al., 2013). Anti-inflammatory cytokines assist with the mechanical removal of PMNs, enhanced production of prostaglandins, and removal of luminal fluid (Schjenken & Robertson, 2014). Macrophages and lymphocytes are also recruited to the endometrium to clear debris and release cytokines of their own following the initial influx of neutrophils (Skarzynski

et al., 2020). The complement system is also activated by spermatozoa exposure at the site of the endometrium, assisting in the chemotaxis of PMNs into the uterine lumen (Troedsson et al., 2001). This robust anti-inflammatory response is crucial to halting the initial response to semen and initiating the clean-up process, depressing the pro-inflammatory response within 6 hours from the time of breeding (Woodward et al., 2013). The clearance of fluid from the maternal reproductive tract is essential for pregnancy success, as the equine embryo re-enters the uterus 6 days after fertilization and is dependent on a hospitable uterine environment (Morris et al., 2020). Mares that are susceptible to the accumulation of uterine fluid are diagnosed with the disorder of persistent breeding-induced endometritis (PBIE), which is characterized by the inability of a mare to clear uterine fluid following the initial inflammatory response to breeding that occurs within the equine uterus (Watson & Thomson, 1996). This imposes an obstacle for breeding operations in efforts to rebreed mares and keep them pregnant, as the success of early pregnancy relies on the proper resolution of endometritis (Fedorka et al., 2016).

Comparatively, studies in mouse models demonstrate a similar pro-inflammatory response to semen upon mating, as macrophages, neutrophils, and dendritic cells are recruited to the endometrium from the circulation in response to pro-inflammatory chemokine upregulation and estrogen treatment (Robertson et al., 2018). Within one day of mating, the pro-inflammatory cytokines IL-1α, IL-1β, and tumor necrosis factor alpha (TNFα) are increased; after this timepoint, these cytokines decreased, demonstrating that the pro-inflammatory response resolves quickly (McMaster et al., 1992). This shift from a pro-inflammatory profile to an anti-inflammatory one is supported hormonally, as progesterone surges after ovulation as a direct result of luteal function (Robertson et al., 1994). De et al. found similar findings, noting that numbers of PMNs were increased on days 1 and 2 post-mating, but numbers of PMNs were no

longer significant in the murine uterus by day 3 (De et al., 1991). In mice, DCs are present in the pregnant uterus within by day 1.5 and remain elevated until day 5.5 of gestation, indicative of uterine recruitment at the time of mating and suggestive of a role in stimulating the adaptive immune response within days (Blois et al., 2004). This study specifically aimed to validate research in human models that identified DCs as the primary antigen presenting cell in stimulating naïve T cells and instructing the antigen-specific response.

In the human model, intercourse, and thus the innate immune response to semen, has been shown to induce a similar pro-inflammatory response in terms of cytokine expression. A clinical study enrolled women who underwent regular cervical biopsies into three groups to observe the immunological changes that occur when women had penetrative intercourse with and without condoms, in addition to a group that abstained. At the 12 hour time post-coitus, pro-inflammatory cytokines CXCL8 and IL-1α were significantly increased in the group without condoms in comparison to both couples that abstained and those who used condoms upon intercourse (Sharkey, 2012a). This study also found that the leukocytes responding to these cytokines were comprised mainly of macrophages and DCs, but the number of PMNs in the uterus were unchanged at the 12-hour timepoint. Earlier studies contradict this finding, as Thompson et al. first described the leukocytic response to coitus as primarily neutrophilic at the site of the cervix (Thompson et al., 1992). This recruitment of PMNs was attributed as a response to the upregulation in inflammatory cytokines expressed by human cervical cells.

In the equine model, the innate immune response to semen is characterized by a prolific pro-inflammatory response lead by PMNs, which is followed by a significant anti-inflammatory cytokine response at the site of the uterus (Woodward & Troedsson, 2015). This robust anti-inflammatory response post-mating is less prevalent in terms of cytokine production in both

mouse and human models, where rather a cessation of inflammatory PMNs and macrophages appears to occur (Robertson et al., 1998). These previously highlighted differences between species relative to the innate response to insemination may impact how adaptive immunity to pregnancy develops between equine, murine, and human models.

1.2.3 Adaptive Immune Response to Semen

The mechanisms of adaptive immunity during pregnancy have been primarily characterized in human and murine models, with an emphasis on defining the functional roles of adaptive immune cells. Thus, comparatively less is known about the adaptive response in the equine model. Regardless of species, the adaptive branch of the immune system operates to develop mechanisms against any given antigen that will prepare and maintain the hosts ability to fend off this antigen in the future (Murphy, 2017). Within the adaptive branch, two types of lymphocytes carry out different functions: B cells carry out humoral immunity through antibody production, while T cells carry out cell-mediated immunity upon antigen stimulation (McNeela & Mills, 2001). T cells are the primary cell within the adaptive response that once presented with a non-pathogenic antigen, will proliferate and differentiate into mature immune cells that are specialized to that antigen (Meng et al., 2023). Semen, comprised of both SP and spermatozoa, introduces paternal antigens to the site of the endometrium. While these antigens are not pathogenic, the expression of major histocompatibility complex (MHC) genes by sperm cells is recognized by maternal APCs in the uterus, kickstarting the adaptive immune response (Robertson et al., 2015). The presence of MHC I/II specifically within the ejaculate has been confirmed in the mouse (Guerin et al., 2011), but this has not yet been confirmed in the horse.

In mouse models, the formal adaptive immune response to semen begins within 48 hours from exposure, as dendritic cells capture the seminal paternal antigens and migrate to the lymph

nodes to stimulate naïve T cell recruitment in the mouse model (Shima et al., 2020). Naïve T cells are derived from the bone marrow that migrate to the thymus for positive and negative selection (Takahama, 2006). Then, T cell precursors become either 'helper' (*CD4+*) or 'killer' (*CD8+*) cells depending on antigen presentation (Iezzi et al., 1998). The subsequent proliferation into specific T cell subsets is accompanied by the secretions of cytokines, which determine further differentiation into effector T cells that can carry out more specific functions that impact the rest of the immune system (Curtsinger & Mescher, 2010). Specifically, CD4+ T cells can be further differentiated into the following subsets: Th1, Th2, Th17, and Tregs (Zhu & Paul, 2010). In the presence of IL-12, naïve T cells are skewed towards a Th1 type immunity, which is carried out by the pro-inflammatory cytokine IFN-γ (Robinson & O'Garra, 2002). When anti-inflammatory cytokines IL-4 and IL-10 are dominant, a Th2 response is initiated; this T helper phenotype further promote anti-inflammatory cytokines and plays a role in promoting B cell development (Zhu, 2010). The predominance of IL-6 and TGF-β at the time of dendritic antigen presentation stimulates differentiation towards a Th17 phenotype (Ivanov et al., 2007). Like Th1 cells, Th17 cells are pro-inflammatory in nature but this is modulated through IL-17 production. Finally, the prevalence of TGF-β alone prompts naïve T cells into Tregs, the immunosuppressive T helper phenotype (Eric et al., 2018). While both Th1 and Th2 cells have singular effector functions, Th17 cells and Tregs have been found to demonstrate plasticity, or the ability to alter phenotypes into other effector cell types (Lee et al., 2009).

In terms of the pregnancy-specific effector T cell response, Weggman *et al.* first theorized that the human conceptus downregulates potentially harmful pro-inflammatory actions by promoting a Th2 type response (Wegmann et al., 1993). This was supported by research conducted in both human and mouse models, where Th1 phenotypes were associated with

pregnancy failure (Raghupathy, 1997). More specifically, the introduction of the Th-1 associated cytokine, IFN-γ in abortion-prone mouse models increased rates of fetal loss (Chaouat et al., 1990). Similar findings were established in the human model, as Th2 cells predominated over Th1 cells during early pregnancy at the site of the decidua (Miyazaki et al., 2003). Aluvihare *et al.* put forth a novel hypothesis, proposing that rather than a predominant Th2 response to pregnancy, Treg populations play a larger role in carrying out the long-term immunotolerance towards the pregnancy in the mouse model (Aluvihare et al., 2004). Following studies confirmed Treg dominance during murine pregnancy (Polanczyk et al., 2005), and even suggested a potential impact of seminal plasma in modulating a prolific Treg response (Guerin et al., 2011). In human models, researchers investigated how a balance between each effector cell type may be required to sustain pregnancy (Saito, 2010).

Studies in the equine model are far more limited, with most simply identifying primary immune cell populations rather than elucidating specific effector phenotypes. Watson and Dixon first characterized populations of T lymphocytes in the endometrium of the cycling mare in 1993, in which numbers of lymphocytes did not change significantly between estrus and diestrus (Watson & Dixon, 1993). A later study further investigated specific endometrial T cell subsets in cycling mares and reported both CD4+ and CD8+ T cells present throughout various uterine sections, and similarly reported no difference between cycling groups (Watson & Thomson, 1996). These studies demonstrate a consistent lymphocytic presence of both CD4+ and CD8+ T cells at the site of the endometrium in cycling mares, indicating that these cell populations play a role in maintaining the uterine immune environment during cyclicity. In terms of studies that specifically studied T cell responses following breeding and early pregnancy, Lawler *et al.* (1999) explored lymphocyte expression specifically at the site of the corpus luteum (CL) during

diestrus and early pregnancy and found a significant decrease in the number of CD4+ cells from the CL between late diestrus and the progression to early pregnancy (Lawler et al., 1999). Another study examined the endometrial expression of T lymphocytes immediately after AI, in which both CD4+ (T helper) and CD8+ (cytotoxic T) cells were identified at the level of the endometrial epithelium in the body of the uterus post-breeding (Tunón et al., 2000). Researchers reported a significant increase in the number of *CD4+* cells in the uterine body both 6 hours and 48 hours following insemination, indicative of an influx of helper T cell populations in response to seminal deposition. However, this marker is expressed in all helper T cells and by itself does not further characterize the balance of T helper phenotypes.

Other adaptive immune cells have been shown to play a role in early pregnancy in mouse and human models, and this includes monocytes, including natural killer (NK) cells and macrophages, within the uterine microenvironment (Kayisli et al., 2002). NK cells specific to the uterus (uNK) proliferate during early pregnancy, and this has been demonstrated by day 10 of human gestation. These cells serve to carry out the circulatory changes necessary to support early placentation (Cartwright et al., 2017). UNK cells are antigen presenting cells (APCs) that are involved in capturing the newly introduced antigens at the time of implantation, in which the embryo first makes direct contact with the maternal blood flow and influences peripheral immune responses (Laskarin et al., 2007). Additionally, uNKs are able to signal with dendritic cells, which ultimately capture the paternal antigen and stimulate the formal adaptive immune response through the differentiation of T cells specific to the embryo (Meng et al., 2023). In the human, other APCs are recruited to the endometrium, including decidual macrophages which play a vital role in establishing a tolerant immune environment for the embryo (Hunt & Robertson, 1996). Specifically, this population of macrophages are immunosuppressive in action,

producing the anti-inflammatory cytokine IL-10 in response to the paternal and embryonic antigens (Heikkinen et al., 2003). The establishment of an anti-inflammatory phenotype towards the developing embryo and placental membranes is essential to successful placentation. The leukocytes involved in continued decidualization, in addition to adaptive lymphocytes, go on to modulate the fetal-maternal connection throughout the remainder of the pregnancy (Norwitz et al., 2001).

In the equine model, the role of NKs in pregnancy has been described, but only once endometrial cups form around day 34 of pregnancy (Noronha et al., 2012). The relationship between NK cells, DCs, and Treg stimulation was proposed by Antczak *et al.,* suggesting that previous findings in human and mouse models may be applicable in the horse (Antczak, 2012). A more recent study portrayed the presence of both NKs and Tregs with flow cytometry in cycling non-pregnant mares, establishing that Tregs were increased in diestrus and decreased in estrus, but NK cell populations did not differ (Jaworska et al., 2022). Additional studies are needed to elucidate how these cell populations change over time during early pregnancy in the horse.

1.3 MATERNAL TOLERANCE TO PREGNANCY

The successful establishment of pregnancy dependents on maternal immune tolerance towards the embryo, a process that begins once the paternal antigen is introduced into the uterus by way of ejaculation (Robertson et al., 2015). Because paternal antigens express major histocompatibility complex (MHC) genes that are considered foreign to the maternal immune system, downregulation of these genes is required to prevent the immune system from destroying the developing fetus (Aluvihare et al., 2004). Upon embryonic implantation into maternal tissues, the embryo, further expressing both paternal and fetal antigens into maternal circulation, must avoid immunological rejection as fetal antigen exposure increases (Seavey & Mosmann, 2008). In humans and mice, implantation occurs quickly after fertilization, generating an increased immune response to paternal antigens alongside the development of the fetal-placental unit (Norwitz et al., 2001); this coincides with the onset of Treg dominance during the first trimester (Robertson et al., 2018). The most recent research suggests that Tregs modulate this ongoing feto-maternal tolerance as pregnancy progresses, through the regulation of other effector populations and the mediation of cross communication between monocytes like DCs, NKs, and macrophages (Cheng et al., 2022).

1.3.1 The Original Hypothesis: The Th1/Th2 Ratio

As previously mentioned, the adaptive immune response to pregnancy was first classified primarily by an anti-inflammatory Th2 response, dampening potential inflammatory Th1 mechanisms (Wegmann et al., 1993). This was affirmed in human studies, where Th2 cells were elevated during normal pregnancy (Miyazaki et al., 2003), whereas Th1 predomination resulted in early pregnancy failure (Raghupathy, 1997). In another study, pre-term labor was associated

with an altered Th1/Th2 ratio that favored a Th1 response through upregulated peripheral *IFN-γ* expression (Sykes et al., 2012). As advancements in immunological research continued and the role of the more-recently discovered plastic Th17 and Treg cells were established, researchers considered how all four effector populations changed throughout pregnancy in various models. Saito *et al.* introduced the paradigm between all of these phenotypes, proposing that a fine-tuned balancing act may more-accurately describe the temporal changes in T lymphocytes during gestation (Saito, 2010). Tregs suppress other T helper responses through the production of IL-10 and TGF-β to maintain a tolerant immune response towards both the human and murine fetus (Robertson et al., 2015). This has not been fully explored in the equine model.

1.3.2 The Role of Tregs in Pregnancy Maintenance

Tregs suppress immune responses from other immune cells by inhibiting potential pro-inflammatory pathways through the upregulation of the cytokine TGF-β (Chen et al., 2003). Tregs can be identified by various T cell markers, including CD4, FoxP3, CD25, and CD39. Cells that are positive for $CD3^+$ $CD4^+$FoxP3+ but negative for $CD8^-$ can then be identified as Treg populations. CD25 is the T cell surface marker that characterizes activated CD4+ T cells, specifically playing a role in maintaining self-tolerance and preventing autoimmune disorders (Sakaguchi et al., 1995). The CD25 marker is expressed upon the IL-2 receptor alpha (IL-2Ra), resulting in the names for this marker being used interchangeably to describe expression. Aluvihare et al. demonstrated that the successful fetal-maternal tolerance was reliant on the proliferation of CD25+ Tregs populations (Aluvihare et al., 2004).

The *FoxP3* gene is specifically expressed in Tregs inducing immune suppression of other T effector cells, and has been recognized as the primary transcription factor that is required for the successful differentiation of the naïve T cell into a regulatory T cell (Fontenot et al., 2003).

The *FoxP3* gene was first discovered by Brunkow *et al.* in 2001, which was found to be defective in scurfy mice (Brunkow et al., 2001). Shortly after, the same group determined that X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome (IPEX) in humans is similarly linked to *FoxP3* mutations through genetic sequencing (Wildin et al., 2001). These findings initiated research into elucidating the genetic mechanisms of the *FoxP3* transcription factor; Hori, Nomura, and Sakaguchi first confirmed that the *FoxP3* gene is crucial to the development of CD4+ regulatory T-cells (Tregs) (Hori et al., 2003). Interestingly, Tregs have been identified as the primary T helper cell subset involved in various cancers, including: gastric (Mizukami et al., 2008), colorectal (Saito et al., 2016), and breast (Plitas et al., 2016).

A recent study compared the mechanism of immuno-tolerance to pregnancy to that of solid tumor development, and found the function of Tregs to be similar (Wienke et al., 2020). The precursor to Treg cells, TGF-β, is a crucial cytokine for this suppressive action. Tumor cells recruit Tregs to protect them from the body's immune system by suppressing other immune cells, which is carried out by cytokines IL-10 and TGF-β to suppress other effector T cells from initiating a pro-inflammatory response to the foreign antigens of the tumor cells. The immunosuppressive action of tumor Tregs is similar to the function of uterine Tregs, which allows for the suppression of other immune cells, providing tolerance towards the embryo and preventing a pro-inflammatory environment. In human research, TGF-β has been identified within the seminal fluid of the ejaculate, suggestive that this may play a role in stimulating uterine Tregs (Ibrahim et al., 2019).

Extensive research has been conducted in the mouse model relative to how the pregnancy is supported immunologically, with a focus on the impact of antigen exposure at the time of mating. As paternal antigen expression exposure increased in mouse pregnancy, an increase in

the Treg proliferation was noted (Ruocco et al., 2014). Under the influence of progesterone and various immunosuppressive cytokines, murine naïve T cells are differentiated into a regulatory phenotype that have been assigned to the newly presented paternal antigen (Tilburgs et al., 2008). Treg cells are then released to the periphery before recruitment to the endometrium to carry out cell-mediated immunity. In another murine study, Teles *et al.* (2013) investigated the origins of FoxP3+ Tregs during early murine pregnancy by isolating immune cells from the thymus and uterine lymph nodes and noted the expansion of thymic Tregs as pregnancy progressed until day 10 (Teles et al., 2013). In this study, a significant expansion of peripheral Tregs both at day 2 and until day 5 before declining by day 10. This rise was attributed to both the already-present expansion of Tregs at the lymph nodes by day 2 and embryonic implantation occurring by day 5.

Over the past decade, research in both human and mouse models indicates that Treg cells play a role in modulating this maternal immunotolerance to newly introduced paternal antigens, but few studies in the horse have elucidated this. Recently, a study in horses aimed to establish the Treg profile during pregnancy, noting that the proportion of specific lymphocyte populations varied relative to gestational length. When mid to late gestation was assessed, researchers saw increased Treg-related transcripts from 120-300 days of gestation before declining prepartum (Fedorka et al., 2020), similarly to what has been noted in mouse models relative to late pregnancy. This dynamic shift in Treg dominance over other T helper cell populations throughout gestation reiterates the importance of the timing of upregulated Treg expression and the maintenance of pregnancy. This study focused on mid-late gestation but the establishment of the normal Treg response during early pregnancy, immediately following breeding, has yet to be identified.

1.3.3 Consequences of Impaired Treg Proliferation

Interestingly, two studies have evaluated how Tregs are associated with pregnancy failure in the horse. When assessing Treg transcripts during early pregnancy, mares that continued with successful pregnancies had a significantly increased Treg response in comparison to that of those mares who underwent early pregnancy loss (Aurich et al., 2014). Researchers reported that CD4+CD25+FoxP3+ populations were elevated specifically in the pre-breeding cells in comparison to mares that underwent early embryonic loss, and this difference in Treg populations persisted until days 14, 20, and 40 of gestation. In pregnant mares that were inoculated with *S. zooepidemicus* to induce placentitis during late pregnancy, the Treg response was diminished in comparison to noninfected controls (Fedorka et al., 2021). The impact of disease challenge on Treg expression could indicate that dysregulation of Treg responses as detrimental to subsequent pregnancy success.

In mouse models, researchers linked abnormal Treg development with skewed antigen presentation and spontaneous abortion. The use of abortion-prone CBA/J mouse models allows researchers to compare pregnancy outcomes to that of normal pregnant mice, and a study by Zenclussen *et al.* (2005) found that Tregs were significantly elevated during normal pregnancy but were depleted in abortion mice by day 14 (Zenclussen et al., 2005). A different case-controlled study mated BALB/c mice to investigate if the depletion of Treg cells using treatment with anti-CD25 monoclonal antibodies impacted implantation rates. When administered 2.5 days post-coitus, implantation failure was induced in pregnant mice, but pregnancy status was not impacted when Treg depletion was induced at 4.5 and 7.5 days post-coitus (Shima et al., 2010). These results indicate the importance of Treg dominance specifically during the pre-implantation period of embryonic development. Additionally, the transfer of pregnancy-induced Tregs from

phenotypically normal mice reverses abortion rates in abortion-prone mouse models, suggestive of a potential therapeutic treatment upon further elucidation (Wang et al., 2014).

A study demonstrated that Tregs were significantly downregulated in women with miscarriages in comparison to those with successful pregnancy outcomes, suggesting that the establishment of this lymphocyte population is crucial to maintaining early pregnancy (Winger & Reed, 2011). Levels were assessed prior to coitus and around the end of the first trimester (38.0 ± 19.7 days). Patients with lower Treg levels (<0.7%) experienced a significantly lower success rate at 12 weeks than those with a higher Treg level. We similarly reported a relationship between Treg expression and pregnancy success, although our data was relative to specific timepoints and noted a difference specifically prior to mating and at d7. Another study compared differences in gene expression between women who became pregnant and underwent early pregnancy loss; specifically, immune cells were isolated from both peripheral and decidual blood samples. *FoxP3* expression was only significantly increased in pregnant women in comparison to non-pregnant women in decidual samples, whereas expression from peripheral circulation did not differ between groups (Dimova et al., 2011). This finding demonstrates an important insight, as Tregs isolated directly from the uterus were enriched ten-fold in comparison to those isolated from the peripheral blood flow. Additional research found a relationship between Treg expression and transfer outcomes in women undergoing ART procedures, where the percentage of naïve $CD45RA^+$ Tregs within the total Treg pool was decreased ($p < 0.05$) in women with unsuccessful outcomes (Schlossberger et al., 2013). More recently, immunofluorescence data shows accumulating evidence that disruption of the *FoxP3* gene is directly related to women who experienced early pregnancy failure and pre-eclampsia (Moldenhauer et al., 2024). The relationship between Treg dysfunction and pre-eclampsia has been well-explored, but the

extensive complexities of this systemic metabolic condition limit future research in this area (Hsu et al., 2012).

1.4 PHYSIOLOGY OF PREGNANCY

Early pregnancy is generally defined as the period from conception to the development of the temporary placental organ, but the timing of placentation varies greatly between species. In mice, the transition from early to mid-gestation occurs by day E9.5, roughly halfway throughout the gestational period. The murine embryo undergoes implantation by day 4.5 and the placental unit is further established and considered functionally developed by day 9.5 (Woods et al., 2018). In humans, the definitive chorioallantoic placenta is present by Day 21 of pregnancy, at which time maternal blood invades the well-developed placental tissues (Malassiné et al., 2003). Unique to the horse, the equine conceptus avoids true implantation with maternal tissues until chorionic girdle cells develop on the outer surface of the embryo before making contact with the endometrium. This process begins around Day 25, in which the glandular tissue undergoes cleavages before sloughing off and allowing underlying trophoblast cells to finally invade the endometrium at approximately Day 36 (Allen & Stewart, 2002). Between species, the relationship between the onset of embryonic events and gestational timeline may impact the maternal immune response. Both human and mice are considered altricial species, with offspring born in an immature state requiring significant postnatal development (Flannery, 1989). Horses, however, are a precocious species, as newborn offspring need to be able to stand within hours of birth in order to evade predation (Rossdale, 1993). Denoting key differences between species is crucial to investigating the immunological events during early pregnancy, as research in varying

models demonstrate that both fetal and paternal antigen exposure increases once placentation initiates the invasion of fetal-derived tissues into the maternal endometrium (Shima et al., 2015).

1.4.1 Equine

The horse is seasonally polyestrous, cycling multiple times during the long days of the year (Senger, 2012). The average gestational timeline is 340 days in the horse, which is relatively long in comparison to other domestic species (Ginther, 1992). Additionally, equine placentation differs from that of human and murine, as it is far less invasive in terms of chorionic attachment to the endometrium (Noronha & Antczak, 2010). Because of this distinct fetal-maternal interface, the equine model offers opportunities to investigate reproductive mechanisms that cannot be easily examined in species with invasive placentation. While studies in other livestock models also demonstrate less invasive models of placentation, the horse is unique in its combination of prolonged a well-characterized post-breeding inflammatory response, pre-implantation mobility, and delayed embryonic attachment (Noronha & Antczak, 2010). These features make the equine system particularly valuable for studying early immune recognition of paternal antigens, as well as the transition from innate inflammation to adaptive immune tolerance within the uterus.

When mares are bred, whether via natural mating or AI, the ejaculate is deposited into the uterine lumen, and motile spermatozoa are able to be transported to the oviduct (Troedsson et al., 1998). At the site of the uterus spermatozoa undergo capacitation, resulting in hyperactive motility action necessary for successful penetration of the oocytes zona pellucida (Barańska & Tischner, 1995). While select spermatozoa remain in the sperm reservoir within the oviduct prior to fertilization, a strong pro-inflammatory response occurs within the uterus in response to semen deposition (Troedsson et al., 2001). Neutrophils and macrophages are recruited from the uterine

stroma to the glandular endometrium in response to the spermatozoa, proteins, and SP within the ejaculate (Troedsson et al., 1998). The quickly recruited pro-inflammatory wave is soon resolved, as an anti-inflammatory response begins within 6 hours of insemination to resolve the resulting fluid from the inflammatory response (Woodward et al., 2013). The clearance of this fluid is imperative to preparing the uterus for the receival of an embryo in the following days.

Once spermatozoa reach the oocyte, they must bind to zona pellucida (ZP) proteins to initiate the acrosome reaction leading to the block to polyspermy, or eliminating potential for other sperm cells to penetrate the zona (Töpfer-Petersen et al., 2000). Following acrosomal initiation, the male and female gametes fuse to create the zygote, which will then undergo cell cleavage while remaining in the oviduct until developing into a blastocyst. The ZP remains as the external layer of the blastocyst, but the embryonic capsule begins to develop between the trophectoderm and the ZP by day 6 (Oriol et al., 1993a). Simultaneously, the blastocyst begins to release prostaglandins, allowing for uterine relaxation and embryonic passage through the utero-tubule junction and into the uterine lumen (Oriol et al., 1993b). Rather than immediately undergoing placentation, the equine embryo remains mobile and migrates across the endometrial surface (Ginther, 1985). By Day 9, the zona pellucida is dissolved and the capsule becomes the primary external layer surrounding the embryo. The glycoprotein capsule plays a vital role in supporting the embryo throughout this period of mobility, mediating between the maternal environment and the embryo (Herrler et al., 2000).

Mobility of the embryo increases around day 10 due to continued embryonic release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) as the embryo migrates across the surface area of both uterine horns (McDowell et al., 1988). Communication between the embryo and the endometrial surface via prostaglandin production is pertinent to initiating the process of maternal recognition of

pregnancy (MRP) in the horse by distributing pro-luteolytic signals around day 12 (Klein et al., 2010). Prostaglandins bind to the endometrial surface, in which hormone concentrations enter the peripheral circulation before traveling back to the corpus luteum to promote prolonged luteal function (Allen & Stewart, 2002). Although the exact mechanisms of MRP in the horse have not been fully established, researchers believe that the embryonic and endometrial production of $PGF_{2\alpha}$ plays a role in this process (Klein & Troedsson, 2011). During this period of embryonic mobility, the embryo expands rapidly in diameter between days 11 and 16 while maintaining its spherical shape (Ginther, 1992). Around day 16 of pregnancy, the embryo ceases mobility and fixates in either of the caudal uterine horns. At this time, the embryo temporarily halts the production of $PGF_{2\alpha}$, but the impact this has on MRP has yet to be fully determined (Klein & Troedsson, 2011). Still, true implantation of the embryo is not yet initiated upon fixation; rather, the embryo settles near a specific spot against the endometrium but does not adhere to it (Enders & Liu, 1991). This has been attributed to the increase in uterine tonicity observed around day 17, assisting in embryonic stability (Allen & Stewart, 2002). The capsule still surrounds the embryo, but the trophoblast cells begin to expand towards the endometrial surface. Additionally, sialic acid is lost from the glycan structures making up the capsule, which is necessary for the successful fixation of the conceptus (Arar et al., 2007). The glycoprotein capsule remains intact until approximately day 21 of pregnancy, as the loss of this membrane allowing for the formal implantation process to occur in the following several days (Enders & Liu, 1991).

Following fixation, the equine embryo undergoes rapid growth (Chavatte-Palmer et al., 2022). At this time, the conceptus begins to develop its circulatory system and is enveloped into the amniotic cavity by day 21 (Betteridge, 2000). Additionally, the early placental membranes begin to form, as the allantoic cavity emerges and is vascularized in preparation for chorionic

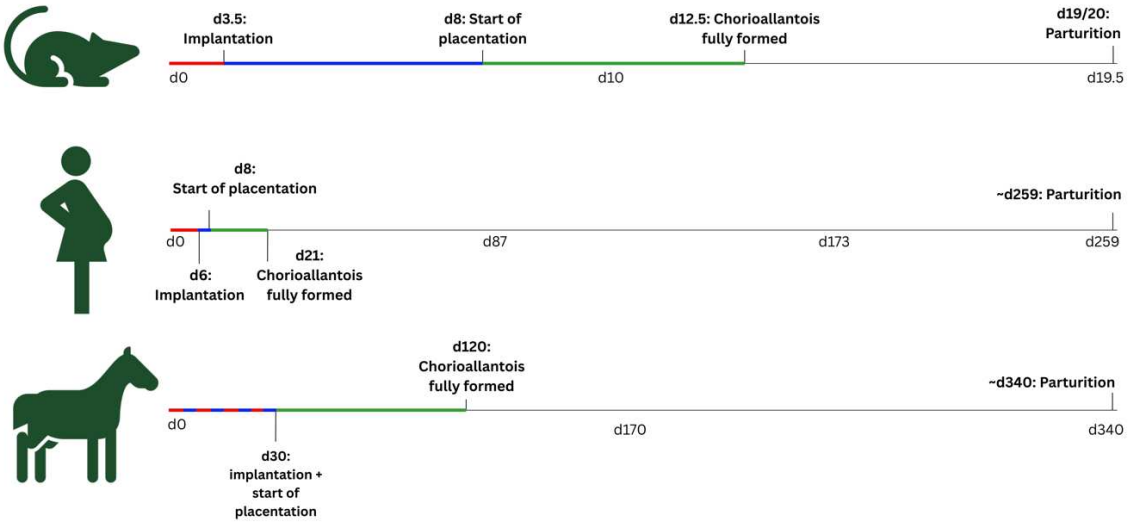
attachment (Ginther, 1985). Clinically, the equine fetal heartbeat can typically be detected by day 24 via ultrasound, a result of the developed fetal circulatory system. In the following days, the allantoic membrane rapidly expands, engulfing the amnionic cavity and the fetus within it (Ginther, 2022). By day 33, the trophoblast cells give rise to the chorionic girdle, a chorionic band surrounding the fetus (Allen & Stewart, 2002). Upon chorionic girdle formation, it detaches from fetal tissues and the trophoblast cells invade the endometrium in select areas, forming endometrial cups (Allen & Moor, 1972). In the horse, the invasion of endometrial cups into select areas of the maternal decidua is the first instance of true fetal implantation; up until this point, the equine embryo remains separate from the maternal tissues in terms of vasculature (Enders & Liu, 1991). Following cup development, equine chorionic gonadatrophin (eCG) can be detected by approximately day 35, indicative of successful placentation (Allen & Stewart, 2002). As the formation of the fetal-placental unit is established, the placental membranes expand and develop attachments to the uterus through microcotyledons that regulate fetal-maternal gas exchange (Ginther, 2022). Several layers of chorion modulate fetal-maternal circulation in the horse, providing a relatively non-invasive connection in comparison to that human or murine models where maternal blood directly contacts the fetal-placental unit (Hemberger et al., 2020).

1.4.2 Murine

The majority of our understanding of early pregnancy physiology stems from mouse models, as this species has paved the way for reproductive research in other species. Both mice and women undergo hemochorial placentation, in which maternal blood is directly in contact with the fetus and placental membranes (Soares et al., 2018). Thus, advancements in human medicine specific to investigating pregnancy outcomes, placental development, and the dynamic

feto-maternal relationship often originate from research within the murine model. In the mouse, gestation averages 20 days in length, and this rapid gestational timeline is ideal for researchers to conduct reproduction studies to investigate complex biochemical aspects of pregnancy with the downstream goal of application in human clinical models (**Figure *2***).

Figure 2. Comparative timeline of murine, human, and equine gestation with an emphasis on implantation and placentation.



The murine embryo quickly undergoes implantation by day 3.5 but placentation begins around day 8, almost halfway through the pregnancy. The chorioallantois, or the full fetal-placental unit, is fully formed by day 12.5. In humans, the gestational length averages 259 days; implantation also occurs relatively quickly, by day 6. But unlike in mouse models, placentation begins soon after implantation by day 8 and the chorioallantois is fully formed by day 21. In the equine model, gestation is longer, averaging 340 days in length. The equine conceptus avoids true implantation with maternal tissues until chorionic girdle cells develop around Day 25. However, the chorioallantois is not fully developed until day 120, roughly 1/3 of the way through gestation.

The mating process in estrogen-primed mice induces a pro-inflammatory response to semen, as macrophages, neutrophils, and dendritic cells are recruited to the endometrium from the peripheral circulation in response to pro-inflammatory chemokine upregulation (Robertson et

al., 2018). Within one day of mating, the number of cells secreting pro-inflammatory cytokines decreases, demonstrating that the anti-inflammatory response quickly suppresses the inflammatory mating response (McMaster et al., 1992). Upon successful fertilization at day 0, the zygote undergoes multiple rounds of cellular divisions until it becomes a morula at day 2.5 post-mating. Subsequently, the blastocyst exits the oviduct and enter the uterus around day 4, followed by implantation of the early embryo by day 4.5 (Hemberger et al., 2020). At this stage, the trophectoderm cells line the outer layer of the embryo, which is the group of cells that specifically carry out embryonic implantation into the endometrial stroma. While murine embryo implantation occurs relatively promptly in terms of number of days post-mating in comparison to species with longer gestations, this occurs almost a quarter of the way through the mouse's gestational period; thus, rapid embryogenesis occurs upon endometrial invasion of the trophectoderm cells of the embryo (Malassiné et al., 2003). The invasive trophectoderm cells are transformed into trophoblast giant cells (TGCs) that remain in contact with the maternal decidua throughout the remainder of the pregnancy (Enders & Welsh, 1993). As fetal trophoblasts initiate contact with the endometrium, lymphocytes are recruited to the maternal decidua to carry out uterine remodeling specifically at the site of embryonic implantation. uNKs secrete interferon gamma (IFN-γ), which is imperative for initiating proper decidual artery remodeling (Ashkar et al., 2000). The successful implantation of the embryo stimulates a process called decidualization, in which the endometrial stroma undergoes a series of cellular, immunological, and vascular changes in preparation for supporting proper placentation; this occurs under the influence of both estrogen and progesterone (Ramathal et al., 2010). Unlike in human and equine models, the mouse does not produce a species-specific hormone that signals the maternal recognition of

pregnancy, but is rather marked by successful endometrial decidualization and embryonic implantation alongside adequate progesterone and prolactin secretion (Dey et al., 2004).

By day 6.5, the embryo undergoes gastrulation and early placental membranes are formed. Specifically, the chorionic ectoderm attaches to the allantois on day 8, triggering chorionic folding to support the developing blood vessels from the allantois (Lu et al., 2011). The fusion of the chorioallantois enables further morphogenesis of the primary chorionic villi, promoting fetal blood vessel expansion into the chorionic folds by day 8.5. This initiates the development of the labyrinth zone of the placenta, the primary site of nutrient and gas exchange that connects the placenta to the umbilical cord (Cross et al., 2003). Additional placental layers are developed at this time between the labyrinth zone and the maternal decidua, in which this region is summarized as the junction zone. This consists of two distinct layers of syncytiotrophoblast cells, from the differentiation of chorionic trophoblast cells. Specifically, these layers are covered by the spongiotrophoblast layer which separates from the labyrinth zone (Woods et al., 2018). By day 10.5, the fetal-placental unit has been established with all placental layers complete structurally. During the final half of gestation, the labyrinth zone undergoes expansion, spiral arteries grow in diameter, and an intricate circulatory relationship is established between the maternal and fetal tissues through the placental layers (Adamson et al., 2002). Simultaneously, the murine fetus continues to grow exponentially and develop within the amnionic cavity.

1.4.3 Human

Human medicine largely differs from animal research primarily due to the ethical standards, regulations, and patient-rights protections that regulate studies involving human subjects (Rugg-Gunn et al., 2023). Thus, advancements in human medicine specific to

investigating the dynamic fetal-maternal relationship often originate from findings observed in murine models. The majority of women become pregnant outside of clinical research settings, which makes understanding mating, conception, and early embryology difficult. Upon a woman's own suspicion of pregnancy or preliminary detection of human chorionic gonadotropin (hCG), clinical reproductive care is often sought, whether that be through pre-natal care or elective pregnancy termination. In a risk assessment survey conducted in 2022, 85% of respondents were certain of their pregnancy status by 8 weeks of gestation (Krukowski et al., 2022). Thus, most clinical research studies on coitus and early pregnancy are carried out in women who are prone to miscarriages or undergoing in-vitro fertilization procedures rather than those undergoing pregnancy via natural conception. Numerous systemic reviews have demonstrated that reproductive success outcomes via IVF are significantly influenced by socioeconomic disparities, including factors such as income, education, and race (Smith et al., 2011); women that have difficulty achieving and maintaining pregnancy have various assisted reproductive technologies to choose from, bearing financial restraints (Peterson et al., 2025) and legal restrictions by state (Rugg-Gunn et al., 2023). As scientific advances have been made and ARTs become more clinically accessibly, these treatments have become more popular in the United States in recent years; between the years 2013 to 2022, the CDC reports that the number of ART cycles undergone by women yearly increased by 128% (CDC, 2024). Even so, the percentage of ART-derived pregnancies resulting in live births is low, at only 37.5% success, as of 2022.

Human pregnancy is typically divided into trimesters, with an average gestational period of 280 days, or 40 weeks in length. The site of semen deposition in humans is the anterior vagina, in which the ejaculate migrates towards the cervix where spermatozoa navigate through

cervical mucus and folds to access the uterus (Suarez & Pacey, 2006). Similar to other species, the human ejaculate induces an acute pro-inflammatory response within the female reproductive tract, but this occurs specifically at the site of the cervix rather than at the endometrium like in mouse and horse models. A recent *in-vitro* study demonstrated that when cervical epithelial cells were stimulated with semen, the secretion of the cytokines IL-6 and CXCL8 were significantly elevated within 8 hours of administration, indicative of a pro-inflammatory wave induced by the ejaculate (Rametse et al., 2018). Fertilization occurs in the ampulla of the oviduct, upon the fusion of a motile spermatozoan and oocyte, giving rise to the zygote (Kalousek et al., 2013). By day 3 post fertilization, the zygote undergoes mitotic cell divisions becoming a morula just prior to entering the uterine cavity. The pre-implantation embryo is influenced by various growth factors and cytokines as it travels through the oviductal environment and into the uterus, influencing embryonic cleavage and development (Suarez & Pacey, 2006). The structure fills with fluid, forming the blastocyst by day 4 that is composed of the outer trophectoderm layer surrounded by the zona pellucida containing the inner cell mass (ICM). The trophoblast cells will then differentiate over the following weeks to become the early placental membranes that connect with the maternal endometrium, whereas the ICM gives rise to the embryo proper that later develops into the fetus (Schoenwolf et al., 2014). Upon blastocyst formation, the zona layer sheds as the embryo settles in the body of the uterus and initiates the process of embryo implantation around day 6 (Schiewe et al., 1995). At this time, trophoblast cells lining the embryo begin to invade the endometrial decidua, establishing a primary connection between the embryo and the maternal interface (Norwitz et al., 2001).

Successful implantation of the early embryo requires proper decidualization, a process marked by significant biochemical and hormonal changes within the uterine environment

(Ochoa-Bernal & Fazleabas, 2020). Decidualization regularly occurs as part of the menstrual cycle in humans, in which the glandular endometrium undergoes stromal enlargement and increased edema during the mid-luteal phase, regardless of prior mating or conception (Noyes et al., 1950). Johannison *et al.* demonstrated that this occurs in response to the progesterone surge that occurs post-ovulation in regularly cycling women, establishing that decidualization occurs independent of embryo implantation (Johannisson et al., 1987). This finding confirmed a crucial difference in the endometrial phenotype prior to conception between human and mouse models, as decidualization is only triggered upon the completion of implantation in the murine model (Enders & Welsh, 1993). Upon adequate decidualization of the human endometrium, implantation of the embryo introduces invasive trophoblast cells and further stimulates remodeling of the uterine stroma and spiral arterioles in preparation for supporting placentation (Craven et al., 1998). Specifically, the trophoblast layer rapidly differentiates to form the villous syncytiotrophoblast cells, which initially penetrate the uterine lining and stimulates the production of human chorionic gonadotrophin (hCG) (Pierce & Midgley, 1963). The circulatory levels of hCG are indicative of maternal recognition of pregnancy in the human model. Cytotrophoblast cells also originate from this primitive trophectoderm layer but serve to later carry out further interstitial and endovascular invasion to give rise to the extravillous connections that bridge the maternal interface to the villous placental membranes (Handwerger, 2010).

Embryonic implantation is completed by day 9, in which leukocyte recruitment can be observed in response to the introduction of fetal tissue to maternal circulation and the generation of early placental membranes (Pijnenborg et al., 1981). Around day 10, the maternal decidua is characterized by the upregulation of uNK cells that carry out the circulatory changes necessary to support early placentation (Cartwright et al., 2017). Other APCs, including decidual

macrophages, play a vital role in establishing a tolerant immune environment for the embryo upon the initiation of placentation (Hunt & Robertson, 1996). Specifically this population of macrophages is immunosuppressive in action, producing the anti-inflammatory cytokine IL-10 in response to the paternal and embryonic antigens (Heikkinen et al., 2003). The establishment of an anti-inflammatory phenotype towards the developing embryo and placental membranes is essential to successful placentation; the leukocytes involved in continued decidualization, in addition to adaptive lymphocytes, go on to modulate the fetal-maternal connection throughout the remainder of the pregnancy (Norwitz et al., 2001).

1.5 SEMINAL PLASMA AS AN IMMUNOREGULATORY SIGNAL

Seminal plasma (SP) is the fluid portion of the ejaculate, made up of secretions from the rete testes, caudal epididymides, and the accessory sex glands for the primary purpose of assisting in sperm transport (Senger, 2012). While some SP is secreted alongside the development and storage of spermatozoan within the tail of the epididymis, the majority of the seminal volume is derived from the accessory sex glands of the male reproductive tract (Weber & Woods, 1993). In the stallion, the accessory sex glands include the ampulla, seminal vesicle, prostate, and bulbourethral glands, from which the fluidic secretions are stored prior to ejaculation. Although most of the volumetric SP fraction is made up of water, SP contains a variety of biologic components that are amongst spermatozoa at the time of ejaculation, including proteins, hormones, prostaglandins, cytokines, and sugars (Amann et al., 1987).

1.5.1 Composition and Biological Role of SP

Historically, scientists believed the primary function of SP lay in its role as a transportation medium for spermatozoa within the female tract (Loomis, 2001). Advancements

in proteomic analysis in other species indicated that elements of SP play a role in modulating metabolic and immunologic interactions at the site of the uterine epithelium, rather than strictly by interacting with spermatozoa (Magistrini, 2000). The theory that SP may be modulating the expression of cytokines at the maternal environment has been further investigated in many species, including in the human, murine, and porcine models (Robertson, 2007). Initial studies in the porcine model demonstrated that the addition of SP to the endometrium regulated the leukocytic immune response, follicular development, and influenced cytokine and progesterone production (O'Leary et al., 2004). Bromfield *et al.* (2014) conducted a study in the murine model by comparing outcomes in females bred with intact males and to those with seminal vesicle excision (SVX) to assess the impact of SP exposure on the phenotypic and metabolic outcomes of offspring. Early pregnancy rates were significantly reduced in the SVX group in comparison to the males with intact seminal vesicles, indicative that SP may play an integral role in modulating pregnancy acceptance and early embryonic development (Bromfield et al., 2014).

Extensive research has been conducted in the area of characterizing the immediate leukocytic response to insemination in the horse model, which is related to the need for clinical solutions for treating breeding-induced endometritis in academic and commercial breeding operations (Woodward & Troedsson, 2015). Before fertilization can occur, spermatozoa must avoid phagocytosis by maternal immune cells that respond to the newly introduced foreign antigens within the mare's uterus. A majority of the ejaculate will be engulfed by PMNs, reducing the number of spermatozoa in the mare's reproductive tract significantly within the first few hours of breeding (Troedsson et al., 1998). Doty *et al.* (2011) found that when sperm were exposed to SP, they demonstrated differing binding rates to PMNs in comparison to limited or no SP exposure; this introduced the idea that elements within SP could be modulating sperm

survivability through interactions at the level of the endometrium (Doty, 2011). In addition to the impact that SP has on sperm binding to neutrophils in the female tract, the role of SP in regulating early pregnancy and embryonic development has been demonstrated in various species. Following insemination with either raw semen or centrifuged semen in the horse, Fedorka *et al.* (2024) found that 158 genes were differentially expressed in the group with reduced SP exposure. These differentially expressed genes were relative to immune cell signaling, antigen presentation, and embryo growth and development, which is indicative that SP plays a role in the presentation of paternal antigen to the maternal immune system following insemination (Fedorka et al., 2024). While the impact of SP on the innate immune response to spermatozoa has been well-described in the equine model, the long-term relationship between SP exposure and the resulting adaptive immune response is under-investigated.

1.5.1.1 Proteins

SP proteins play various roles in interactions with spermatozoan, and not only in assistance with modulation through the female reproductive tract. These molecules within the SP interact with both the spermatozoa, as well as the maternal uterine epithelium on a cellular level to assist in the transportation towards the oviduct (Rodríguez-Martínez, 2011). Amann *et al.* (1987) first formally analyzed SP proteins in the stallion, identifying a total of 27 proteins through gel electrophoresis. Later studies further characterized 14 groups of proteins that were common between stallions (Frazer & Bucci, 1996). The proteins found within stallion SP can be most simply categorized into three groups: the fibronectin type II molecules (FN-2 type proteins), cysteine-rich secretory proteins (CRISPs), and spermadhesin molecules (Töpfer-Petersen et al., 2005). The FN-2 type proteins are species-specific; in the bull, these proteins are called bovine heparin binding proteins (BSP), long known to play a role in sperm capacitation.

Horse SP proteins (HSP-1 and HSP-2) are also known as SP-1 and SP-2, as they are the most abundant FN-2 type proteins identified in the horse. Calvete *et al.* (1995) first reported the amino acid sequences of HSP-1 and HSP-2 from SP in 1995 through affinity chromatography (Calvete et al., 1995). HSP-1/2 bind to choline phospholipids of the sperm membrane, removing cholesterol and subsequently initiating the acrosome reaction, which was first demonstrated with bovine SP proteins (Thérien et al., 1995). The efflux of phospholipids and cholesterol is imperative to triggering sperm capacitation. In more recent years, Kumar *et al.* demonstrated that HSP-1 and HSP-2 exhibit chaperone-like activity, protecting spermatozoa from oxidative stress and thermal or chemical damage (Kumar et al., 2018). Both HSP-1 and HSP-2 bind to sperm membranes after ejaculation to assist in processes critical to sperm capacitation and survival during the journey through the female reproductive tract.

The third equine SP protein was initially named HSP-3, but it was later described to additionally be a member of the CRISP protein family; thus, it is more commonly known as equine CRISP-3 in the horse (Magdaleno et al., 1997). The CRISP-3 gene was first linked to fertility success in the horse by Hamann *et al.*, as genotyping revealed a polymorphism amongst Hanoverian stallions that was associated with lower fertility outcomes in comparison to stallions without the polymorphism (Hamann et al., 2007). In a study investigating *in vivo* biomarkers of fertility from stallion SP, Novak *et al.* found a correlation between increased amounts of equine CRISP-3 in the ejaculate with increased first-cycle conception rates upon breeding (Novak et al., 2010). Both studies demonstrated a need for further research in elucidating potential mechanisms in which CRISP-3 impacts fertility parameters. Fedorka *et al.* further characterized CRISP-3 expression in both the pre- and post-pubertal stallion, finding most originated from the ampulla of the vas deferens and remaining from the seminal vesicle, concurrent with previous literature

(Fedorka et al., 2017). An *in vitro* study supplemented equine CRISP-3 to PMNs and live spermatozoa, resulting in decreased binding between PMNs and spermatozoa, demonstrating protective characteristics for spermatozoa against neutrophilia (Doty, 2011). Sperm bound with CRISP-3 are protected from phagocytotic leukocytes, but the mechanism in which this is carried out has yet to be identified. Most recently, Doty *et al.* described the selective interaction of CRISP-3 with sperm and neutrophils based on sperm viability (Doty et al., 2024). Researchers reported that live sperm were protected from binding to PMNs, but this did not impact the necessary binding to dead sperm to clear the post-breeding inflammatory response to semen.

Another protein found within stallion SP is lactoferrin, which does not fall into any of the previously mentioned categories. Similarly to CRISP-3, lactoferrin modulates how PMNs bind to spermatozoa; as lactoferrin-bound spermatozoa are tagged to undergo phagocytosis or apoptosis by way of the mare's innate immune response (Troedsson et al., 2014). Although the exact manner in which this occurs in the stallion has not been identified, the use of this protein as an anti-inflammatory agent as treatment for mares with persistent breeding-induced endometritis (PBIE) has been studied. Fedorka *et al*. published the safety of the infusion of exogenous human recombinant lactoferrin at 6 h post-breeding for the clinical treatment of prolonged inflammation by way of PBIE (Fedorka et al., 2018).

5.1.1.2 Cytokines

While many cytokines exist within the SP fraction, the most well investigated in terms of modulating immunotolerance in human models is transforming growth factor-beta (TGF-β). This cytokine is an immune signaling molecule that was originally described as 'sarcoma growth factor' in the observed role in transforming murine fibroblasts (De Larco & Todaro, 1978). The first human amino acid of TGF-β was identified by Derynck *et al.* through protein sequencing,

revealing the isoform TGF-β1 (Derynck et al., 1985). Within years, two additional isotypes of TGF-β in mammals were reported: TGF-β2 and TGF-β3. Since the discovery of TGF-β, potential functions of this cytokine in human medicine and animal research models have been extensively studied, revealing numerous roles in fibroblast differentiation in mice (Ignotz & Massagué, 1985), tumor suppression in mice (Pierce et al., 1995), and spermatogenesis in humans (Dobashi et al., 2002).

Active TGF-β ligands bind to the TGF-β transmembrane receptor types I and II, which are serine/threonine kinases. The phosphorylation of the type II kinases initiates the subsequent transphosphorylation of the type I kinases, resulting in the phosphorylation of Smad proteins. Receptor-activated Smads (R-Smads) translocate to the nucleus, regulating gene transcription (Derynck & Zhang, 2003). This TGF-β/Smad cytokine signaling pathway is unique to the TGF-β superfamily, which also includes groups such as bone morphogenic proteins (BMPs), activins, and inhibins. The TGF-β signaling cascade differs from other pathways of cytokine regulation, primarily in the novel immunosuppressive mechanism of action of TGF-β (Chen & Wahl, 2003). TGF-β can also modulate these other pathways through R-Smad activation, including JAK/STAT, NF-κB, and MAPK/ERK (Derynck & Zhang, 2003). Since the discovery of the TGF-β signaling pathway, researchers have investigated how TGF-β plays a role in modulating a multitude of physiological mechanisms, including embryogenesis, wound healing, and other immune processes (Deng et al., 2024).

In the context of reproduction, seminal-derived TGF-β has been extensively studied in murine models, establishing that this cytokine that is found within the ejaculate may serve to alter the immune response to semen (Robertson et al., 2002). Once at the site of the uterus, TGF-β stimulates the endometrium, resulting in a cascade of responses within the female's

reproductive tract and subsequent immune system (Balandya et al., 2012). This cytokine plays a direct role in recruiting dendritic cells to the site of inflammation to begin the formal antigen presentation process (McGrath et al., 2009). In the mouse model, Tremellen *et al.* (1998) demonstrated that TGF-β from murine SP stimulates the production of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the mouse uterus, which is crucial in uterine remodeling prior to successful embryo implantation (Tremellen et al., 1998). When GM-CSF was knocked out in mice, the expression of uterine dendritic cells were reduced and antigen presentation through MHC I/II expression was altered (Moldenhauer et al., 2010). This finding shows that GM-CSF is crucial to establishing pregnancy through antigen presentation in the mouse. Recently, *in vitro* studies found the addition of SP to maternal T cells in mice resulted in an enhanced regulatory phenotype of effector T cells (du Fosse et al., 2022), consistent with the hypothesis that Treg cell expansion is required for the successful establishment of pregnancy (Aluvihare et al., 2004).

In the human model, Sharkey *et al.* evaluated human SP and found that all three isoforms of TGF-β had high concentrations (TGF-β1, TGF-β2, and TGF-β3) within all ejaculates assessed from twenty fertile men (Sharkey et al., 2012). This group then collected female ectocervical epithelial cells to culture and treat with recombinant human TGF-β1, TGF-β2, and TGF-β3 and determined that exposure to TGF-β significantly impacted the cervical epithelial production of IL-6 and GM-CSF. Impacting the acute inflammatory response to spermatozoa, SP exposure within the uterine epithelium stimulates the production of GM-CSF and IL-6 (Tremellen et al., 1998). These cytokines, once at the site of epithelial cells, regulate leukocyte migration into the lumen (Sharkey, 2012b). Ibrahim *et al.* found that SP-derived TGF-β has an immunosuppressive effect on the female inflammatory response to semen (Ibrahim et al., 2019). This has been

reported numerous times in the human (Ingman & Robertson, 2002), as the theory that the TGF-β present within SP plays a role in subsequent pregnancy development has been explored as the advancements in fertility research continue. Recently, a systematic review considered numerous biomarkers identified within human SP that could be predictive of IVF and ICSI fertility outcomes (van den Berg et al., 2024). Reviewers found that significantly higher ratios of TGF-β/IL-18 within SP of successful pregnancies in women undergoing IVF/ICSI procedures. These findings indicate a potential role of TGF-β within SP at the time of exposure to the maternal tract, as differing pregnancy rates occur between SP with varying TGF-β concentrations (Nikolaeva et al., 2016).

In the majority of domestic species, TGF-β has been commonly described within the SP fraction of the ejaculate upon collection (O'Leary et al., 2011a). In the rat, the production of TGF-β isoforms was noted within the accessory sex glands and the epididymis (Desai et al., 1998). Similarly, clinical studies in the human model found that latent isomers of TGF-β exist within fractioned human SP (Nocera & Chu, 1995). In porcine SP, immunoassay quantification revealed high levels of TGF-β isomers in all boars tested (O'Leary et al., 2011b). Unfortunately, the presence of TGF-β within SP has not been documented in the horse; this has been attributed to the lack of TGF-β immunoassays that are specific to equine reactivity. Increasing evidence in the before mentioned species demonstrates a clear relationship between seminal-derived TGF-β and successful pregnancy, indicating that it is likely that TGF-β is present within stallion SP, potentially playing a role in equine pregnancy modulation.

5.1.1.3 Prostaglandins

Alongside these proteins and cytokines that are present within the ejaculate are hormones, which are chemical messengers that bind to specific receptors in the body as part of

the neuroendocrine system. Hormones regulate the majority of reproduction, as localized concentrations of various steroid hormones from both the brain and reproductive organs directly regulates follicular recruitment, ovulation, development of the corpus luteum, and sexual receptivity in the female; in males, spermatogenesis is regulated by the conversion of steroid hormones in all mammalian species (Senger, 2012). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is secreted at the site of the endometrium before acting at the ovary to begin luteolysis, or the lysis of the corpus luteum. Endogenous prostaglandins actively play a role in clearing uterine fluid following breeding through initiating adequate muscle contractility throughout the reproductive tract following breeding (Samper, 2008). Prostaglandin E_2 (PGE_2) is another dominant prostaglandin that has been identified within the horse ejaculate, which also plays a role in stimulating uterine contractility (Kareskoski et al., 2011). In the stallion, both estrogens and prostaglandins were identified in semen samples, and researchers found that the mean seminal concentration of $PGF_{2\alpha}$ was higher than that of estrogens (Claus et al., 1992). While $PGF_{2\alpha}$ has been quantified within the stallion ejaculate, it is unclear whether the presence of this hormone alongside spermatozoa triggers further $PGF_{2\alpha}$ production by the endometrium, or whether other elements within equine SP also contribute to this increase in endometrial prostaglandin production. In other species, the impact of seminal prostaglandins at the uterus has been studied; in the sow, both $PGF_{2\alpha}$ and PGE_2 concentrations were increased in SP-treated gilts in comparison to controls (Kaczmarek et al., 2013). Similar findings have been found in the ewe, as prostaglandins have been shown to assist with sperm transport at the site of the uterus, along with stimulating uterine blood flow (Bollwein et al., 2003).

5.2 Reduction of SP

Although the reduction of SP has been shown to enhance the cryo-survival of spermatozoa for both animal and human ART operations (Aurich et al., 1996), the potential consequences of this reduction on embryo, fetal, and offspring development have yet to be ascertained in the equine model. Similarly to other previously discussed areas of equine research that are lacking adequate elucidation in areas, this theory was initially researched in pregnant mice. Bromfield *et al.* (2014) conducted a study in the murine model in which BALB/c mice underwent seminal vesicle excision (SVX) to investigate the impact of SP elimination on pregnancy outcomes and various phenotypic offspring measures. Female CBFA1 mice were bred with both SVX and control males, and early pregnancy rates were significantly reduced in the SVX group in comparison to the males with intact seminal vesicles (Bromfield et al., 2014). Interestingly, late pregnancy rates were not significantly impacted, indicative that only early implantation and fertilization rates were decreased with SP elimination from the ejaculate, rather than impacting viability rates during the late term. Following the parturition of offspring, fetal weight was not impacted between SVX-sired and control-sired offspring, but litter size was reduced in the SVX group. Additionally, placental hypertrophy was noted in the SVX-sired group, with a significant increase in placental weight and surface area. These findings are described in the literature as a compromise to placental function and fetal growth, which can have long-term ramifications on offspring growth and metabolic status (Burton & Fowden, 2012). Bromfield analyzed how offspring growth was impacted between the two groups throughout the first 14 weeks of life; in terms of offspring birth weight, it was noted that male SVX offspring were disproportionately larger than their controls, as well as the SVX female offspring. Around the time that these findings were published, an immunogenetic study used

abortion-prone mouse models (CBA/J) to challenge how pre-immunization with SP from normal (BALB/c) males prior to mating impacted abortion rates (Clark et al., 2013). Researchers found a significant relationship between seminal-treated cells and decreased embryonic resorption rates, suggesting that antigen specific peptides that were isolated from SP samples may play a role in modulating the cross presentation of the paternal antigen at the time of mating.

Specific to the theory that Tregs are impacted by SP reduction, Robertson *et al.* (2009) mated female mice with both intact and vasectomized males to characterize how SP priming might alter T lymphocyte expression and ultimately the adaptive immune response to progressive embryonic development. Females mated by intact males demonstrated an increase in *FoxP3* expression, indicative of Treg expansion, but this increase was not reflected in the females bred by males without seminal vesicles. This indicates a direct relationship between SP exposure at the time of mating and the proliferation of Treg cells (Robertson et al., 2009). A later study from the Robertson lab identified the mechanism by which Tregs are recruited by analyzing the maternal uterine lymph node following insemination and at the time of embryonic implantation; the expression of Treg-specific chemokine *CCL19* was upregulated, which influences the peripheral Treg recruitment and mediates additional antigen presentation by the semi-allogenic embryo (Guerin et al., 2011). These findings, along with those in other species, are summarized in **Table 1**.

Table 1. Seminal Plasma Reduction & Impact on Pregnancy by Species Model.

FINDINGS	MODEL	STUDY
Females mated by intact males demonstrated an increase in Tregs, but this increase was not reflected in the females bred by males without seminal vesicles	Murine	(Robertson et al., 2009)
CBFA1 mice bred with both SVX and control males; early pregnancy rates were significantly reduced in the SVX group in comparison to those of intact males	Murine	(Bromfield et al., 2014)
Women with recurrent IVF failure had downregulated Treg-related transcripts in comparison to those with successful outcomes; addition of SP induced increase in endometrial TGF-β	Human	(Azad et al., 2017)
In IVF/ICSI trials, Tregs were decreased in women with unsuccessful pregnancy outcomes	Human	(Schlossberger et al., 2013)
More than twice as many mares bred with cooled semen became pregnant by day 45 (92%) in comparison to mares bred with frozen semen (53%)	Equine	(Gaspardy et al., 2020)

A study in humans described the immediate inflammatory response to 'pure sperm,' in which women underwent AI with concentrated motile sperm with SP reduction (Thompson et al., 1992). Cervical mucus samples underwent immunocytochemical staining and researchers reported that neutrophils represented the largest population of leukocytes that responded to the upregulation in pro-inflammatory cytokines. Additionally, the leukocytic responses between the raw ejaculate and concentrated spermatozoa differed significantly, suggestive that SP mediates the leukocytic endometrial response to spermatozoa. Subsequent studies focused on components within the SP fraction that could contribute to paternal antigen presentation, maternal immune tolerance, and both fetal and maternal metabolic outcomes (Ingman & Robertson, 2002). Specifically, Robertson suggested that SP may prime the maternal immune system through the introduction of TGF-β to the maternal decidua (Robertson et al., 2003). Upon breakthroughs in murine models relative to investigating the mechanisms of maternal immunotolerance in the late 2000s, translational research studies were established in human models. Within the following decade, researchers had begun establishing the relationship between SP-derived TGF-β, maternal Treg proliferation, and pregnancy outcomes (Robertson et al., 2013). This was demonstrated

when human SP was used to stimulate female peripheral blood mononuclear cells (PBMCs) in-vitro, inducing the proliferation of CD4+ Treg populations (Balandya et al., 2012).

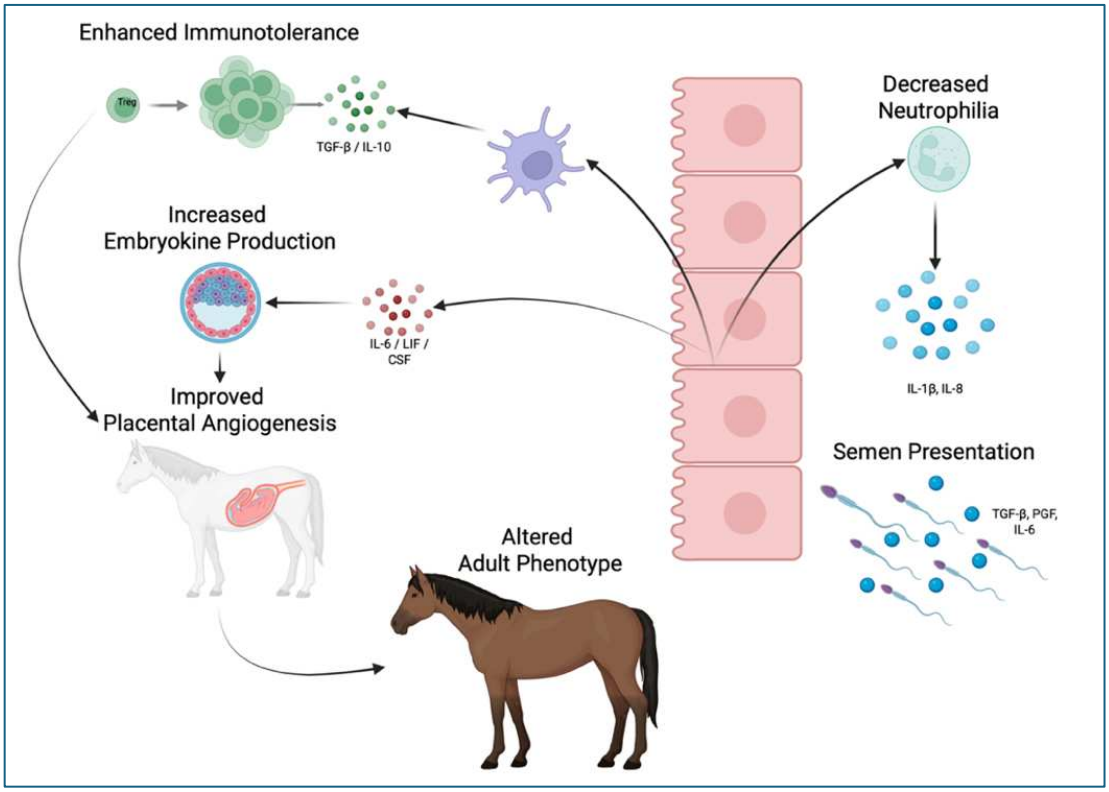
Additional studies have investigated the role of reduced SP exposure and pregnancy loss in women (Schjenken & Robertson, 2015), suggesting that the removal of SP associated with ARTs may be impeding the successful immunotolerance of embryonic development in the human model. This was further explored through the establishment of T helper cell subsets before and after SP exposure; in women with recurrent IVF failure, Treg-related transcripts were significantly downregulated in comparison to those with successful outcomes, even prior to SP exposure, suggestive of a imbalanced T helper response (Azad et al., 2017). After the addition of SP, these transcripts were upregulated in both groups, and TGF-β expression was increased in those with successful outcomes. As in-vitro fertilization (IVF) continues to rise in popularity as a clinical option for women who are unable to conceive through natural copulation efforts, researchers have considered that the lack of SP exposure involved when fertilization is initiated in-vitro may be playing a role in the subsequent conception rates (Kanannejad et al., 2020).

1.6 ASSISTED REPRODUCTIVE TECHNOLOGIES

Advancements in the equine reproduction and breeding industry have drastically increased the clinical use of assisted reproductive technologies (ARTs). In the horse, assisted reproductive technologies (ARTs) include AI (AI), embryo transfer (ET), intracytoplasmic sperm injection (ICSI), and more recently in vitro fertilization (IVF). The use of ARTs in the equine breeding industry have become increasingly popular (Squires, 2019). AI differs from natural mating's primarily in terms of gamete collection and handling, but also in the routine reduction of the SP prior to storage or in both cooled and frozen semen insemination (Pickett et al., 1975)

(Amann et al., 1987). The centrifugation and removal of much of the SP fraction is crucial for maintaining the integrity of the spermatozoa within the breeding dose in preparation for cryopreservation (Brinsko et al., 2000). Although pregnancies are established when SP fractions are reduced, success rates are lower when comparing rates of natural breeding to AI (Cuervo-Arango, 2019) with both cooled and frozen semen (Cochran et al., 1983), embryo transfer, and other methods of in-vitro embryo maturation (Panzani, 2014). These lower success rates from ART-produced pregnancies in comparison to natural breeding rates have long been attributed to the manipulative process of gamete handling and cryopreservation on sperm integrity (Loomis, 2001). However, the ramifications of this removal on subsequent maternal immunity and offspring phenotype have largely gone uninvestigated in the horse. It is unknown if this reduction in SP when ART is used leads to impacts affecting the pregnancy or subsequent offspring (Proposed in **Figure *3***).

Figure 3. Schematic representation of the proposed impact of seminal plasma on the maternal immune response to breeding and pregnancy.



Seminal plasma is believed to impact the maternal immune response to breeding, embryo growth and development, in addition to immunotolerance of the developing fetus, all of which are believed to improve pregnancy outcomes and optimize the growth and development of offspring. Proteins, cytokines, and paternal antigen within seminal plasma modulates the immune response to breeding, leading to the production of various cytokines which assist with the innate immune response to breeding, in addition to the cell-mediate immune response to pregnancy. Breeding techniques in which seminal plasma is reduced or eliminated are believed to alter the endometrial and placental transcriptome, in addition to altering cell-mediated immunity both peripherally in addition to the reproductive tract. Modified from Bromfield et al. (2016).

1.6.1 Overview of ARTs in the Horse

Initial clinical research investigations into the collection, handling, and cryopreservation of equine semen began in the 1930s with the development of the artificial vagina (AV) for the boar by Dr. McKenzie, who then went on to report successful pregnancies from equine semen stored short-term after collection with the use of a modified Cambridge pattern AV (McKenzie,

1939). B.W. Pickett of Colorado State University pioneered the application of these preliminary ART techniques in other species to the horse and dedicated research efforts towards the development of novel techniques that defined the principles of equine semen cryopreservation (Pickett et al., 1975). This study contributed to establishing the Colorado method of frozen semen handling and processing, as researchers published that the reduction of the SP fraction to 10% was required to avoid impacting post-thaw motility parameter on semen. Later studies identified a negative interaction between semen extenders and SP when cooling and freezing semen for later use, impacting both total motility and progressive motility rates on spermatozoa post-thaw (Bedford et al., 1995). Thus, subsequent studies determined that reducing the SP fraction via centrifugation improved post-thaw total motility and progressive motility when semen was cooled (Brinsko et al., 2000). Today, a variety of assisted reproductive technologies (ARTs) are used in equine clinical practices. Benefits of ART include the ability to ship semen and embryos from anywhere to the site of the mare (Squires, 2009). The process of AI begins with collection of the ejaculate within an artificial vagina. Semen is composed of two portions: spermatozoa; the gametes that are capable of fertilization, and the seminal plasma (SP) portion. This gel-fraction of the semen originates from various accessory sex glands, contributing the fluid volume to the total semen (Amann et al., 1987). When semen is collected, the concentration of spermatozoa relative to the entire volume of the ejaculate is calculated; if the concentration is low, this may require the centrifugation of the semen to remove a portion of the SP to achieve the appropriate breeding-dose volume (Lindsey et al., 2001). When semen is not used immediately for on-site fresh AI, it is cooled for short-term storage or cryopreserved for long-term storage. In both cases, the majority of the SP fraction is pipetted off and replaced with milk-based extender to provide the sperm with a cryoprotective agent before cooling or freezing

(Bowen, 1969). Initial studies analyzing techniques of stallion semen cryopreservation attributed the quick deterioration of semen after collection to the antioxidant enzymes found specifically within the SP fraction of the ejaculate (Ball et al., 2001).

An additional ART procedure available to the equine industry is embryo transfer. This procedure presents an opportunity for a donor mare to produce more than one offspring per year by way of a recipient mare who can carry the donor's genetic offspring to term (Squires, 2009). This appeals to mares in competition, in which the donor can resume performance training. Alternatively, clients may opt for ET if the donor mare has difficulty carrying pregnancies to term and still pass on her genetics by transferring the embryo to a recipient who has been deemed fit to carry based on reproductive evaluations. Embryos can be flushed from the donor mare approximately 8 days after ovulation to then be transferred to a recipient upon hormonal induction to prepare the uterus to accept the embryo. Of the ARTs involving embryo or oocyte collection and manipulation, ET is the most commonly used worldwide (Hinrichs, 2012). More invasive techniques involve the capture of oocytes directly from the donor ovary, whether obtained laparoscopically or surgically. Oocyte transfer (OT), also known as gamete intrafallopian transfer (GIFT), refers to the surgical transfer of a donor's oocyte, or a single female gamete, to the recipient's oviduct for subsequent insemination of the recipient (Carnevale, 2004). More commonly utilized clinically, ovum pick-up (OPU) involves the laparoscopic retrieval of oocytes directly from the ovary; but rather than placing the oocyte within the recipient mare, it is cultured within an incubator. Oocytes are then fertilized through ICSI, in which an embryologist injects a single spermatogonia to inject directly into the immature egg before maturing within culture medium to later be transferred to a recipient (Squires & Blaszczyk, 1996).

1.6.2 Pregnancy Outcomes Following ART in the Horse

While these clinical advances in technologies have increased the mechanisms by which practitioners can acquire gametes and embryos, the fertilization and pregnancy rates vary greatly by clinics and populations. A retrospective analysis conducted at Utrecht University analyzed various reproductive parameters of mares bred via natural mating, AI (with either cooled or frozen semen), embryo flush/transfer, and OPU/ICSI to ultimately determine the impact of advanced maternal age on these parameters (Cuervo-Arango, 2019). Mares bred via natural mating had the best success rates in comparison to mares bred using the various ARTs, and a significant impact of increasing maternal age influenced efficiency in all breeding techniques exempting the in-vitro produced group (**Table *2***).

Table 2. Rates of pregnancy at Days (d) 14 and 45 post-ovulation with varying ART methods.

	Natural Mating	Artificial Insemination	Embryo Transfer	In-Vitro Produced
Pregnancy at d14	67.5%[a]	46.6%[b]	47.8%[b]	40.4%[b]
Pregnancy at d45	63.3%[a]	43.9%[b]	45.8%[b]	37.4%[c]

Amended from Cuervo-Arango et. al (Cuervo-Arango et al., 2019)

In the above study, mares bred via natural mating averaged pregnancy rates at 68% in comparison to the significantly lower rates (47%) when bred via AI and ET. No significant difference was noted in the day 45 pregnancy rates between mares used for ET (43.9%) compared to the AI group (45.8%), but both groups were more efficient than the OPU/ICSI group (37.4%). In a different study comparing the conception rates between cooled and frozen semen via AI in a practical clinical setting, more than twice as many mares bred with cooled

semen became pregnant by the 45th day of the breeding season (92%) in comparison to mares bred with frozen semen (53%) (Gaspardy et al., 2020).

The collection rates of individual immature follicles, or oocytes, reported by clinical breeding practices and/or research facilities across the globe are not consistent, appearing to depend on many factors. A study in Argentina yielded 23% success to foaling per oocyte captured via oocyte transfer following OPU procedures with a sample of 25 mares (Riera et al., 2016). In a study conducted at Utrecht University in the Netherlands, researchers reported decent success with in vitro embryo production (IVEP) methods, OPU and ICSI. Blastocyst capture rates averaged 78% in terms of initial capture success; odds for subsequent pregnancy success remained high at 77.7% (Claes & Stout, 2022). Of the transferred blastocysts, 60% resulted in live births. The influence of ART on pregnancy outcomes varied immensely based on which clinics reported, which demonstrates the need for additional research in the area. The reduced early pregnancy rates associated with ARTs may be associated with reduced seminal plasma exposure at the time of breeding.

1.6.3 SP Reduction and Consequences

Consistent between these ART techniques, the SP fraction of the ejaculate is significantly reduced or fully eliminated. This practice of SP reduction has become routine across the equine industry for the preparation of frozen semen, largely in the theory that dilution of SP to spermatozoa to a 1:4 ratio is crucial to the cryosurvival of semen in preparation for long-term cryopreservation (Pickett et al., 1975). This was later investigated in the use of cooled semen, where centrifugation significantly improved both total and progressive sperm motility (Bedford et al., 1995). With the use of techniques such as embryo transfer and ICSI, the recipient mare does not gain access to any SP as only the embryo itself is transferred directly to the uterus. It is

important to note that the recipient mare will be exposed to the early blastocyst that expresses paternal antigens, but without the SP exposure prior (Moldenhauer et al., 2009).

While the decreased pregnancy rates following these procedures is commonly attributed to gamete handling, recent studies have investigated if the reduction or elimination of SP, and therefore poor priming of the maternal immune system, impacts fertility rates associated with ARTs in the horse. A 2018 study analyzed endometrial biopsies for Treg-related transcripts following exposure to raw semen, SP, or phosphate buffered saline (PBS) in the horse. Researchers found that endometrial Tregs were not influenced by exposure to semen and SP (Hartmann et al., 2018). However, the process of antigen presentation takes at least 36 hours to initiate effector T cell differentiation specific to the paternal antigen and the cytokines found within the SP fraction. Transcripts of naïve Treg differentiation would not necessarily be detectable within 48 hours, and not if biopsies were collected 24 hours post semen/SP exposure. Additional work by Fedorka *et al.* (2024) assessed endometrial biopsies taken 7 days after ovulation/insemination and found 158 differentially expressed genes following insemination with either raw semen or centrifuged semen (Fedorka et al., 2024) Alteration of biological pathways include immune cell signaling, antigen presentation, and embryo growth and development – indicating that SP pays a role in the presentation of paternal antigen to the maternal immune system following insemination.

While equine-specific findings are limited, murine data clearly demonstrates the impact of SP reduction on Treg expansion. Robertson et al. found a prolific antigen-specific Treg response when females were mated by intact males, but mating with SVX males curbed Treg expansion, potentially altering maternal tolerance (Robertson et al., 2009). This theory was later applied in human models, where a similar relationship between Tregs and SP signaling was

identified (Schjenken & Robertson, 2015). A clinical study considered women with unexplained infertility who were undergoing IVF treatments and characterized how effector T cell populations differed between successful and unsuccessful outcomes; Th1-associated transcripts were upregulated in the unsuccessful IVF group whereas Treg-associated transcripts were upregulated in the successful IVF group (Azad et al., 2017). Considering these findings in other species, SP exposure in the horse may significantly impact the resulting effector T cell response during early pregnancy.

1.7 KNOWLEDGE GAPS AND RATIONAL FOR CURRENT STUDY

Successful establishment of pregnancy requires a finely tuned balance between immune activation and immune tolerance. Across species, maternal exposure to paternal antigens at the time of mating initiates a cascade of innate and adaptive immune events that ultimately support embryonic survival (Morelli et al., 2015). While the acute inflammatory response to semen deposition is well characterized in the horse, the adaptive immune mechanisms that promote long-term tolerance remain incompletely defined. Evidence from murine and human models consistently demonstrates that Treg expansion, driven in part by SP-derived immunomodulatory factors such as TGF-β, is critical for implantation, placentation, and maintenance of early pregnancy (Bromfield, 2016). Impaired Treg proliferation has been linked to early pregnancy loss, recurrent implantation failure, and pregnancy-associated pathologies, reinforcing the central importance of immune regulation during the peri-implantation period in human (Winger & Reed, 2011) and mouse models (Zenclussen et al., 2005).

In contrast, equine research has primarily focused on innate post-breeding inflammation and clinical management of breeding-induced endometritis, with comparatively limited investigation into adaptive immune programming and maternal immunotolerance (Woodward & Troedsson, 2015). Although Treg-related transcripts have been described in mid- to late-gestation mares, the establishment of Treg dominance during early pregnancy, and particularly in the context of SP reduction associated with assisted reproductive technologies (ARTs), remains largely unexplored (Fedorka et al., 2020). Given that ART use in both human and equine medicine frequently involves substantial reduction or elimination of SP, understanding how this practice may influence maternal immune priming represents a significant knowledge gap.

Collectively, the literature highlights a compelling need to define the temporal development of Tregs during early equine pregnancy and to determine whether SP exposure contributes to this process. Addressing these gaps will not only improve understanding of reproductive immunology in the horse but may also provide translational insight into maternal–fetal tolerance mechanisms relevant across species. Therefore, we aimed to assess the expression of transcripts relating to Treg cell development in mares during early pregnancy. Additionally, we aimed to assess the impact of SP exposure on this outcome. In doing so, we may be able to predict pregnancy success, plan for pregnancy loss, and improve pregnancy outcomes in our mares undergoing ART techniques.

CHAPTER TWO: DEFINING THE DEVELOPMENT OF T CELLS DURING EARLY PREGNANCY IN THE HORSE

2.1 INTRODUCTION:

Pregnancy acceptance requires immune tolerance of the embryo, which is initiated upon maternal exposure to the paternal antigen into the uterus (Robertson et al., 2015). The immune system of the female must recognize the foreign paternal antigen to accept the conceptus and to preventing the maternal immune system from destroying the developing fetus (Munoz-Suano et al., 2011). The mechanisms in which this is modulated have yet to be fully elucidated, but data from various species have demonstrated a role between an immunosuppressive population of regulatory T cells (Tregs) during early pregnancy and successful gestational outcomes, specifically in the human (Dimova et al., 2011), the mouse (Shima et al., 2010), and the horse (Aurich et al., 2014). In contrast, pro-inflammatory effector T cells, particularly T helper 1 (Th1) cells, promote inflammatory immune responses through cytokines such as interferon- γ (IFN-γ) and interleukin-12 (IL-12), which may contribute to embryo rejection if not tightly regulated (Sykes et al., 2012). Therefore, the balance between tolerogenic Treg responses and inflammatory Th1 cell responses is considered a critical determinant of early pregnancy success.

T cells can be identified by various cell surface markers, transcription factors, and secretory cytokines. When assessing Tregs, this includes cell surface markers CD4 and CD25, alongside transcription factor FoxP3, and secretory cytokine TGF-β. Tregs are known for their ability to suppress immune responses from other T cells, inhibiting potential pro-inflammatory actions triggered by an antigen (Tilburgs et al., 2008). FoxP3 has been recognized as the primary

transcription factor that is required for the successful differentiation of the naïve T cell into a regulatory T cell (Fontenot et al., 2003). Tregs suppress immune responses from other immune cells, by inhibiting potential pro-inflammatory pathways through the upregulation of TGF-β which is crucial to maintaining tolerance (Ibrahim et al., 2019). Through these mechanisms, Tregs function to limit the activation and expansion of effector T cell populations, including Th1 cells, thereby preventing excessive inflammatory responses against paternal antigens (Samy et al., 2006). Th1 type immunity is pro-inflammatory in nature, secreting IFN-γ which has been shown to be harmful to pregnancy outcomes (Raghupathy, 1997). The primary transcription factor for Th1 cells is TBX21 and secretory protein is either IFNγ or IL-12 (Robinson & O'Garra, 2002).

When pregnancy is initiated, paternal antigens are expressed by spermatozoa in addition to being present in seminal plasma (SP) within the ejaculate (Moldenhauer et al., 2009). Upon the deposition of semen into the female reproductive tract, paternal alloantigens are captured by uterine dendritic cells, activating mature tolerogenic dendritic cells (tDC) (Laskarin et al., 2007). These tDCs initiate the expansion of Treg populations, which is modulated by the upregulation of progesterone, TGF-β, and other immunosuppressive mechanisms (Robertson et al., 2015). In turn, maternal tolerance to paternal antigens is enhanced by early embryonic development due to the rapid increase of semi-allogenic antigens (Seavey & Mosmann, 2008). Failure to establish this regulatory environment may allow Th1 cell responses to predominate, potentially compromising embryo implantation and survival (Saito, 2010).

In both mouse and human models, the importance of Treg dominance during the first trimester to pregnancy outcomes has been well-established (Robertson et al., 2018). During early pregnancy, immunosuppressive Treg cell populations are elevated in pregnant women during

early pregnancy (8-12 weeks) in comparison to non-pregnant controls (Dimova et al., 2011). In the murine model, Tregs continue to proliferate from the time of embryo implantation to mid gestation before declining prepartum (Ruocco et al., 2014). Antczak *et al.* noted that the equine model presents a unique research opportunity in which to investigate the role of Tregs and pregnancy, as cited research in human and murine models demonstrate increased *FoxP3* expression reported in both maternal peripheral circulation and at the site of the fetal-maternal interface (Antczak, 2012). In the equine model, Treg-related transcripts trended down from 45 days to 120 days gestation, followed by an upregulation to 300 days before declining prepartum (Fedorka et al., 2020). However, the regulation of Tregs during early equine pregnancy has not been fully described.

SP is the fluid portion of the ejaculate, made up of secretions from the rete testes, caudal epididymides, and the accessory sex glands. Historically, scientists have recognized the primary function of SP in its role as a transportation medium for spermatozoa once deposited at the site of the female tract (Amann & Pickett, 1987). Further investigations into other impacts of SP indicated that elements within SP play a role in modulating metabolic and immunologic interactions within the maternal environment, rather than strictly in the transportation of spermatozoa (Rozeboom et al., 2000). In human studies, researchers have investigated how SP exposure initiates immunological changes in the female tract, specifically by way of cytokines that 'condition' the maternal immune system to tolerate the newly presented paternal antigen (Sharkey et al., 2007). Reduced SP exposure at the time of mating has been associated with decreased Treg expression in humans, potentially highlighting a relationship between these two factors and successful pregnancy outcomes (Benammar et al., 2021).

In recent decades, the clinical use of assisted reproductive techniques (ART) in human medicine has increased (Sunderam, 2022). The percentage of ART-derived pregnancies resulting in live births is low, at only a 37.5% success rate, thus, research has focused on the causality of ART-derived pregnancy failure (CDC, 2024). Studies have investigated the role of reduced SP exposure and pregnancy loss in women (Azad et al., 2017), with a focus on how Treg populations differ between these two factors (Kanannejad et al., 2019). This evidence suggests that the removal of SP associated with ARTs may be impeding the successful immunotolerance of embryonic development in the human model, which may be an impact of decreased Treg expression (Schjenken & Robertson, 2015).

Investigating this theory in the equine model may offer unique insights into applications in the human model, as SP is frequently reduced in breeding doses prepared in commercial breeding operations since semen doses are often cooled and shipped domestically (Brinsko et al., 2000). The primary aims of this study were to a) evaluate Treg and Th1-related transcripts during early pregnancy, b) determine the influence of pregnancy on Treg and Th1-related transcripts, and c) consider the impact of ARTs on Treg and Th1-related transcripts in early pregnancy. We hypothesize that Treg expression will increase following semen deposition while Th1-associated transcripts will be suppressed, and that Treg expression will be increased in pregnant mares in comparison to mares found open, while Th1-associated transcripts will be elevated in non-pregnant mares. Additionally, seminal plasma exposure at the time of breeding will heighten Treg development.

2.2 MATERIALS AND METHODS:

2.2.1 Animal Usage

Twenty-nine (n=29) client-owned mares of mixed breeds, age, and weight (5-20 yr; 450-600 kgs) were used over two years for the study. Mares were kept on dry lots with free choice hay and owner-provided grain supplementation with access to water and minerals *ad libitum* at Colorado State University's Equine Reproduction Laboratory. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Colorado State University (protocol #2023-5968).

2.2.1.1 Semen preparation of fresh semen

Semen was collected from 3 stallions (n=3) with an artificial vagina of choice, equipped with a gel filter (Animal Reproduction Systems, Chino, CA, USA). Immediately after, volume and concentration of semen was assessed via graduated cylinder and densimeter (590B Densimeter, ARS Equine; Ontario, CA, USA). For fresh semen, $500x10^6$ of sperm was taken from the total volume, extended with the semen extender of stallion owner's choice. Upon confirmation of the presence of a preovulatory follicle (>35mm) combined with reduced uterine tone, increased endometrial edema, and a relaxed cervix, mares were inseminated within 30 min of fresh semen collection.

2.2.1.2 Semen preparation of cooled semen

For cooled semen, samples were collected from 6 client-owned stallions (n=6). Following, $500x10^6$ of sperm was taken from the total volume centrifuged at 600xg for 20 minutes. The SP/supernatant was reduced following centrifugation, and sperm samples were extended with pre-warmed semen extender of stallion owner's choice. Semen was then cooled to

4°C using Equine Express II Cooled Semen Transport System (Next Generation; Beverly, Massachusetts, USA) before being transferred to long-term refrigerated storage at 4°C. before being inseminated into the mare within 72 hours. Upon confirmation of the presence of a preovulatory follicle (>35mm) combined with reduced uterine tone, increased endometrial edema, and a relaxed cervix, mares were inseminated within 72 hours of cooled semen collection.

2.2.1.3 Semen preparation of frozen semen

Frozen semen was prepared according to industry standards. In brief, semen was collected from four client-owned stallions (n=4) and diluted at a 1:1 ratio (semen: extender) before being subjected to cushioned centrifugation at 600×*g* x 20 min, as described previously (Ramírez-Agámez et al., 2023). Following centrifugation, the SP/supernatant was reduced, and the sperm pellet was resuspended using a freezing extender of stallion's owner choice at a sperm concentration of 25×10^6 sperm/mL. Sperm diluted with the freezing extender was loaded into 0.5 mL plastic straws, sealed ultrasonically, and frozen in a controlled rate freezer (CBS 2100; Custom Biogenic Systems, Bruce Township, MI, USA) using the following freezing program: −2.0 °C/min from 25 to 22 °C; −0.3 °C/min from 22 to 10 °C; −0.2 °C/min from 10 to 4 °C; hold for 5 min; a freezing rate of −60 °C/min from 4 to −140 °C (Salazar et al., 2011). The straws were then plunged into liquid nitrogen for storage until thawing prior to insemination upon confirmation of the presence of a preovulatory follicle (>35mm) combined with reduced uterine tone, increased endometrial edema, and a relaxed cervix.

2.2.1.4 Artificial Insemination

Mares were examined daily via transrectal palpation and ultrasonography of their reproductive tracts for follicular development, endometrial edema, as well as uterine and cervical

tone. When the presence of a preovulatory follicle was noted (>35mm) combined with reduced uterine tone, increased endometrial edema, and a relaxed cervix, mares received 3000 IU of human chorionic gonadotropin (hCG; Intervet International B.V., Boxmeer Holland) intravenously to standardize the interval between insemination and ovulation. Insemination occurred 24-36 hours after hCG administration.

2.2.1.5 Peripheral immune response to insemination with fresh semen

To assess the impact of fresh semen on lymphocyte responses to insemination, fifteen mares (n=15) were examined daily via transrectal palpation and ultrasonography of their reproductive tract for follicular development, endometrial edema, as well as uterine and cervical tone. Of the fifteen mares bred with fresh semen, ten mares became pregnant and five remained open. When estrus was detected, blood was obtained aseptically via jugular venipuncture for the isolation of peripheral blood mononuclear cells (PBMCs) as previously described by Fedorka *et al.* (2019). In brief, three red top vacutainer tubes were retrieved from each mare, and 100 USP heparin sodium injection (Porcine; Henry Schein Animal Health; Dublin, OH, USA) was added to each as an anticoagulant for retrieval of plasma (Fedorka et al., 2019). Blood was taken to the laboratory at ambient temperature for further processing and analysis. Following, mares were inseminated with 500 x 10^6 spermatozoa with full SP fraction. Ovulation (d0) was confirmed by transrectal ultrasonography. Pregnancy determination was performed using trans-rectal ultrasonography at 14 days following ovulation. Additional PBMCs were collected on day 7, 14, 21, and day 28 following ovulation.

2.2.1.6 Peripheral immune response to insemination with various insemination methods

To compare the peripheral immune response to breeding based on insemination method, 14 additional mares that became pregnant were bred with either cooled (n=8) or frozen (n=6)

semen to be compared to mares bred with fresh semen that became pregnant (n=10). When estrus was detected, blood was obtained aseptically via jugular venipuncture for the isolation of peripheral blood mononuclear cells (PBMCs). In brief, three red top vacutainer tubes were retrieved from each mare, and 100 USP heparin sodium injection (Porcine; Henry Schein Animal Health; Dublin, OH, USA) was added to each as an anticoagulant for retrieval of plasma. Blood was taken to the laboratory at ambient temperature for further processing and analysis. Following, mares were inseminated with prepared doses of either fresh, cooled, or frozen semen following routine clinical management. Ovulation was confirmed by transrectal ultrasonography. Pregnancy determination was performed 14 days following ovulation. Additional PBMCs were collected day 7 and day 14 following ovulation.

2.2.2 Laboratory Work

2.2.2.1 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated from mares as previously described (Fedorka et al., 2019). In brief, heparinized blood was used to isolate PBMCs by Ficoll-Paque PlusTM (Amersham Biosciences, Piscataway, NJ) gradient centrifugation. Following isolation, $1x10^6$ cells were separated and snap frozen in TRIzol®-LS Reagent (Invitrogen, Carlsbad, CA, USA) for the determination of lymphocyte gene expression as previously described (Elzinga et al., 2017).

2.2.2.2 Quantitative Polymerase Chain Reaction Analysis

To assess expression of targets on lymphocyte expression, total RNA from PBMCs was extracted using TRIzol®-LS Reagent (Invitrogen, Carlsbad, CA, USA) as described by the

manufacturer. Total RNA was precipitated using isopropanol and 75% ethanol, resuspended in ddH_2O and DNAse treated (TURBO DNase™, (2 U/μL), Thermo-Fischer) and then analyzed for quantity and quality via a NanoDrop® spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and only samples with a 260/280 ratio greater than 2.0 were assessed. RNA was reverse transcribed and qPCR was performed as previously described by Fedorka *et al.* (Fedorka et al., 2017). Briefly, 0.5 μg of RNA in 85 μL ddH_20 was reverse transcribed using Promega reagents; 0.625 μL AMV Reverse Transcriptase, 2.5 μL 5x RT Buffer, 0.5 μL RNAsin®, 5.5 μL MgCl, 5 μL dNTP, and 1.25μL Random Hexamer Primer (Promega, Madison, WI, USA). Samples were incubated at 42°C for 60 min followed by 95°C for 5 min. cDNA was diluted 1:1 with ddH_20, and qPCR was performed using 4.5 μL of cDNA, 5μL of Sensimix™ II (Bioline, Tauton, MA, USA) and 0.5μL of a custom primer/probe set from Applied Biosystems. Transcripts of interest were chosen based on previous data regarding transcripts relating to T lymphocyte development (Fedorka et al., 2020) and included transcripts relating to regulatory T cell development (*FoxP3, TGF-β, CD25*) and Th1 development (*IFNγ, TBX21*). Primer sequences were designed using the TaqMan® Gene Expression System (Thermo Fischer, Wilmington, DE, USA) as previously described (Fedorka et al., 2020). An overview of the selected transcripts and their Assay IDs is presented in *Table 3*. Reactions were performed in duplicate samples using the ViiA 7 Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA). Samples were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 60°C for 60 sec. Results are expressed as the relative quantification of expression following the the Livak and Schmittgen method (Livak & Schmittgen, 2001).

Table 3. Overview of Transcripts Evaluated

T Cell Type	Related Transcripts	Functions	TaqMan® Assay ID
Treg	FoxP3	Transcription factor	Ec04319945_g1
	CD25	Cell-surface marker	Ec03469221_m1
	TGF-β1	Secretory Cytokine	Ec03468030_m1
Th1	TBX21	Transcription factor	Ec07055855_m1
	IFN-γ	Secretory Cytokine	Ec03468605_g1

Transcripts of interest were chosen based on previous data connecting select genes with T cell development in the equine model (Fedorka et al., 2020). Assay IDs are specific to the TaqMan® Gene Expression System (Thermo Fischer, Wilmington, DE, USA).

2.3 STATISTICS:

Data were analyzed using SAS 9.4® (SAS Institute Inc., Cary, NC, USA). Data was assessed for normality using a Shapiro-Wilkes test and equal variances with a Bartlett's test. Data was analyzed using linear mixed-effects models (*PROC MIXED*) with mare as a random effect and gestational length, pregnancy status, and breeding method as fixed effects. A repeated statement with an appropriate covariance structure was included to model within-subject correlations over time. Post hoc analysis was performed using Tukey's adjustment. Significance set to $p \leq 0.05$, with trends noted as $p \leq 0.10$. Data are presented as the mean ± SEM unless otherwise noted.

2.4 RESULTS:

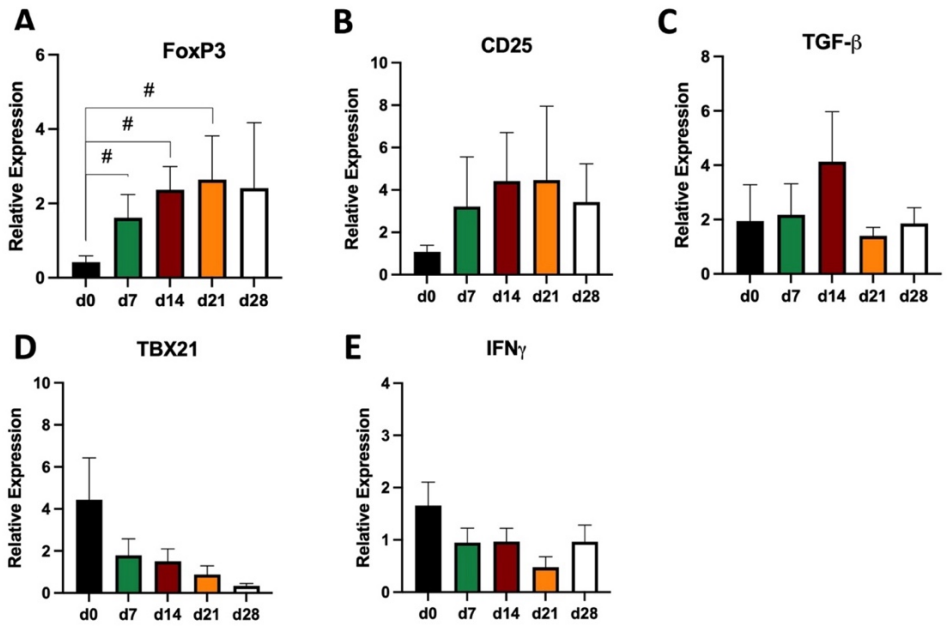
2.4.1 Treg and Th1-related transcripts during early pregnancy

In other models, Treg-related transcripts or populations of Treg cells increase following deposition of semen, while Th1-related transcripts can be embryotoxic or abortogenic

(Raghupathy, 1997). Here, relative expression of Treg-related transcripts (*FoxP3, CD25, TGF-β*) and Th1-associated transcripts (*TBX21, IFN-γ*) were evaluated in peripheral blood mononuclear cells (PBMCs) collected both prior to insemination in addition to weekly for 28 days following ovulation. Gestational length was found to impact *FoxP3* expression ($p = 0.04$), with transcript abundance increasing following breeding and remaining elevated throughout the early post-ovulatory period (Figure 4A). This temporal pattern was most apparent during the second and third weeks following ovulation, which tended to be significantly higher in expression at day 7 (p=0.06) and day 14 when compared to day 0 (p=0.08).

In contrast, expression of cell surface marker *CD25* ($p = 0.67$; Figure 4B) and secreted cytokine *TGF-β* ($p = 0.38$; Figure 4C) did not significantly change across the sampling period. Similarly, no overall impact of gestational length was observed for the Th1-associated transcription factor *TBX21* ($p = 0.39$; Figure 4D) or the pro-inflammatory cytokine *IFN-γ* ($p = 0.14$; Figure 4E).

Figure 4. Temporal changes in Treg- and Th1-associated transcript expression in peripheral blood mononuclear cells (PBMCs) following breeding.



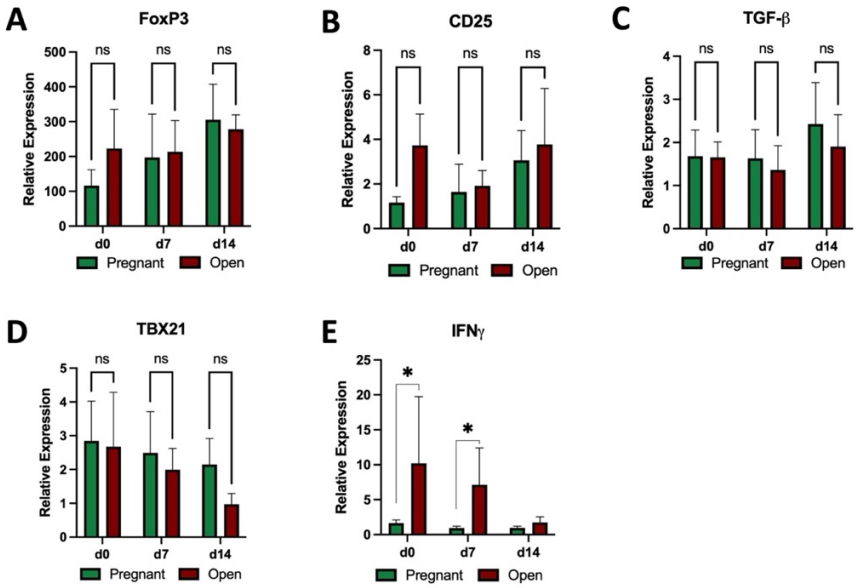
Relative gene expression of (A) FoxP3, (B) CD25, (C) TGF-β, (D) TBX21, and (E) IFN-γ was quantified by qRT-PCR from PBMCs collected from mares in the estrus prior to insemination (d0) and at 7, 14, 21, and 28 days following ovulation. A significant effect of time was observed for FoxP3 expression ($p = 0.04$), with increased expression noted as pregnancy progressed following breeding. No significant temporal changes were detected for CD25 ($p = 0.67$), TGF-β ($p = 0.38$), TBX21 ($p = 0.39$), or IFN-γ ($p = 0.14$). Bars represent mean ± SEM relative expression levels normalized to housekeeping genes and pre-breeding samples. Symbols (#) denote timepoints that differed significantly from baseline (d0).

2.4.2 Influence of pregnancy on Treg and Th1-related transcripts

Pregnancy is routinely diagnosed in the equine model at 14 days after ovulation (Ginther & Utt, 2004). Therefore, we assessed T cell-related transcripts retrospectively based on this outcome. Mares are defined as pregnant (vesicle seen) versus non-pregnant (open). Pregnancy status did not significantly influence expression of the Treg-associated transcripts *FoxP3* ($p = 0.61$; Figure 5A), *CD25* ($p = 0.29$; Figure 5B), *TGF-β* ($p = 0.66$; Figure 5C), nor did it impact expression of the Th1-associated transcription factor *TBX21* ($p = 0.93$; Figure 5D).

In contrast, pregnancy status was found to influence expression of *IFN-γ* ($p = 0.05$; Figure 5E). This was primarily noted prior to breeding ($p = 0.02$) in addition to at day 7 following ovulation ($p = 0.04$), where inseminations which resulted in pregnancy had higher *IFN-γ* expression at these times in comparison to inseminations that did not. This was no longer significant at day 14 ($p = 0.15$).

Figure 5. Temporal changes in Treg- and Th1-associated transcript expression in peripheral blood mononuclear cells (PBMCs) following breeding.



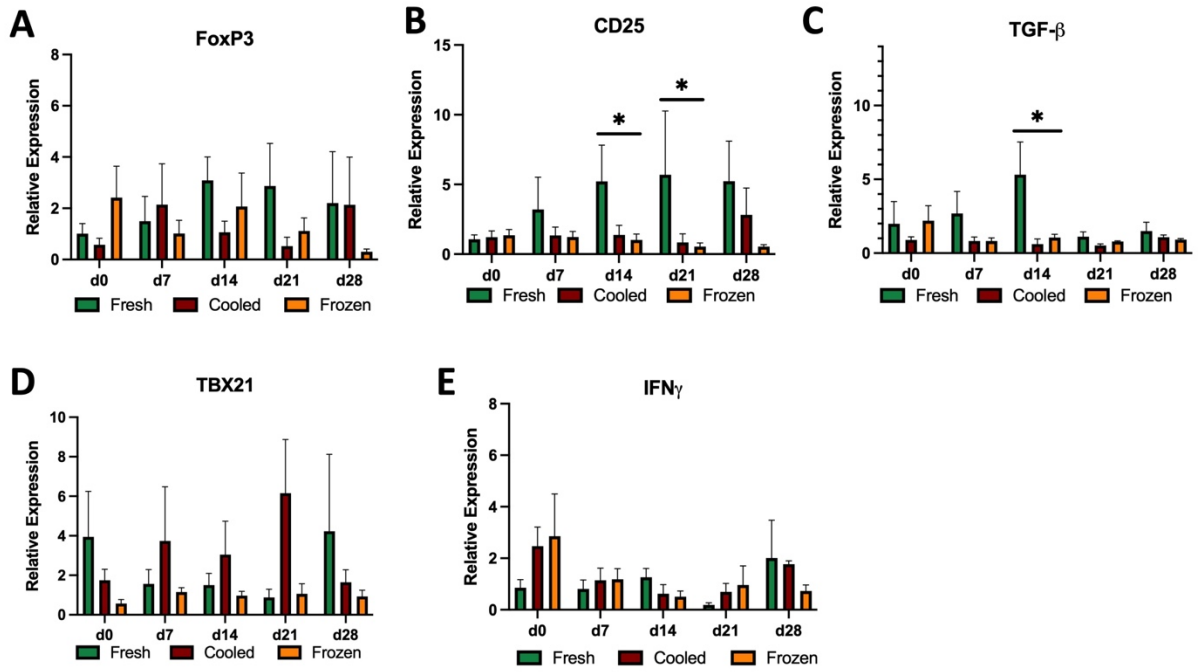
*Relative expression of (A) FoxP3, (B) CD25, (C) TGF-β, (D) TBX21, and (E) IFN-γ was quantified by qRT-PCR in PBMCs collected from mares following insemination. Data are presented according to pregnancy outcome (pregnant vs. open) as assessed by day 14 pregnancy diagnosis by transrectal ultrasonography. Pregnancy status significantly influenced IFN-γ expression ($p = 0.05$)**,** with greater expression observed in non-pregnant mares, suggesting increased Th1-associated inflammatory signaling in animals that failed to establish pregnancy. No significant effect of pregnancy status was detected for FoxP3 ($p = 0.61$), CD25 ($p = 0.29$), TGF-β ($p = 0.66$), or TBX21 ($p = 0.93$). Bars represent mean ± SEM relative gene expression normalized to housekeeping genes and pre-breeding samples. Symbols (*) denote timepoints that differed significantly $p<0.05$.*

2.4.3 Influence of ARTs on Treg and Th1-related transcripts in early pregnancy

To evaluate the influence of SP exposure on maternal immune responses, T cell-related transcript expression was compared amongst mares bred using fresh semen (full SP exposure), cooled semen (reduced SP exposure), and frozen semen (reduced SP exposure). Additionally, no effect of breeding method was noted when assessing expression of *FoxP3 (p*=0.40; Figure 6A), *TBX21 (p*=0.99; Figure 6D). or *IFN-γ* (*p*=0.85; Figure 6E). Additionally, gestational length also did not alter expression of various transcripts, and this included *FoxP3* (*p*=0.94; Figure 6A), *CD25* (*p*=0.86; Figure 6B), *TGF-β* (*p*=0.67; Figure 6C), *TBX21* (*p*=0.30; Figure 6D) or *IFN-γ* (*p*=0.15; Figure 6E).

In contrast, breeding method was found to alter expression of Treg-related cell surface marker *CD25* (*p* = 0.04), with highest expression detected in mares bred with fresh semen in comparison mares were bred with either cooled or frozen semen (Figure 6B). This was noted at significant levels on day 14 (*p*=0.05) and day 21 (*p*=0.04) after ovulation. A similar pattern was observed for expression of Treg-secreted cytokine *TGF-β*, which demonstrated a trend toward significance (*p* = 0.06). Similar to CD25, highest expression of *TGF-β* was noted in mares bred with fresh semen, and this was specifically noted on day 14 following ovulation, where significantly higher found in mares bred with fresh compared to cooled (*p*=0.01) and frozen semen (*p*=0.04; Figure 6C).

Figure 6. Influence of insemination method and seminal plasma exposure on Treg- and Th1-associated transcript expression in peripheral blood mononuclear cells (PBMCs) during early pregnancy.



Relative gene expression of (A) FoxP3, (B) CD25, (C) TGF-β, (D) TBX21, and (E) IFN-γ was quantified by qRT-PCR from PBMCs collected from mares following insemination. Expression levels are presented according to insemination method, representing differing levels of seminal plasma (SP) exposure: fresh semen (green; full SP exposure), cooled semen (red; reduced SP exposure), and frozen semen (orange; minimal SP exposure). Breeding method was found to significantly effect expression of CD25, and had a tendency to alter expression of TGF-β. For both Treg-related transcripts, mares bred with fresh semen had highest expression levels compared to mares bred with cooled or frozen semen. No effect of breeding method was found to FoxP3, TBX21, or IFN-γ. Collectively, these results indicate that the level of seminal plasma exposure associated with different insemination methods may influence systemic immune signaling related to regulatory and effector T cell balance during early pregnancy. Bars represent mean ± SEM relative gene expression normalized to housekeeping genes. Symbols (*) denote timepoints that differed significantly $p<0.05$.

2.4 DISCUSSION:

The present study aimed to characterize systemic immune responses associated with early pregnancy in the mare by evaluating transcriptional markers of immunomodulatory Treg and pro-inflammatory Th1 cells in peripheral blood mononuclear cells following breeding. Collectively, our findings suggest that successful early equine pregnancy is associated with subtle shifts toward a more regulatory immune environment. Specifically, expression of the Treg-associated transcription factor *FoxP3* increased over time following breeding, while the Th1-associated secretory cytokine *IFN-γ* was elevated in mares that ultimately failed to establish pregnancy. These findings support the concept that successful pregnancy establishment requires a balance between immunotolerance and inflammatory signaling, where expansion of regulatory immune pathways occurs alongside suppression of potentially embryotoxic Th1 responses (Sykes et al., 2012). Additionally, breeding method influenced expression of select Treg-associated transcripts, with mares bred with fresh semen demonstrating greater *CD25* and *TGF-β* expression compared to mares bred with cooled or frozen semen. Together, these results suggest that both pregnancy outcome and breeding method contribute to shaping early maternal immune responses in the horse and highlight the potential importance of Treg-mediated immunotolerance during the peri-implantation period.

Our findings characterized equine T cell populations from PBMCs before breeding and during early pregnancy as measured by mRNA expression of Treg and Th1-related transcripts. In the present study, relative *FoxP3* expression was found to gradually increase over time, and this was noted specifically on day 7, 14, and 21 when compared to pre-breeding values. A similar trend in expression has been described in the mouse model, in which *FoxP3* is upregulated by Tregs between day 7 and day 14 of pregnancy in estrogen-treated mice (Polanczyk et al., 2005).

While we saw an increase in *FoxP3* expression on day 7, 14, and 21 when compared to pre-breeding values, the equine Treg response to pregnancy may not be fully established until further embryonic antigen presentation presumably occurs. The timeline of species-specific embryonic events may impact Treg-related transcripts at varying time points, suggesting a role between time of implantation and placentation with Treg proliferation. In humans and mice, implantation occurs quickly after fertilization, generating an increased immune response to paternal antigens alongside the development of the fetal-placental unit relatively soon after the initial mating (Norwitz et al., 2001). In contrast, the equine conceptus avoids true implantation into maternal tissues until chorionic girdle cells develop on the outer surface of the embryo, connecting fetal and maternal tissues around Day 25 (Allen & Stewart, 2002). Considering the impact of gestational timelines between species is crucial to investigating the immunological events during early pregnancy, because fetal antigen exposure increases exponentially once placentation initiates the invasion of fetal-derived tissues into the maternal endometrium and further stimulates paternal antigens (Seavey & Mosmann, 2008). Further investigation into expression until day 35 may provide insights into how the onset of equine placentation impacts Treg-related transcripts.

In contrast to Treg-related transcripts, the expression of both Th1 associated transcripts, *TBX21* and *IFN-γ,* did not significantly change over time as early pregnancy progressed. In contrast, an increase in Th1-related *IFN-γ* was found to be elevated prior to breeding in mares that did not become pregnant based on day 14 trans-rectal ultrasound assessment. This was specifically observed prior to breeding and at day 7, but this difference was no longer significant at day 14. These findings support previous literature in mouse models, as Zenclussen *et al.* used abortion-prone (DBA/2J x CBA/J) and normal pregnant mouse models (CBA/J x BALB/c) to

illustrate embryonic implantation rates and corresponding decidual T cell phenotypes (Zenclussen et al., 2005). Researchers found that in decidual samples, abortion mice expressed higher *IFN-γ,* indicative of Th1 expansion. Other studies demonstrated spontaneous abortion rates in the CBA x DBA/2 murine model, where the administration of IFN-γ increased fetal resorption rates, but neutralizing IFN-γ levels in pregnant animals decreased rates of resorption and improved pregnancy outcomes (Clark et al., 1998). More recent findings suggest that Tregs may play a part in preventing the overstimulation of IFN-γ levels that result from Th1 type immunity (Saito, 2010). Additionally, Th1 cells have been shown to be abortogenic and incompatible with successful pregnancy (Raghupathy, 1997). In human models, dominance of pro-inflammatory Th1 populations over anti-inflammatory Th2 populations is associated with pre-term labor, pre-eclampsia, and adverse neo-natal outcomes (Sykes et al., 2012). It is well described that systemic inflammation is harmful to reproductive outcomes in the equine model, as inflammatory condition such as pituitary pars intermedia disorder, laminitis, and equine metabolic syndrome are all associated with poor breeding outcomes (Burns, 2016) Additionally, the leading cause of subfertility in mares is inflammation within the endometrium, deemed endometritis (Troedsson 1993). Therefore, while the etiology of this increase in *IFN-γ* could not be determined within the confines of this study, it is interesting to note.

No changes in Treg-related transcripts *(FoxP3, CD25, TGF-β)* were noted retrospectively based on pregnancy outcomes as diagnosed by ultrasonic detection on days 0, 7, and 14 when assessed in the present study. When Aurich *et al.* (2014) isolated PBMCs from mares, FoxP3+ cells were identified through flow cytometry staining in comparison to our RT-qPCR expression analysis. Researchers reported that CD4+CD25+FoxP3+ populations were elevated specifically in the pre-breeding cells in comparison to mares that underwent early embryonic loss, and this

difference in Treg populations persisted until day 14, day 20, and day 40 of gestation (Aurich et al., 2014). Another study used an ELISA with a human TGF-β1 immunoassay (citing 95% human/equine compatibility) and found that serum levels of TGF-β in mares with successful pregnancy outcomes to be significantly higher in comparison to levels in mares that experienced early embryonic death (Krakowski et al., 2010). Based on these previous findings in the horse model, we expected to see a corresponding difference in Treg-related transcripts (*FoxP3, CD25, TGF-β*) between pregnant and non-pregnant mares, but the sole use of gene expression analysis without flow cytometry validation may have contributed to this. Additionally, the collection of peripheral blood immune cells rather than local uterine immune cells likely contributed to the non-significant changes between groups in *FoxP3* expression that we observed. In a paired study with both peripheral and decidual blood samples, Dimova *et al.* demonstrated that local Tregs were enriched 10-fold in comparison to those isolated from circulation (Dimova et al., 2011). Interestingly, there was no difference in peripheral Treg populations between pregnant and non-pregnant women, suggesting that changes in circulating Tregs may not be representative of local Tregs that are specific to the uterus.

In the present study, breeding method significantly impacted the overall expression of Treg-related *CD25* **and TGF-B** from isolated peripheral immune cells. A significant difference in *CD25* expression was noted between mares bred with fresh semen in comparison mares were bred with either cooled or frozen semen. This higher expression of *CD25* was observed specifically at day 14 and day 21 following ovulation. This is in agreement with our hypothesis that SP exposure at the time of mating would enhance Treg-related profiles. In murine studies, mating with intact males has been shown to induce the expansion of CD25+FoxP3+ Tregs at the para-aortic lymph nodes that drain to the uterus (Robertson et al., 2009). When seminal vesicles

were excised in males, thus reducing the seminal plasma fraction of the ejaculate, this expansion of CD25+FoxP3+ Tregs was not observed. While we did not observe an impact of seminal plasma reduction on *FoxP3* expression, the expression of Treg cell-surface marker *CD25* differed based on breeding method which suggests that Tregs may be altered by breeding method in the equine model. Similarly, the expression of Treg-secreted cytokine *TGF-β* tended to increase in mares bred with fresh compared to cooled and frozen semen. When specific timepoints were assessed, a similar pattern to that of *CD25* was observed with *TGF-β*, as mares bred with fresh semen experienced heightened *TGF-β* expression particularly at the day 14 timepoint. In human and murine studies, seminal-derived TGF-β impacts the inflammatory response to semen (Nocera & Chu, 1993). SP has been shown to be necessary for uterine Treg cell expansion, as mating with seminal vesicle-deficient males failed to elicit an increase in uterine Tregs (Guerin et al., 2011). In the human, seminal-derived TGF-β has been shown to mediate the pro-inflammatory response induced by insemination (Sharkey et al., 2012), but the impact of SP factors on embryo implantation and the downstream immunoregulation of pregnancy has not been fully explored in the human model (Ahmadi et al., 2022). Interestingly, clinical studies have highlighted a relationship between TGF-β from SP and subsequent IVF/ICSI success, reporting that TGF-β was higher in women that became pregnant in comparison to those who did not (Nikolaeva et al., 2016). The data in other species supports the potential relationship between SP and TGF-β in the equine model, but unfortunately, the presence of TGF-β within SP has not been specifically documented in the horse. Advancements in equine-specific immunoassays may provide future opportunities for investigating the seminal-derived TGF-β theory for programming maternal immunotolerance in the equine model.

2.5 LIMITATIONS:

This study further defined equine T cell populations from peripheral circulation before breeding and during early pregnancy by measuring mRNA expression of the Treg-related transcripts *FoxP3, CD25,* and *TGF-β*, in addition to expression of the Th1-related transcripts *TBX21* and *IFN-γ*. T cell populations, whether isolated locally or from the periphery, can be identified through molecular assessment by flow cytometry, protein quantification, or transcriptional analysis. Because this study characterized Treg-related gene expression rather that describing Treg function through flow cytometry, protein quantification should be performed in future studies to validate the biological significance of these findings. Additionally, we were not able to measure the T cell response to pregnancy specifically at the site of the uterus in this study, as all mares were client-owned and in pursuance of generating viable pregnancies. In the future, a study conducted on research mares may provide opportunities to explore how the local immunotolerance to pregnancy is maintained.

Additionally, samples were collected from client-owned mares bred to multiple commercially available stallions. Stallion information as far as the extent of SP reduction and stallion-specific cryosurvival was not obtained. Additional studies are warranted to gauge the impact of stallion on the relationship between SP, breeding method, and the resulting T cell tolerance towards the developing conceptus. Initial aims included sampling until day 35 of pregnancy to assess the impact of placentation/implantation, but there was difficulty obtaining complete sample sets from outpatient mares. In the mouse, Tregs proliferate from the time of implantation until late in gestation; this was demonstrated by Ruocco *et al.* and is suggestive of the role of embryonic implantation and Treg increase (Ruocco et al., 2014). The successful integration of the d35 timepoint may have elucidated the impact of embryonic implantation and

placentation on Treg expression in the horse. Additionally, all mares had extensive breeding histories, but the majority were multiparous and therefore had carried previous pregnancies. Previous histories of endometritis and/or endometrial health were not provided. Ideally, maiden mares would be used for future studies to truly assess naïve antigen exposure and maternal response to first inseminations. Gene expression was extremely variable between mares, reiterating the importance of increasing statistical power in future studies. The present study lacked power in the cooled and frozen groups, the extent of this trend was not fully demonstrated. However, a future study with an increased population of mares in each group may further elucidate the relationship between breeding method and pregnancy outcomes.

This study evaluated peripheral Treg-related transcripts in mares before breeding and throughout early pregnancy to investigate an association with pregnancy establishment. Relative *FoxP3* mRNA expression in PBMCs demonstrated a gradual increase during early pregnancy, plateauing by day 28. Although these increases did not reach statistical significance across timepoints, pregnant mares exhibited higher *FoxP3* expression compared to mares later determined to be non-pregnant, suggesting that elevated peripheral Treg-associated transcription prior to and immediately following breeding may contribute to successful pregnancy establishment. In contrast, peripheral *TGF-β* transcript levels did not differ across timepoints or by pregnancy outcome. Pregnancy status was influenced by Th1-related *IFN-γ*, with mares unable to achieve pregnancy having heightened *IFN-γ* prior to and following breeding, further supporting the negative impact of systemic inflammation prior to breeding (Troedsson et al., 2001). Breeding method significantly altered *TGF-β* and CD25 expression; both of which were heightened in mares bred by fresh semen in comparison to frozen. This further supports the hypothesis that SP exposure at the time of breeding influences immunotolerance to pregnancy

and development of regulatory environment. Collectively, these findings support a role for peripheral Treg-associated transcription in early equine pregnancy, particularly prior to and shortly after breeding, while highlighting the need for further investigation of uterine immune dynamics and protein-level validation to clarify mechanisms of maternal immunotolerance in the mare.

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