The role of Nodal in the modulation of the maternal immune system in early pregnancy

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August 2021

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

ABSTRACT

Pregnancy is an essential biological process of mammalian reproduction comprised of complex sequential events including implantation, decidualization, placentation and parturition. Pregnancy-specific complications and disorders can arise at numerous critical events during pregnancy causing issues with gestation, miscarriage, or infertility.

The first and most crucial part of pregnancy is the peri-implantation period where the fertilized blastocyst will transvers through the fallopian tubes and enter the uterine lumen where it will implant into the uterine endometrium. The process of implantation is highly regulated and requires the synchronization between the acquisition of implantation competency by the blastocyst and a receptive state in the uterine endometrium. Numerous factors such as cytokines, growth factors, and morphogens are known to be implicated in this molecular cross-talk. One of the most crucial components to the process of implantation is an adequate maternal immunological response to the semi-allogenic blastocyst. This response must be balanced between pro-inflammatory factors; promoting implantation, tissue remodeling, and vascularization, and immune tolerant factors; protecting the development and survival of the blastocyst. While the physiological role of the immune system during implantation has been characterized, the specific molecular pathways involved in modulating this response have yet to be elucidated.

Nodal, a morphogen in the transforming growth factor-beta (TGF-β) superfamily has been shown to play a critical role in early pregnancy in mice. Females devoid of Nodal signalling in the reproductive tract experience infertility due to embryo destruction and impaired implantation. In late pregnancy, these mice are sensitive to infection and experience preterm birth. Investigations into the causality of this phenotype revealed that Nodal mutant mice have a higher basal level of proinflammatory cytokines and increased macrophage infiltration in the maternal decidual tissue in late pregnancy compared to control mice. These results suggest that the Nodal signalling pathway plays a role in the modulation of the inflammatory state present in the uterus later on in pregnancy.

We hypothesized that similar to late pregnancy, Nodal plays an important role in modulating the inflammatory response present in the uterus during early pregnancy and in its absence leads to decreased fertility. Using a maternal reproductive-tract specific Nodal knockout mouse strain, we characterized the state of the immune system in the uterus during the peri-implantation period. We found that the maternal immune response was altered in the Nodal

deficient mice. These mice had increased infiltration of leukocytes within the uterine endometrium localizing near the uterine lumen. Investigations into the uterine immunological profile revealed that Nodal mutants had altered infiltration of eosinophils, macrophages, and natural killer cells. Finally, these mutants had altered expression of genes relating to immune cell migration, differentiation and response to inflammation.

Our findings therefore suggest that the Nodal signaling pathway plays an important role in modulating the maternal immune response during early pregnancy.

RÉSUMÉ

La grossesse est un processus biologique essentiel à la reproduction des mammifères, qui comprend des événements séquentiels complexes tels que l'implantation, la décidualisation, la placentation et la parturition. Une perturbation dans ces événements peut entraîner différents problèmes cliniques, notamment de l'infertilité et des complications de grossesse.

La première et la plus cruciale des étapes de la grossesse est la période péri-implantatoire. Pendant cette période, le blastocyste fécondé traverse les trompes de Fallope et pénètre dans la lumière utérine où il s'implante dans l'endomètre utérin. Le processus d'implantation est hautement régulé et nécessite la synchronisation entre l'acquisition de la compétence d'implantation par le blastocyste et un état réceptif dans l'endomètre utérin. De nombreux facteurs tels que les cytokines, les facteurs de croissance et les morphogènes sont connus pour être impliqués dans ce dialogue moléculaire. L'un des éléments les plus cruciaux au processus d'implantation est une réponse immunologique maternelle adéquate au blastocyste semi-allogénique. Cette réponse doit être équilibrée entre des facteurs pro-inflammatoires; favorisant l'implantation, et des facteurs de tolérance immunitaire; protégeant le développement et la survie du blastocyste. Bien que le rôle physiologique du système immunitaire pendant l'implantation ait été caractérisé, les voies moléculaires spécifiques impliquées dans la modulation de cette réponse doivent encore être élucidées.

Il a été démontré que Nodal, un morphogène de la superfamille du facteur de croissance transformant bêta (TGF-β), joue un rôle critique au début de la grossesse chez la souris. Les femelles transgénique, dans laquelle Nodal est supprimé conditionnellement dans les tissus reproductif, sont infertiles en raison de la destruction des embryons et d'une implantation déficiente. En fin de grossesse, ces souris sont sensibles aux infections et connaissent des naissances prématurées. Des recherches sur la causalité de ce phénotype ont révélé que les souris mutantes Nodal ont un niveau basal plus élevé de cytokines pro-inflammatoires et une infiltration accrue de macrophages dans le tissu décidual maternel en fin de grossesse par rapport aux souris contrôles. Ces résultats suggèrent que la voie de signalisation Nodal joue un rôle dans la modulation de l'état inflammatoire présent dans l'utérus plus tard dans la grossesse.

Nous avons postulé que, comme en fin de grossesse, Nodal joue un rôle important dans la modulation de la réponse inflammatoire présente dans l'utérus en début de grossesse et que son absence entraîne une baisse de fertilité.

En utilisant un modèle murin avec une inactivation conditionnelle de Nodal, nous avons caractérisé l'état du système immunitaire dans l'utérus pendant la période péri-implantatoire. Nous avons constaté que la réponse immunitaire maternelle était altérée chez les souris déficientes en Nodal. Ces souris présentaient une infiltration accrue de leucocytes dans l'endomètre utérin, localisée près de la lumière utérine. L'étude du profil immunologique de l'utérus a révélé que les mutants de Nodal présentaient une infiltration modifiée d'éosinophiles, de macrophages et de cellules tueuses naturelles. De plus, ces mutants présentaient une expression altérée des gènes impliqués dans la migration, la différenciation et la réponse à l'inflammation des cellules immunitaires.

Ainsi, nos résultats suggèrent que la voie de signalisation Nodal joue un rôle important dans la modulation de la réponse immunitaire maternelle en début de grossesse.

ACKNOWLEDGMENTS

First, I would like to express my gratitude to my supervisor and mentor, Dr. Daniel Dufort, for providing me with guidance and moral support throughout my graduate studies. Thank you for your continual encouragement and for providing a positive and caring work environment. I also want to thank the members of my advisory committee Dr. Indra Gupta, Dr. Ciriaco Piccirillo, and Dr. Elham Rahme for their expert advice and feedback throughout this project.

I would like to attribute a special thanks to my colleagues and friends which I had the privilege to work alongside during my studies. I am very grateful to Shiva Shafiei, Taghreed Ayash, Luz Esther Ramos Ballesteros, and Suba Rana for providing assistance, support, and contributing to a great work environment. Importantly, I would like to thank my best friend and partner Marc-André for his unwavering support throughout all my studies. Thank you for your friendship, empathy, patience, and love.

Lastly, I would like to dedicate this manuscript to my parents Joyce and Sylvain, who have always encouraged me, challenged me, and supported me every step of the way. Thank you for your love, sacrifice, and dedication. I am very grateful to have an amazing support system that I could not have succeeded without.

CONTRIBUTION OF AUTHORS

The candidate Godin Pagé M., performed the experiments presented in this manuscript.
Godin Pagé M. performed the data analysis and figure preparation for all experiments.
Godin Pagé M. and Dufort D. wrote the manuscript.

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1 INTRODUCTION

1.1. Female reproduction and infertility

Reproduction is an essential biological process by which genetic material of the proceeding parental generation is passed on to produce offspring. Since reproduction is responsible for the existence of all living organisms, this process is vital for the success and survival of a species. In mammals, haploid gametes from a fertile female (oocyte) and male (spermatozoa) will fuse, giving rise to a diploid embryo. This process of fertilization occurs internally in the fallopian tubes of the female reproductive tract. The developing embryo will then transverse through the oviduct and enter the uterine lumen, where it will implant into the endometrial wall of the uterus. Thereafter, the developing placenta will mediate the exchange of resources such as nutrients, gases, and waste between the maternal and fetal blood circulation. Moreover, the placenta will also provide immunological protection to the fetus throughout pregnancy (Georgiades et al., 2002).The establishment of a healthy pregnancy and the success of this pregnancy throughout the term is dependent on many factors. Pregnancy-specific complications and disorders can arise at numerous critical events during pregnancy, causing issues with gestation, miscarriage, or infertility.

Infertility, defined as the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse, is a common clinical issue affecting approximately 48 million couples and 186 million individuals worldwide (WHO, 2018). Infertility can be caused by many different factors in either the male or female reproductive systems. In females, causes include *tubal disorders* such as blocked fallopian tubes, *uterine disorders* such as endometriosis, *disorders of the ovaries* such as polycystic ovarian syndrome, *hormonal disorders*, *genetic disorders*, or even *environmental toxicology factors* (WHO, 2018). However, there is evidence showing that most pregnancy loss arises from defects that occur immediately before or during the process of embryo implantation (Wang & Dey, 2006). Despite extensive research on causes of infertility, approximately 15-30% of cases remain idiopathic (Quaas & Dokras, 2008).

Many causes of infertility can be overcome through the use of assisted reproductive technologies (ART), such as in-vitro fertilization (IVF); however, the success rates of these techniques are still low. The main reason for this low success rate is issues arising with the embryo's implantation into the uterine endometrium. For this reason, many patients experience recurrent implantation failure (RIF), where women have three failed IVF attempts with good-quality embryos (Bashiri, 2018). Many known risk factors have been associated with RIF, including advanced maternal age, smoking status of both parents, elevated body mass index, and stress levels (Bashiri, 2018). However, there are more complex risk factors that have been shown to affect the incidence of RIF. Immunological factors such as cytokines levels, the presence of endometrial leukocytes, and HLA compatibility between partners have been shown to play a role in the occurrence of RIF (Bashiri, 2018; Simon & Laufer, 2012; Timeva et al., 2014). Despite advancements in ART, the role of the maternal immune system during implantation and the regulatory mechanisms involved in this process remain unclear. Understanding the role of the maternal immune system and its modulation in early pregnancy can allow for the development of new therapeutic targets to decrease the incidence of RIF and infertility.

1.2. The peri-implantation period

The first and most crucial part of pregnancy is the peri-implantation period which encompasses the time from fertilization to implantation. During this time, the fertilized ovum (zygote), encased in a non-adhesive protective coating called the zona pellucida, will undergo several rounds of mitotic cell division, becoming a mass of 12 to 16 cells (morula) (Cockburn & Rossant, 2010; Norwitz et al., 2001). These initial stages of development take place as the embryo passes through the fallopian tube. The morula will then enter the uterine cavity, where it will further develop into a blastocyst (Fig.1)(NIH, 2016). The early blastocyst stage is marked by the emergence of a fluid-filled inner cavity within the mass of cells and the appearance of different cell lineages within the blastocyst: the surface cells become the trophectoderm (progenitors of trophoblast cells which will give rise to extraembryonic structures such as the placenta), and the inner cell mass which will give rise to future cell lineages of the embryo proper (Norwitz et al., 2001; Wang & Dey, 2006).

This blastocyst will then hatch from the zona pellucida and gain implantation competency. The cells of the trophectoderm will make the first physical and physiological connection with the luminal epithelium of the maternal uterus, eliciting the process of implantation (Wang & Dey, 2006).

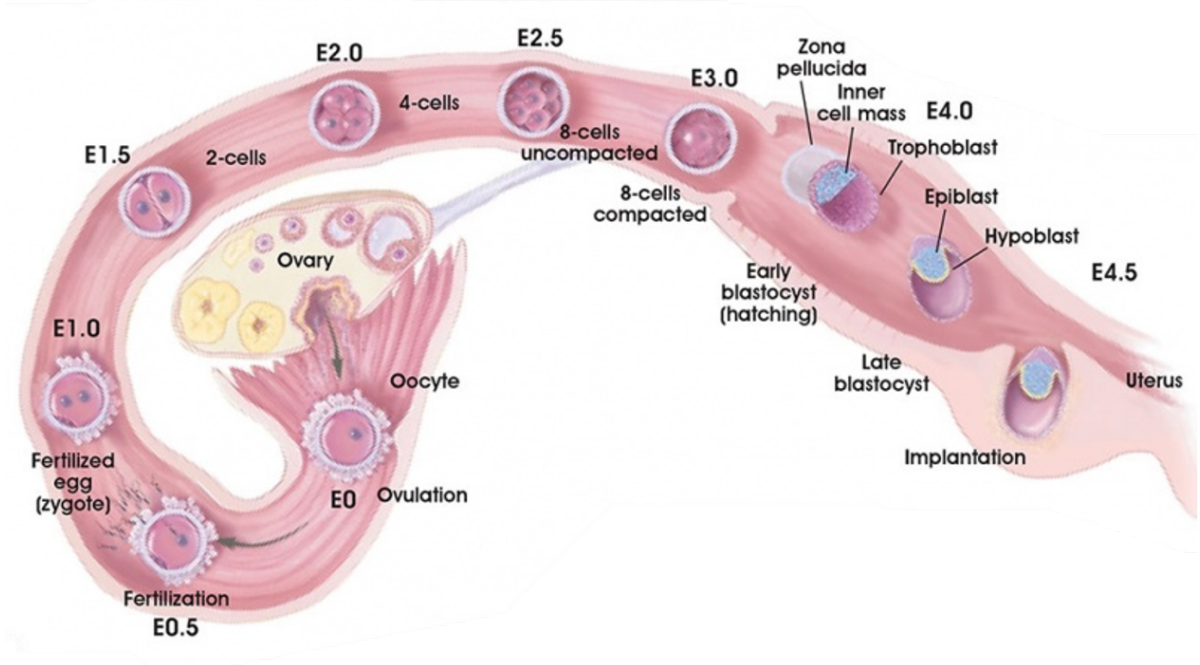

Figure 1 – Pre-implantation embryo development and implantation in mice. Oocytes are fertilized in the oviduct forming a zygote. The embryo undergoes several rounds of mitotic cell division while moving through the fallopian tube, ultimately forming a mass of compacted cells called the morula. This developing morula will enter the uterine cavity and develop into a blastocyst containing a cavity (blastocoel) and two distinct cell populations; the inner cell mass and the trophectoderm. This early blastocyst will hatch and escape from the zona pellucida and differentiate to produce additional cell types. The process of implantation will be initiated when the trophectoderm of the competent blastocyst attaches to the epithelial lining of the uterus. The figure is from NIH, 2016.

1.3. Embryo implantation

Implantation is defined as the process by which a blastocyst attaches to the endometrial surface of the uterus and invades the epithelium (Kim & Kim, 2017). During this time, the uterine endometrium undergoes various morphological and physiological alterations that will delineate the fate of the pregnancy. This process is highly organized and involves the interaction between a receptive uterus and a competent blastocyst. Uterine receptivity is established under the primary control of ovarian steroid hormones progesterone and estrogen. This is accompanied by histological changes, including an increase in endometrium vascularization and edema, an increase in the secretory activity of endometrial glands, and the development of pinopdes on the luminal surface of the epithelium (Fig.2) (Fujiwara et al., 2020; Norwitz et al., 2001). Then, reciprocal signaling between the receptive uterus and implanting embryo must render the blastocyst competent to endometrial attachment and penetration. The period during which the uterus is receptive and the blastocyst is competent to implantation is termed the 'window of implantation' (Su & Fazleabas, 2015; Wang & Dey, 2006).

Uterine receptivity is divided into three stages, namely, *pre-receptive*, *receptive*, and *refractory*. In humans, these stages are regulated by the ovarian-endometrial (menstrual) cycle (Kim & Kim, 2017). Following ovulation, the uterus enters the secretory phase characterized by increasing progesterone and decreasing estrogen concentrations. From the time of ovulation to approximately seven days after, the uterus is considered pre-receptive and is unable to initiate implantation. However, the luminal environment of the uterus is less hostile to the blastocyst allowing for its survival (Wang & Dey, 2006). From day 7-10 post-ovulation, the uterus enters a receptive state that is sensitive to embryo implantation. This is marked by the continued progesterone production by the corpus luteum, which will stimulate proliferation and differentiation of uterine epithelial and stromal cells (Norwitz et al., 2001). Moreover, downstream effectors of steroid hormones, including peptide hormones, growth factors, cytokines, and morphogens, underlie uterine receptivity and partake in the molecular cross-talk (Fig. 2) (Singh et al., 2011). Cytokines such as leukemia inhibiting factor (LIF) have been shown to have increased expression in the luminal and glandular epithelium of the uterus during this time, and diminished secretions of this factor are associated with RIF (Norwitz et al., 2001; Wang & Dey, 2006). Numerous growth factors, such as transforming growth factor-β (TGF-β), epidermal growth factor (EGF), heparin binding-

epidermal growth factor (HG-EGF), and insulin-like growth factor (IGF) are essential for receptivity and exhibit various implantation defects when disrupted (Singh et al., 2011). At the end of receptivity, the uterus enters the refractory stage approximately ten days after ovulation. At this time, the uterus is non-receptive to implantation, and the uterine environment is unfavorable to blastocyst survival (Wang & Dey, 2006). If fertilization did not occur, the uterus would remain refractory throughout the menstrual cycle until the next ovulation and secretory phase.

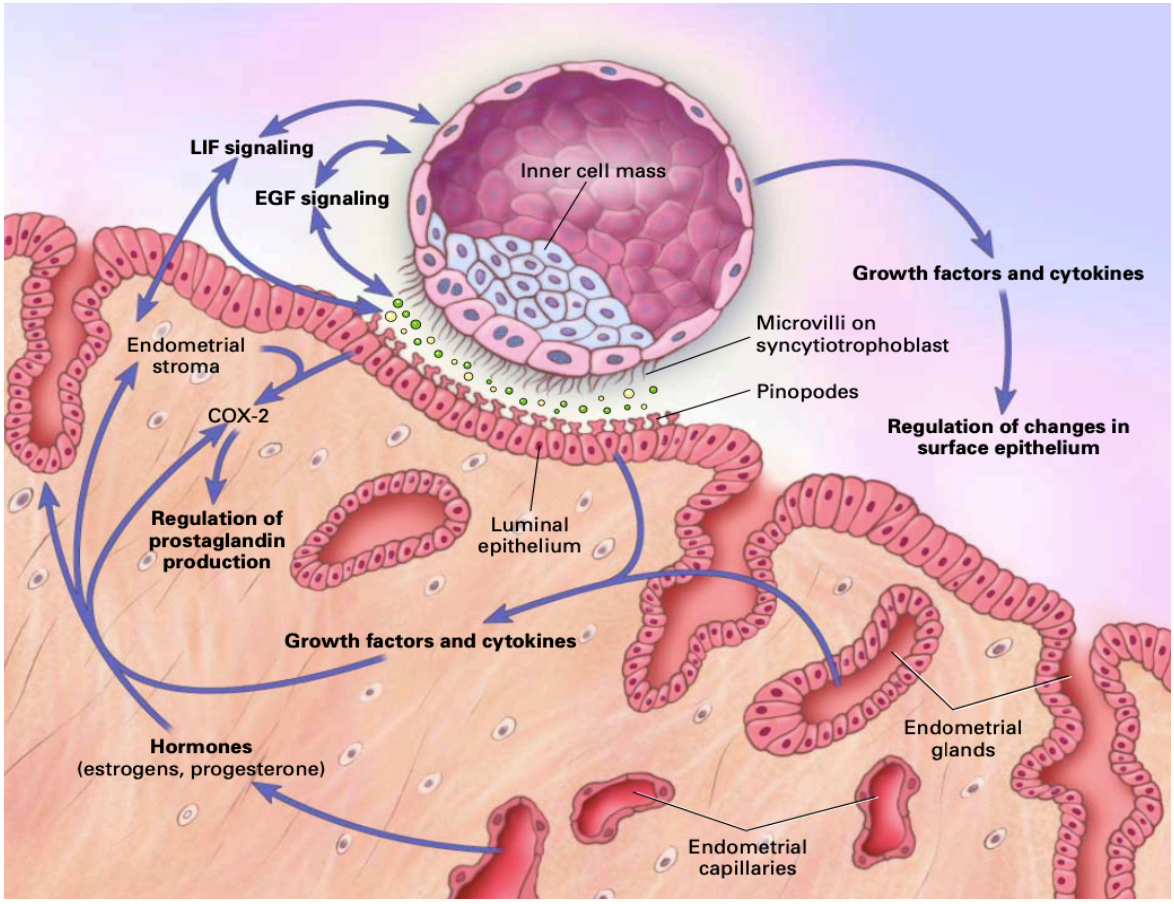

Figure 2 – Molecular cross-talk between pre-implantation embryo and receptive uterus. The diagram shows a preimplantation-stage blastocyst and the factors that play role in uterine receptivity and blastocyst implantation. Figure from Norwitz et al., 2001.

The physical process of embryo implantation consists of three stages that occur after the molecular cross-talk is initiated. First, implantation begins with *apposition* of the blastocyst at the uterine epithelium, where the luminal closure of the uterus positions the blastocyst adjacent to the uterine wall (Singh et al., 2011). Moreover, the trophoblast cells of the blastocyst will adhere loosely to the endometrial epithelium through interactions with pinopodes. Subsequently, during *attachment*, the blastocyst will form a stable adhesion to the uterine endometrial wall. Cell adhesion of the blastocyst and endometrial epithelial cells is mediated by cell adhesion molecules, including integrins, cadherins, selectins, and immunoglobulins (Kim & Kim, 2017). Finally, the fetal trophoblast cells will *invade* and penetrate through the luminal epithelium into the underlying stromal compartments of the maternal decidua. This invasion will allow for the establishment of a vascular relationship between the implanted embryo and mother (Kim & Kim, 2017). A critical factor in endometrial vascularization and tissue remodeling during implantation is the presence of an active immunological response. Many leukocytes will infiltrate into the endometrium during peri-implantation to facilitate embryo attachment and invasion. Evidence suggests that successful implantation depends on various immune cell types and the fine balance between inflammatory and anti-inflammatory processes (Zenclussen & Hämmerling, 2015). However, the regulatory mechanism(s) that modulates the maternal immunological reaction is not adequately understood.

1.4. The maternal immune system during pregnancy

There is a dynamic immunological landscape present in the uterine tissue during the course of pregnancy. The first phase of the female response resembles a classic inflammatory reaction often compared to a wound healing process, where a large population of leukocytes infiltrate the uterine endometrium and produce high levels of pro-inflammatory cytokines (Schjenken & Robertson, 2020). This initial inflammatory response is short-lived and has been shown to be necessary for implantation, tissue remodeling and vascularization (Dekel et al., 2014; Schjenken & Robertson, 2020). After implantation, there is an immunological shift to an anti-inflammatory state. During this time, the mother, placenta, and fetus are symbiotic, and will undergo of rapid fetal growth and development (Mor et al., 2011).

Finally, in preparation of delivery, another shift back to a pro-inflammatory state occurs to initiate the parturition cascade (Cappelletti et al., 2016). Any alterations in the timing or magnitude of these reactions can cause pregnancy complications such as implantation failure, preterm birth or miscarriage.

1.5. The maternal immune system during the peri-implantation period

The maternal immune reaction during pregnancy is initiated by the presence of seminal fluid in the cervix, where seminal fluid factors interact with epithelial cells and immune cells in the mucosal lining of reproductive tissues (Schjenken & Robertson, 2020). Active factors in the seminal fluid, including TGF-β and Toll-like receptor ligands, will bind to receptors on target cells and activate transduction pathways affecting gene expression and cell function in the uterine tissues (Schjenken et al., 2015; Sharkey et al., 2012). This will induce the synthesis of pro-inflammatory cytokines and chemokines such as tumor necrosis factor-alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and interleukin 12 (IL-12) (Fig. 3) (Schjenken & Robertson, 2020; Zhang et al., 2017). Increased expression of these factors by the uterine cells will recruit and activate various inflammatory leukocytes, drawing them into the stromal tissue. Moreover, considering that the implanting blastocyst is semi-allogenic (expressing both maternal and paternal antigens), the maternal immune system must have dual functions: one, support implantation, tissue remodeling, and vascularization through the expression of pro-inflammatory factors, and the other, prevent the maternal immune system from attacking the semi-allogenic blastocyst through immune tolerance (Yoshinaga, 2012). Therefore, various mechanisms mediate an active state of functional tolerance, permitting fetal and maternal cells to coexist (Robertson et al., 2018). Ultimately, the immunological profile (which types of immune cells are present), their abundance (relative quantities of each immune cell type), and their respective response (expression of different cytokines and chemokines) will determine the overall immunological state of the uterine tissue and will have an impact during the peri-implantation period.

1.5.1 Neutrophils

Neutrophils are short-lived and are recruited from the circulation into the female reproductive tract after mating. These cells will enter the uterine lumen, clear excess sperm, and seminal debris, and maintain the microbial balance of the uterus (Fig. 3) (Schjenken & Robertson, 2020). Neutrophils are mostly known for their cytotoxic role and their ability to efficiently clear microorganisms and tissue debris (Ley et al., 2018). However, decidual neutrophils (dNs) are less cytotoxic and produce more angiogenic cytokines than peripheral blood neutrophils (PMNs) (Smith et al., 2006). It was shown that the angiogenic effects of dNs are partly mediated indirectly through T cell induction, revealing a new role of neutrophils as antigen-presenting cells that can regulate the adaptive immune response (Nadkarni et al., 2016). In fact, dNs exposed to pregnancy hormones were shown to promote the establishment of maternal tolerance through the induction of a specific population of T cells called T regulatory cells (Tregs) (described further below) (Nadkarni et al., 2016). Overall, these results suggest that neutrophils, while still having some cytotoxic behavior, have a role in angiogenesis and immune tolerance. However, limited research has been done to understand the role of dNs in implantation. Mouse models and functional studies with specific depletion of dNs will be required to further understand the role of neutrophils in pregnancy.

1.5.2 Eosinophils

Eosinophils, similar to neutrophils and basophils, are granulocyte cells of the innate immune system derived from a common myeloblast progenitor cell. Systemically, eosinophils are known for their cytotoxic effector functions, causing damage to parasitic pathogens and host tissues in allergic diseases (Marichal et al., 2017). These cells can migrate to the site of infection, releasing cytotoxic granule proteins, cytokines, and lipid mediators. However, increasing evidence suggests that eosinophils can also play a role in regulating inflammation and presenting antigens (Shamri et al., 2011; Wen & Rothenberg, 2016). Due to the low abundance of eosinophils located in the uterine tissue during early pregnancy, they do not seem to play an important role in the establishment and maintenance of pregnancy. For this reason, limited research has been done evaluating the impacts of eosinophils in early pregnancy.

1.5.3 Macrophages

Macrophages are one of the most abundant cell types present in the uterus during early pregnancy. Low levels of decidual macrophages are found in the human and rodent uterus before mating. Following copulation, there is a significant infiltration of macrophages into the endometrium, which will thereafter decline after implantation (De et al., 1991; Robertson et al., 1996). At this time, decidual macrophages are activated and will carry paternal antigens to the draining lymph nodes to present antigens to T cells. Moreover, macrophages are known for their plasticity and heterogeneity and therefore have many roles throughout pregnancy. M1 macrophages or classically activated macrophages are more effective at antigen clearance and express pro-inflammatory cytokines. Conversely, M2 macrophages are alternatively activated and have an immunosuppressive phenotype, and will contribute to tissue remodeling (Lee et al., 2011; Zhang et al., 2017).

It has been shown that the activation state and function of decidual macrophages are dependent on the local tissue microenvironment. Therefore, macrophage polarization often fluctuates throughout pregnancy and can be disrupted by changes in the uterine microenvironment (Zhang et al., 2017).

After mating, macrophages are induced toward M1 activation due to the presence of foreign paternal antigens in the cervix (Jaiswal et al., 2012; Zhang et al., 2017). However, as macrophages interact with invading trophoblast cells during implantation, these cells switch to a mixed M1/M2 profile and predominantly become M2 activated thereafter (Heikkinen et al., 2003; Jaiswal et al., 2012; Zenclussen & Hämmerling, 2015; Zhang et al., 2017). These cells secrete enzymes and signaling molecules that modify the structure of the luminal epithelial glycocalyx and stromal extracellular matrix to facilitate embryo attachment (Jasper et al., 2011; Schjenken & Robertson, 2020). Moreover, decidual macrophages also promote vascularization at the implantation site through the synthesis of vascular epithelial growth factor (VEGF) (Zhang et al., 2017). These crucial roles of macrophages make them necessary in early pregnancy. In fact, mice depleted of decidual macrophages in early pregnancy experience implantation failure resulting in pregnancy loss (Care et al., 2013; Ono et al., 2020). Moreover, the fine balance between M1 and M2 activated macrophages in early pregnancy will also affect the pregnancy outcome. Studies have shown that higher levels of M1 macrophages are observed at the maternal-fetal interface in abnormal pregnancies (Yao et al., 2019)

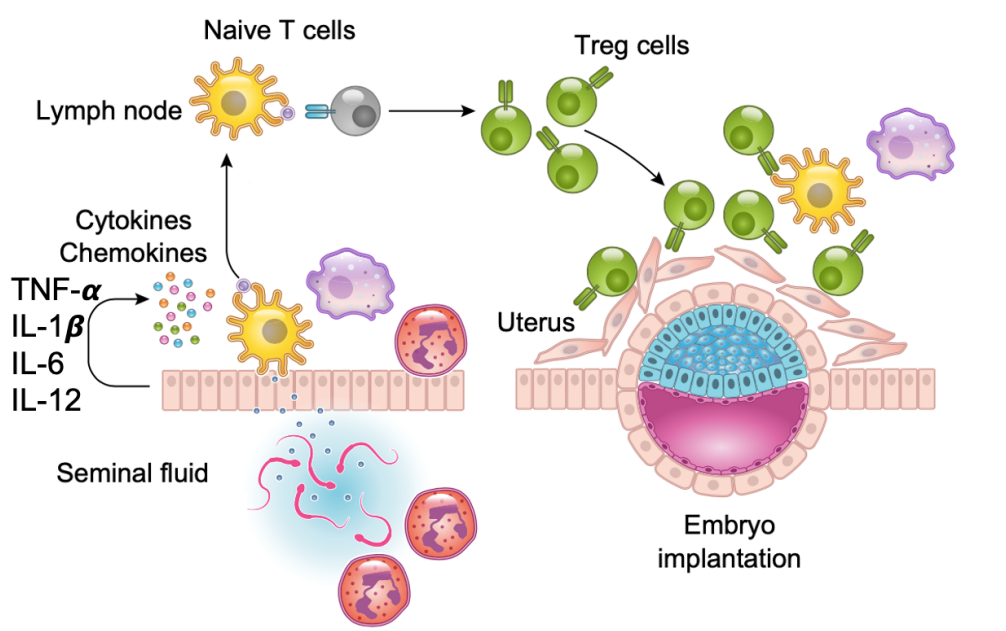

Figure 3 – Maternal immune response to the presence of seminal fluid in the female reproductive tract. Seminal fluid in the cervix and uterus will elicit an immunological response where pro-inflammatory cytokines and chemokines such as TNF-***α***, IL-1***β***, IL-6, and IL-12 will be produced by epithelial cells and residing uterine immune cells. These factors will recruit and activate leukocytes, drawing them into the stromal tissue subjacent to the epithelial surface and into the cervical canal and uterine lumen. Neutrophils (pink) will enter the uterine lumen, clear excess sperm, and seminal debris, and promote angiogenesis and immune tolerance through T cell induction. Macrophages (purple) and dendritic cells (yellow) contribute to endometrial tissue remodeling and receptivity for implantation. These uterine-specific cells will also carry paternal antigens back to the draining lymph nodes to prime naïve T cells (grey) to generate a population of T regulatory (Treg) cells. Treg cells (green) recirculate via the peripheral blood and traffic into the endometrium before implantation to constrain inflammation and mediate immune tolerance of the semi-allogeneic embryo. Figure modified from Schjenken & Robertson, 2020.

1.5.4 Dendritic cells

Similar to macrophages, uterine dendritic cells (uDCs) are also important in early pregnancy due to their ability to present antigens to T cells (Fig. 3). uDCs can be detected in the non-pregnant endometrium of humans and rodents. After mating, there is an accumulation of uDCs located in cluster-like structures along the uterus, indicating future implantation sites (Zenclussen et al., 2013). Like most immune cells found in the uterus during early pregnancy, a unique subpopulation of DCs are present in the uterine endometrium capable of activating inducible Tregs (Hsu et al., 2012). uDCs have also been shown to play an essential role in uterine tissue remodeling and angiogenesis through the expression of soluble factors such as fms-like tyrosine kinase-1 (sFlt-

1) and TGF-β, which promote blood vessel maturation (Plaks et al., 2008). In addition, mice depleted of uDCs are unable to implant due to failures to regulate uterine receptivity, indicating that these cells are necessary for proper implantation (Krey et al., 2008; Plaks et al., 2008).

1.5.5 Natural killer cells

Uterine natural killer (uNKs) cells are the most abundant immune cell population present in the uterus during early pregnancy (Hatta et al., 2011). In humans, uNK numbers fluctuate during the menstrual cycle; however, in mice, they are present in comparable numbers throughout the estrous cycle and will expand if pregnancy occurs (King et al., 1996; Bilinski et al., 2008). Peripheral NKs (pNKs) are known for their cytotoxic role in killing infected cells, protecting against disease, and secreting pro-inflammatory cytokines, which act on other immune cells to enhance the immune response (Abel et al., 2018). However, like most immune cells present in the uterus during early pregnancy, uNK cells have a different phenotype and function than pNK cells. uNK cells do not express the common NK markers such as NK1.1 and DX5 and also binds specifically to Dolichos biflorus agglutin (DBA) (Paffaro et al., 2003; Poli et al., 2009). uNK cells secrete pro-angiogenic factors such as VEGF, placental growth factor (PGF), and Delta-like ligand, making them key regulators of the maternal uterine vasculature remodeling. Moreover, these cells secrete local interferon (IFN)-γ that is necessary and sufficient for the initiation of spiral artery remodeling (Lima et al., 2014; Zenclussen & Hämmerling, 2015). Mice with a depletion of uNK cells have pregnancies exhibiting fetal growth restrictions indicating that uNK cells are required for proper placental development through spiral artery remodeling and adequate trophoblast invasion (Guimond et al., 1997; Rätsep et al., 2015).

1.5.6 Regulatory T cells

An important function of the maternal immune system during early pregnancy is to induce tolerance mechanisms so that the fetus is not rejected by classical immune mechanisms. Due to the bidirectional cell trafficking between mother and fetus, antigen presentation and recognition can occur anywhere in the maternal immune system (Vernochet et al., 2007). Studies have shown that paternal antigens are present in several immune and non-immune maternal organs during the whole period of pregnancy (Mor & Cardenas, 2010; Zenclussen & Hämmerling, 2015). Therefore,

a systemic change in the maternal immune system towards an immunological tolerant state needs to be established early in pregnancy. Primarily, this tolerance is generated through the activation and expansion of a particular T cell type; CD4+Foxp3+ Tregs. These specialized cells are found in clusters in mouse uterine tissues during the receptive phase of the estrous cycle (Teles et al., 2013). After mating, the presence of seminal fluid in the vaginal lumen will elicit an expanded uterine Treg cell pool through antigen presentation by other innate immune cells (Fig. 3)(Robertson et al., 2009).

In early pregnancy, Tregs diminish the inflammation, which otherwise may be too strong and hinder the implantation and survival of the embryo. These cells act through various mechanisms, generally involving suppression of cytokine and effector function in T cells, B cells, NK cells, dendritic cells, and macrophages (Shevach, 2002). Mice depleted of Tregs prior to mating show severely impaired implantation, increased expression of inflammatory mediators and, inflamed and fibrotic uterine tissue (Teles et al., 2013). Thus, Tregs are essential in early pregnancy, and without them, the uterine environment is hostile, hindering embryo implantation and survival.

Much research has been devoted to understanding which cells are essential in early pregnancy and their respective roles during implantation. However, the regulatory mechanisms modulating the duality between pro-inflammatory and immune tolerance are not well understood. There is some evidence that the Nodal signaling pathway might be involved in the modulation of this immune response in early pregnancy.

1.6. Nodal

Nodal is a morphogen in the transforming growth factor-beta (TGF-β) superfamily of signaling molecules. Nodal, along with other signaling molecules in the TGF-β superfamily, including activin, inhibin, bone morphogenetic protein (BMP) and, growth-determining factor (GDF), is involved in various biological processes (Piek et al., 1999).

Nodal was first identified in transgenic mice through a retroviral-induced recessive lethal embryonic mutation (termed 413.d). This mutation caused severe gastrulation defects beginning on day 7.5 post coitum (Conlon et al. 1991, Iannaccone et al. 1992). Insertional mutation of a novel TGF-β-like gene, *Nodal*, which mapped alongside 413.d, recapitulated previously observed gastrulation defects. In addition, these mice experienced hyperplasia of the embryonic and extraembryonic ectoderm, resulting in failed mesoderm induction, leading to embryonic lethality by day 10.5 (Iannaccone et al. 1992, Zhou et al. 1993). Together, these results highlighted the essential role of Nodal during mesoderm and endoderm induction. Since its discovery, many studies have identified Nodal as a critical mediator of processes during embryogenesis, including left-right axis specification, anterior-posterior patterning, and neural patterning (Brennan et al. 2002, Takaoka et al. 2006).

Nodal homologs have been identified in all chordates except in Drosophila and Caenorhabditis Elegans (Schier & Shen 2000). Nodal was identified as a single protein in some species, including humans, mice, and chicks. However, several species, including Zebrafish and Amphioxus, have multiple Nodal-related proteins (Feldman et al. 1998, Takahashi et al. 2000). Subsequently, all Nodal pathway and function discussed in this thesis will focus on the singular mammalian ligand.

1.7. Nodal signaling pathway

Nodal is translated as a precursor pro-protein (38 kDa) that is post-translationally glycosylated (42kD) before exocytosis (Blanchet et al., 2008). The secreted protein has an N-terminal prodomain and a C-terminal ligand-binding domain. To activate the signaling pathway, the secreted Nodal precursor protein must be proteolytically cleaved by convertases Furin (SPC-1) and Pace4 (SPC-4) to release its mature (12 kD) form (Fig.4) (Beck et al., 2002). Due to the instability of the mature Nodal protein, this proteolytic cleavage occurs near the target cell's surface (Le Good et al., 2005; Tian and Meng, 2006).

To activate the Nodal signaling pathway, mature Nodal signals through an extracellular membrane-bound receptor complex comprised of a type I activin receptor-like kinase (ALK4/ALK7) and type II activin receptor (ActRIIA/ActRIIB) (Schier, 2009). Nodal activity also requires a glycosylphosphatidylinositol (GPI)-linked co-receptor, EGF-CFC (Cripto/Cryptic), to

establish signaling transduction (Reissmann et al., 2001; Gritsman et al., 1999). Cripto/Cryptic recruits the convertases Furin and PACE4 to the receptor complex and facilitates Nodal binding to the ALK4/7 (Blanchet et al., 2008). As a result of the complex formation, ActRIIA/B phosphorylates and activates ALK4/7, which in turn phosphorylates intracellular signal transducers, Smad2 and Smad3 (Kumar et al., 2001). The Smad2/3 complex recruits and phosphorylates Smad4, which then translocates into the nucleus and associates with additional transcription factors (FoxH1, Mixer, p53) to facilitate DNA binding and regulate target gene expression (Fig. 4) (Cruz et al., 2015, Kumar et al., 2001, Shen, 2007).

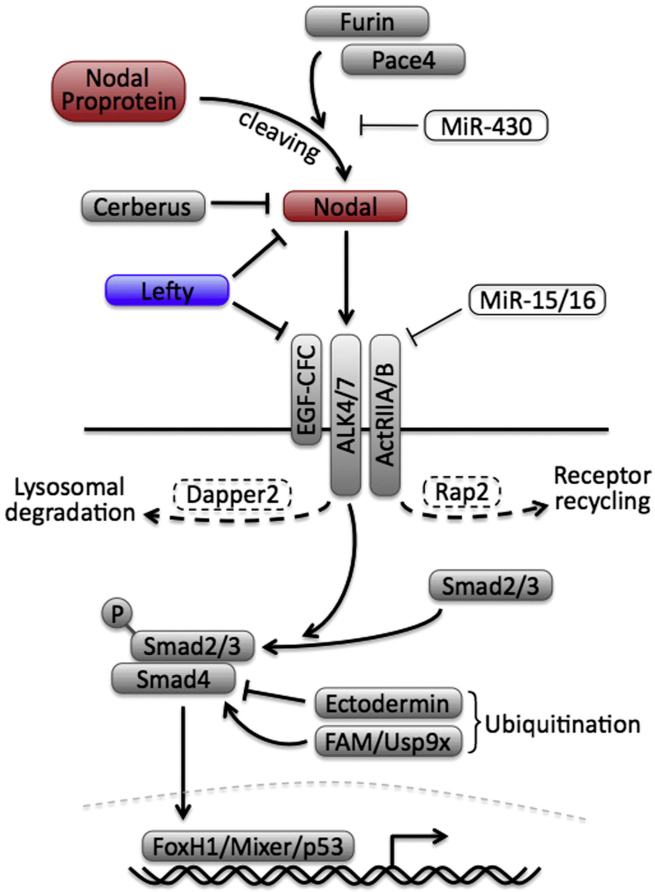

Figure 4 – Nodal signaling pathway. Precursor Nodal pro-protein is cleaved by the convertases Furin and Pace4. Nodal ligand, along with the receptors ALK4/7 and ActIIA/B and the co-receptor EGF-CFC, creates the ligand-receptor complex that phosphorylates Smad2/3, which then phosphorylates Smad 4 and forms a complex that translocates to the nucleus. The activation of the target genes is mediated by different transcription factors, including FoxH1, Mixer, and p53. The extracellular inhibitors Lefty and Cerberus negatively regulate Nodal signaling by binding directly to Nodal and the co-receptor EGF-CFC. At the receptor level, Nodal is positively and negatively regulated by Rap2 and Dapper2, respectively. Other regulators of this signaling pathway include Ectodermin and FAM/Usp9x, which regulate Smad4 activity, and microRNAs, which affect translation of Nodal, Lefty, and ActIIA/B mRNA. The figure is from Park and Dufort, 2011.

In addition to the post-translational modifications and proteolytic cleavage, there are many extracellular regulators of Nodal expression. For example, Lefty, a divergent member of the TGF-β superfamily, antagonizes Nodal signaling by blocking the EGF-CFC co-receptor and binding directly to the Nodal ligand (Fig. 4) (Cheng et al., 2004). Cerberus, a signaling molecule in the Dan family, is also a potent inhibitor of Nodal signaling in mice (Perea-Gomez et al., 2002). At the receptor level, Nodal signaling is positively and negatively regulated by Rap2 and Dapper2, respectively (Fig. 4). Rap2 enhances Nodal signaling by preventing the degradation and increasing the recycling of the activin receptors in the receptor complex (Choi et al., 2008). Conversely, Dapper2 stimulates lysosome-mediate degradation of these receptors limiting Nodal activity (Zhang et al., 2004). Other regulators of this signaling pathway include Ectodermin and FAM/Usp9x, which regulate Smad4 activity, and microRNAs, which affect translation of Nodal, Lefty, and ActIIA/B mRNA (Dupont et al., 2009; Martello et al., 2007).

Nodal, like all morphogens, can diffuse over a long range within tissues and act directly on distant cells in a concentration-dependent manner (Chen & Schier 2001). This morphogenic property gives rise to differing target gene expression at varying Nodal protein concentrations. Moreover, like most morphogens, the most common result of Nodal signaling is the production of more Nodal ligand as well as its inhibitor, Lefty. As a result, Nodal and Lefty generate a complex pattern of positive and negative regulation, adding to the complexity of this signaling cascade (Schier, 2009).

1.8. The role of Nodal in early pregnancy

1.8.1 Nodal expression in early pregnancy

In humans, it has been shown that Nodal is expressed in uterine tissues throughout the menstrual cycle. Nodal mRNA is first detected during the early-proliferative phase, increases during the late-proliferative and early-secretory stages, and then significantly declines at the mid-secretory phase (Papageorigou et al., 2009). Histologically, Nodal was detectable throughout the uterine stroma, luminal epithelium, and glandular epithelium throughout the menstrual cycle.

In addition, Nodal and Lefty protein was detected in uterine fluid samples, indicating that these proteins are secreted into the lumen of the uterus (Papageorigou et al., 2009).

Interestingly, some studies have correlated the incidence of infertility with a disruption in the Nodal signaling pathway, where infertile women had high levels of Lefty protein and low levels of Nodal protein in the uterine fluid during the peri-implantation period compared to fertile women (Tabibzadeh et al., 2000). Similarly, in mice, in vivo gene transfer of exogenous Lefty led to a high incidence of implantation failure. Therefore, increasing Lefty expression in the uterine lumen, thereby affecting Nodal signaling, adversely affects embryo implantation in mice (Tang et al., 2005a). These findings suggested that the dysregulation of the Nodal signaling pathway might be involved in infertility (Papageorgiou et al. 2009, Tabibzadeh et al., 2000). However, due to the logistical and ethical constraints, identifying the mechanistic role of Nodal in early pregnancy in humans is not possible. Therefore, Dufort et al. have developed mice models to identify the potential role of Nodal in pregnancy.

To identify Nodal expression in the mouse uterus, a Nodal-LacZ reporter mouse strain was used, showing that Nodal is not expressed at any point during the estrus cycle in non-pregnant uteri (Fig. 5 a-c)(Park & Dufort, 2011). In contrast, Lefty expression is present in the endometrium at all stages of the estrus cycle in non-pregnant mice (Park & Dufort 2013, Tang et al. 2005b). However, during the peri-implantation period (day 0.5-3.5), Nodal expression is detected throughout the whole uterus. Transverse sections indicate that the expression is restricted to the glandular epithelium (Fig. 5 d-f). Interestingly, on day 4.5 of pregnancy, Nodal is expressed in a band-like pattern along the proximal-distal axis of the mouse uterine horns where regions of high expression occur at the inter-implantation sites and regions of repressed expression arise at implantation sites (Fig. 5 g-i)(Park & Dufort, 2011). This expression pattern was shown to be controlled by the embryo, given that Nodal was not detectable on day 4.5 in pseudopregnant Nodal-LacZ mice mated with vasectomized males (Park & Dufort, 2011). To confirm the embryo-dependent Nodal expression, Park & Dufort transferred healthy blastocysts into pseudopregnant Nodal-LacZ females. These females were able to recover the banding pattern of Nodal expression, indicating that biological factors of the embryo are required for Nodal expression.

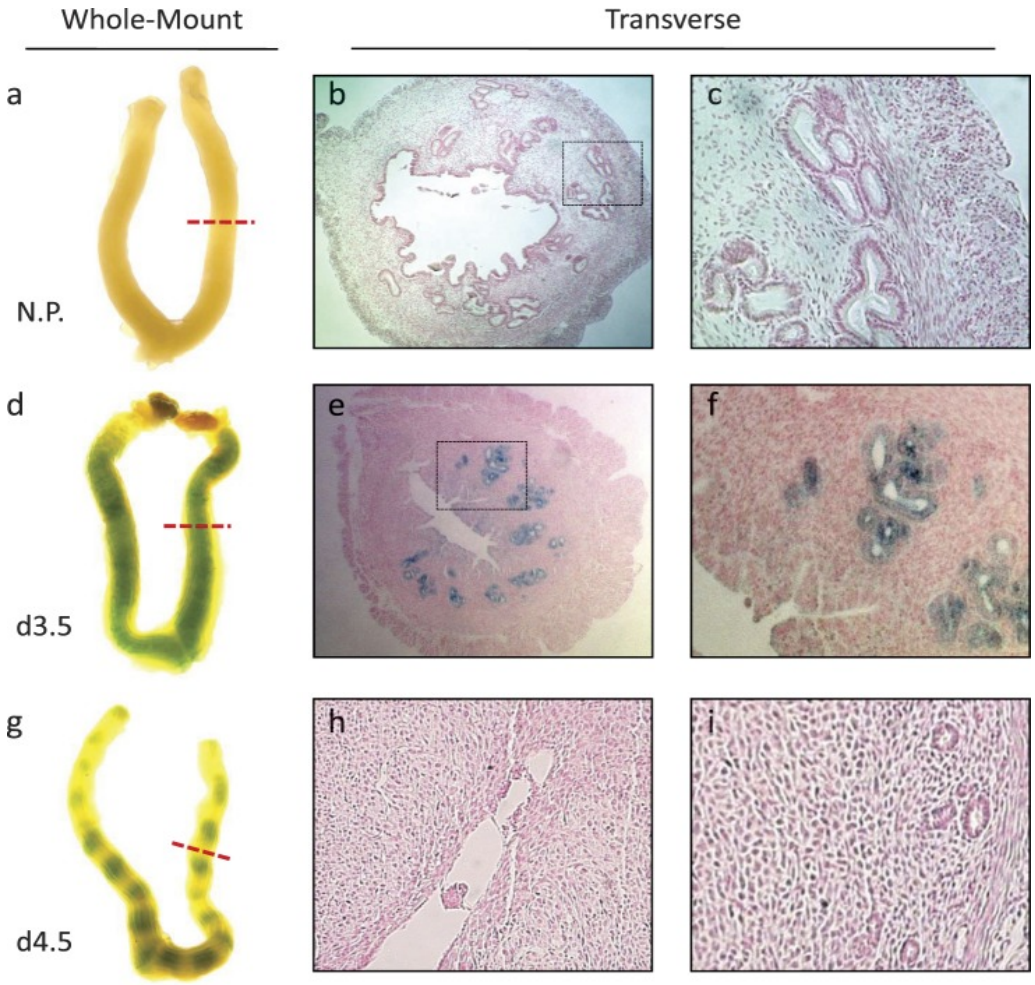

Figure 5 - Nodal-LacZ expression during peri-implantation in a mouse uterus (a) Whole mounted and (b-c) cross-sectioned non-pregnant mouse uterus showing no beta-galactosidase staining. (d) On day 3.5 post-coitum, Nodal is expressed throughout the entire uterine horn. (e-f) Transverse section staining shows that Nodal expression is restricted to the glandular epithelium. (g) On day 4.5 of pregnancy (implantation), the whole mount staining shows a banding pattern along the uterine horn. (h-i) A transverse section through the implantation site showing no staining where the embryo implanted, indicating that Nodal expression is restricted to the inter-implantation site. Figure from Park & Dufort 2011.

1.8.2 Nodal in peri-implantation

To address the function of Nodal in pregnancy, Park & Dufort generated a maternal reproductive-tract specific Nodal knockout mouse strain using the Cre-LoxP system under the control of the progesterone receptor. They evaluated pregnancy outcomes in Nodal heterozygous mutant mice, in which one allele of the Nodal gene is conditionally deleted ($Nodal^{\Delta/+}$), and

knockout mice, where both alleles of the Nodal gene are deleted ($Nodal^{\Delta/\Delta}$). The efficiency of this deletion was confirmed using Western blot analysis and immunofluorescence, where no Nodal protein was detected in the $Nodal^{\Delta/\Delta}$ and reduced expression of Nodal protein was seen in $Nodal^{\Delta/+}$(Park et al., 2012). Interestingly, both $Nodal^{\Delta/\Delta}$ and $Nodal^{\Delta/+}$ females exhibited a reduced ability to establish pregnancy because mice examined after implantation (on day 5.5) rarely contained decidua swellings or developing embryos (Park et al., 2012). Preliminary investigations into the causality behind the reduced fertility suggested that the earliest events that facilitate reproduction are not affected by Nodal deletion. $Nodal^{\Delta/\Delta}$ females exhibit normal estrus cycling, mating behavior, and plugging efficiency. Moreover, oogenesis, folliculogenesis, and ovulation are unaffected in these mutant mice. Lastly, ovarian and uterine morphology and histology appear normal (Park et al., 2012).

When isolating embryos from the oviducts at day 2.5 post coitum, $Nodal^{\Delta/\Delta}$ females contained a similar number of total embryos compared to control ($Nodal^{loxP/loxP}$) females. However, embryos from $Nodal^{\Delta/\Delta}$ were of reduced quality, with a more significant number of under-developed and nonviable embryos (Fig. 6a). Moreover, when embryos are isolated on day 3.5 post coitum, the phenotype observed at day 2.5 appeared to be more severe, where $Nodal^{\Delta/\Delta}$ contained significantly fewer total embryos of which the majority were nonviable (Fig. 6b). To evaluate if this diminished oocyte quality was caused by the Nodal deficient environment, $Nodal^{\Delta/\Delta}$ derived zygotes were transferred into the oviducts of day 0.5 pseudopregnant CD1 wild-type recipients. This transfer gave rise to a normal amount of healthy pups, indicating that zygotes derived from Nodal deficient mice are normal (Park, 2012). Together, these results suggest that the tubal environment in females devoid of uterine Nodal is unfavorable to blastocyst survival, causing the destruction of oocytes and reducing fertility.

Due to the unique banding pattern of Nodal expression at the time of implantation, Park & Dufort evaluated the potential role of uterine-derived Nodal in facilitating implantation regardless of the impaired oviductal environment. Blastocyst from a wild-type female were isolated and transferred into pseudopregnant $Nodal^{\Delta/\Delta}$ females, and implantation sites were asses on day 7.5 of pregnancy. Females that lacked uterine Nodal demonstrated reduced rates of implantation in comparison to the $Nodal^{loxP/loxP}$ controls (Park, 2012). These results further indicate that the uterine

environment is hostile in Nodal deficient females, hindering implantation. Overall, these results emphasize the crucial role of Nodal in early pregnancy; however, the mechanistic action of Nodal giving rise to the observed phenotype has yet to be determined.

One could suspect the involvement of the maternal immune system in this phenotype. It has been shown that Nodal$^{\Delta/+}$ have elevated basal pro-inflammatory cytokine levels and immune cell infiltration at the maternal-fetal interface around the time of parturition (Ayash et al., 2020).

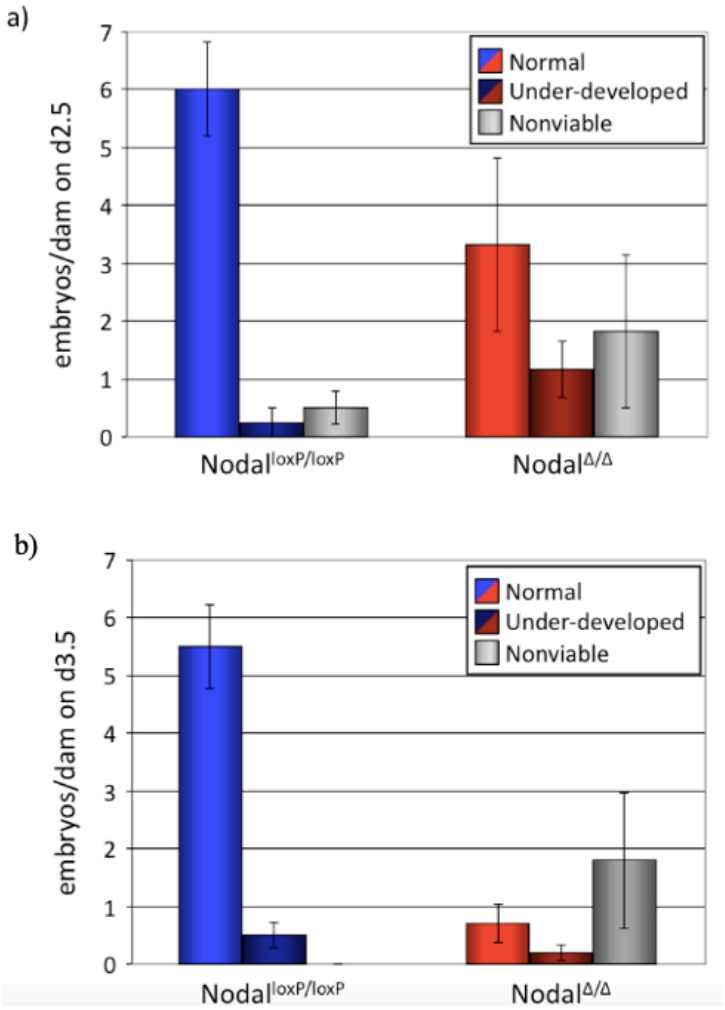

Figure 6 – Embryo quantity and quality in Nodal$^{\Delta/\Delta}$ and $^{NodalloxP/loxP}$ during the peri-implantation period. (a) Day 2.5 and (b) day 3.5 post coitum embryo isolated from the maternal reproductive tract of plugged females. Nodal$^{\Delta/\Delta}$ females exhibit increased instances of underdeveloped and nonviable embryos and decrease in total embryos. Figure from Park 2012.

1.9. The role of Nodal in the modulation of the inflammatory state in late pregnancy

As well as complications in early pregnancy, mice devoid of uterine Nodal signaling also experience problems later in pregnancy, including preterm birth and intrauterine growth restriction (Ayash et al., 2020). Dufort et al. showed that Nodal deficient mice are sensitive to a low dose of the inflammatory mediator LPS and experience preterm birth 12 hours post-injection, while the Nodal control mice were unaffected. Investigations into the causality of this phenotype revealed that Nodal mutant mice have a higher basal level of pro-inflammatory cytokines and increased macrophage infiltration in the maternal decidual tissue in late pregnancy compared to control mice. In vitro studies using a macrophage cell line demonstrated that treatment with recombinant Nodal reduces expression of pro-inflammatory cytokines when these cells are challenged with LPS (Ayash et al., 2020). These results suggest that the Nodal signaling pathway plays a role in modulating the inflammatory state present in the uterus later on in pregnancy.

1.10. Rationale and hypothesis

The peri-implantation period is the first and most crucial step in the establishment of a healthy pregnancy. During this time, an adequate and balanced maternal immunological response is required for proper embryo implantation, tissue remodeling, and vascularization through the expression of pro-inflammatory factors. Moreover, there must be an established state of immune tolerance in maternal reproductive tissues protecting the development and survival of the semi-allogenic blastocyst. This complex environment is obtained through the presence of various immune cell types and their associated molecular networks of cytokines and chemokines. Disruptions in the local uterine microenvironment or regulatory elements modulating this response can cause imbalances in the uterine immune system leading to pregnancy complications and termination.

There is some evidence that the Nodal signaling pathway might be involved in the modulation of this immune response in early pregnancy. Mice devoid of uterine Nodal signaling experience early destruction of blastocysts and implantation failure. Moreover, these mice have altered expression of pro-inflammatory cytokines and increased macrophage infiltration within the maternal decidual tissue in late pregnancy compared to control mice.

Due to the importance of a balanced immunological landscape in early pregnancy and that the phenotypes observed in uterine Nodal deficient mice arise from a hostile uterine environment, we hypothesize that Nodal plays an important role in modulating the inflammatory response present in the uterus during early pregnancy, and in its absence leads to decrease fertility.

We therefore aimed to characterize the state of the immune system in the uterus during the peri-implantation period in $Nodal^{\Delta/\Delta}$ compared to $Nodal^{loxP/loxP}$ to further the understanding of the pathway's role in the mouse model. Moreover, the state of the immune system in $Nodal^{\Delta/+}$ will also be evaluated to identify if Nodal had a dose-dependent effect on the immunological response.

Aim I: Define the uterine immune cell composition during the peri-implantation period.

Aim II: Determine the chemokine and cytokine levels during the peri-implantation period.

Understanding uterine Nodal signaling in early pregnancy and its role in modulating the maternal immune response to pregnancy is essential to contribute to the development of new therapeutic targets to decrease the incidence of RIF and infertility.

2 Materials and Methods

2.1 Mice

All experimental protocols were approved by the Animal Care Committee of the McGill University Health Centre and were in accordance with regulations established by the Canadian Council on Animal Care. Mice with loxP sites flanking exons 2 and 3 of the Nodal gene ($Nodal^{loxP/loxP}$) on a mixed background were previously generated by E. J. Robertson (University of Oxford) (Lu & Robertson, 2004). The generation of these mice has been previously described (Park et al., 2012). Progesterone receptor (PR)-Cre female mice ($Pgr^{Cre/+}$) on a C57BL6/129 background were previously generated by F. J. DeMayo and J. P. Lydon (Baylor College of Medicine) (Soyal et al 2005). Both strains have been shown to have normal fertility and $Pgr^{Cre/+}$ mice have become the standard tool to study uterine-specific gene function (Mukherjee et al., 2007). Homozygous Nodal floxed females ($Nodal^{loxP/loxP}$) were crossed with heterozygous $Pgr^{Cre/+}$ males. Appropriate crossings were set up to finally generate control females ($Nodal^{loxP/loxP}$, $Pgr^{+/+}$ termed $Nodal^{loxP/loxP}$), heterozygous mutant females ($Nodal^{loxP/+}$, $Pgr^{Cre/+}$ termed $Nodal^{\Delta/+}$), and homozygous mutant females ($Nodal^{loxP/loxP}$, $Pgr^{Cre/Cre}$ termed termed $Nodal^{\Delta/\Delta}$). In this study, 8-24 week old mice were used for all experiments.

2.2 Genotyping

Tail snips were collected then digested in 300 µl lysis buffer (100mM tris-HCL pH 8.0, 10mM ETDA pH 8.0, 0.5% Tween 20, 0.5% Nondiet P-40) with 150 µg proteinase K (Roche) at 55°C on a heat block. The DNA was isolated by centrifugation. $Nodal^{loxP-\Delta}$ (290bp) and $Nodal^{+}$ (220bp) alleles were amplified using touchdown PCR (94°C (1 minute), 94°C, 59°C, 72°C (30 seconds each) for 7 cycles, 94°C, 55°C, 72°C (30 seconds each) for 30 cycles; 5'-ATTCCAGCAGTTGAGGCAGA-3'; 5'-GCTATGCCACGCAGAACC-3'). PR^{Cre} (550bp) and PR^{+} (300bp) alleles were amplified using standard PCR (94°C, 60°C (1 minute each), 72°C (2 minutes) for 30 cycles; 5'-ATGTTTAGCTGGCCCAATG-3'; 5'-TATACCGATCTCCCTGGACG-3'; 5'-CCCAAAGAGACACCAGGAAG-3'). Bands were visualized on a 1-2% agarose gel by electrophoresis.

2.3 Mating and manipulation of transgenic mice

$Nodal^{\Delta/\Delta}$, $Nodal^{\Delta/+}$ and $Nodal^{loxP/loxP}$ females were mated overnight with fertile CD1 males. The presence of a vaginal plug the next morning indicated successful copulation and was denoted as day 0.5. The mice were sacrificed on day 1.5, 2.5, and 3.5 respectively and the uterine tissue was dissected. Uterine samples collected on day 3.5 were flushed through the oviducts in the proximal-distal direction using PBS to identify the presence of embryos. Samples were stored for RNA or protein extraction or fixed for immunofluorescence.

2.4 Immunofluorescence

Uterine tissue were dissected into PBS on day 1.5, 2.5, and 3.5 of pregnancy and fixed for 24 hours at 4°C in 10% buffered formalin. Samples were processed using the Leica ASP300S automated vaccume tissue processor. Samples were embedded in paraffin at room temperature and blocks were solidified at -20°C. Five-micrometer sections were cut with a Leica RM2145 microtome and dried overnight. Slides were then washed in xylenes and rehydrated with a decreasing ethanol gradient (100, 95, 85, 75, 50, and 20% for 2 min each). Samples were permeabilized with TBT (0.2% BSA and 2.5% TritonX-100 in TBS) (two times for 5 min each). Slides were then washed in TBS containing 0.1% Tween-20 (two times for 5 min each) and blocked with 10% BSA in TBS for 1 hour within a humidified chamber at room temperature and incubated with primary antibody at 4°C overnight. For detection of leukocytes, a purified rat anti-mouse CD45 antibody was used (1:100, BD Cat No. 553076). Then, slides were washed with TBS containing 0.1% Tween-20 three times and incubated with Alexa Fluor 488-labelled goat anti-rat secondary antibody (1:300, Invitrogen Cat No. A-11006) and DAPI (1:500) for 1 hour at room temperature. To eliminate autofluorescent erythrocytes, slides were treated with TrueBlack® Lipofuscin Autofluorescence Quencher (1X in 70% Ethanol, Biotium Cat No. 23007) for 3 minutes and washed with TBS. Slides were washed and mounted with Mowiol 4-88. Confocal images were obtained on the Zeiss LSM780 laser scanning confocal microscope using the tile-scan function at 20X magnification.

2.5 Semi-quantification of CD45+ cells in immunofluorescent staining

The presence of CD45+ cells in a tissue were semi-quantified using the manual cell counter program in ImageJ. To eliminate the disparities between tissues of different sizes, four areas of a given size (300um x 200um) were selected at given locations throughout the tissue section (mesometrial pole, anti-mesometiral pole, and 2 on both sides). These locations and areas were kept constant between samples. Leukocytes were quantified by manually clicking on all nucleated cells (DAPI stained) surrounded by a positive CD45 stain within each given selected area. The total number of cells in these four boxes were averaged for each sample obtained. To reduce experimenter's bias, samples were blinded during the process of quantification. Samples were identified using non-representative numbering and the genotype of each sample was only determined after the quantification was finalized.

2.6 RNA isolation and RT^2 Profiler PCR Array for uterine tissues

Uterine samples were collected at day 2.5 of pregnancy and kept frozen at -80°C. For RNA extraction, the tissue was removed, placed in 1mL of Trizol solution and homogenization was carried out using a probe. 200μL of chloroform was added to the mixture and the tube was mixed by shaking. The samples were centrifuged at 14,000rpm at 4°C for 15 minutes. The supernatant was isolated, 70%EtOH was added at a 1:1 ratio, and this mixture was run in a Qiagen RNeasy spin column centrifuged at 10,000rpm for 1 minute. The flow-through was discarded. The column was washed with 500μL of RPE wash buffer and centrifuged at 10,000rpm for 2 minutes. The empty column was then centrifuged at 14,000rpm for 1 minute to dry and the flow-though was discarded. 50μL of 55°C RNA-free water was added to the column and allowed to sit for 2 minutes at room temperature. The column was then centrifuged at 14,000rpm for 1 minute and the flow-through containing the RNA was collected and its concentration was measured using a Nano-drop. cDNA was synthesized using the Qiagen RT2 First Strand Kit (Qiagen Cat No. 330404), and RT-PCR was performed using the Mouse Innate & Adaptive Immune Responses RT2 Profiler PCR Array (Qiagen Cat No. PAMM- 052Z) following the manufacturer's protocols.

2.7 Leukocyte isolation and flow cytometry

Mice were sacrificed on day 3.5 of pregnancy and uterine tissue was dissected. Uterine tissues were dissociated mechanically and then chemically for one hour using tissue digestion buffer containing Liberase and DNase I. After dissociation, homogenates were filtered through 70 μm cell strainer and red blood cells were lysed using ACK lysing buffer. The cells obtained were stained with the following antibody-fluorochrome conjugates for antigen detection: CD45 - BUV395, CD11b -efluro450, Siglec F – FITC, CD49b – PE, CD11c - PercpCy5.5, CD19- APC, Ly6G - Alexa Fluor 700, Ly6C- APC-Cy7, F4/80 - Pe-Cy7, CD4- Alexa Fluor 700, CD8-PE-Cy7, CD3- BUV737, and a viability dye – efluro506. Following fixation, cell marker detection was acquired using a BD Biosciences Fortessa X-20 and FACSDiva software. Immunophenotyping analysis was conducted through FlowJo software**.**

2.8 Statistics

Statistical analyses of differences between experimental groups were performed using analysis of variance (ANOVA) or two-sided Student's t-test using GraphPad Prism 9 software. Data represent mean ± SEM. Differences were considered significant if P-value ≤ 0.05. Sample size (N) in each experiment is representative of the number of mice included in the experiment.

3 Results

3.1 Define the uterine immune cell composition during the peri-implantation period

3.1.1 Nodal deficient females have an increase infiltration of leukocytes within the endometrium during the peri-implantation period

To study the role of Nodal in early pregnancy, our lab utilized a tissue-specific conditional knock-out of Nodal in the maternal reproductive tract utilizing the loxP-Cre recombinase system under the control of the progesterone receptor (Park et al., 2012). Mice were mated and dissected on day 1.5, 2.5 and 3.5 of pregnancy. Immunofluorescent staining for CD45, a type I transmembrane protein present on all leukocytes, was performed to identify the localization of immune cells in the uterine tissue. Immunofluorescent staining at day 1.5 of pregnancy showed CD45+ cells located mostly in the myometrium and adjacent to the endometrium in $Nodal^{loxP/loxP}$ uterine samples, with smaller populations of CD45+ cells in the endometrium closer to the uterine lumen (Fig. 7A). Interestingly, in both the $Nodal^{\Delta/+}$ and $Nodal^{\Delta/\Delta}$ uterine samples, a large number of CD45+ cells were located throughout the endometrium including localizing close to the lumen of the uterus (Fig. 7 B-C). Moreover, large clusters of CD45+ cells were identified at the uterine mesometrial pole at the location of the entering uterine blood supply.

To further evaluate the state of the immune system in Nodal deficient mice in early pregnancy, semi-quantification of CD45+ cells present in the uterine tissue was obtained using the immunofluorescent results. At day 1.5 of pregnancy, no significant difference was seen between the approximate number of CD45+ cells in $Nodal^{loxP/loxP}$ compared to $Nodal^{\Delta/\Delta}$ and $Nodal^{\Delta/+}$ uterine samples (Fig. 7 F).

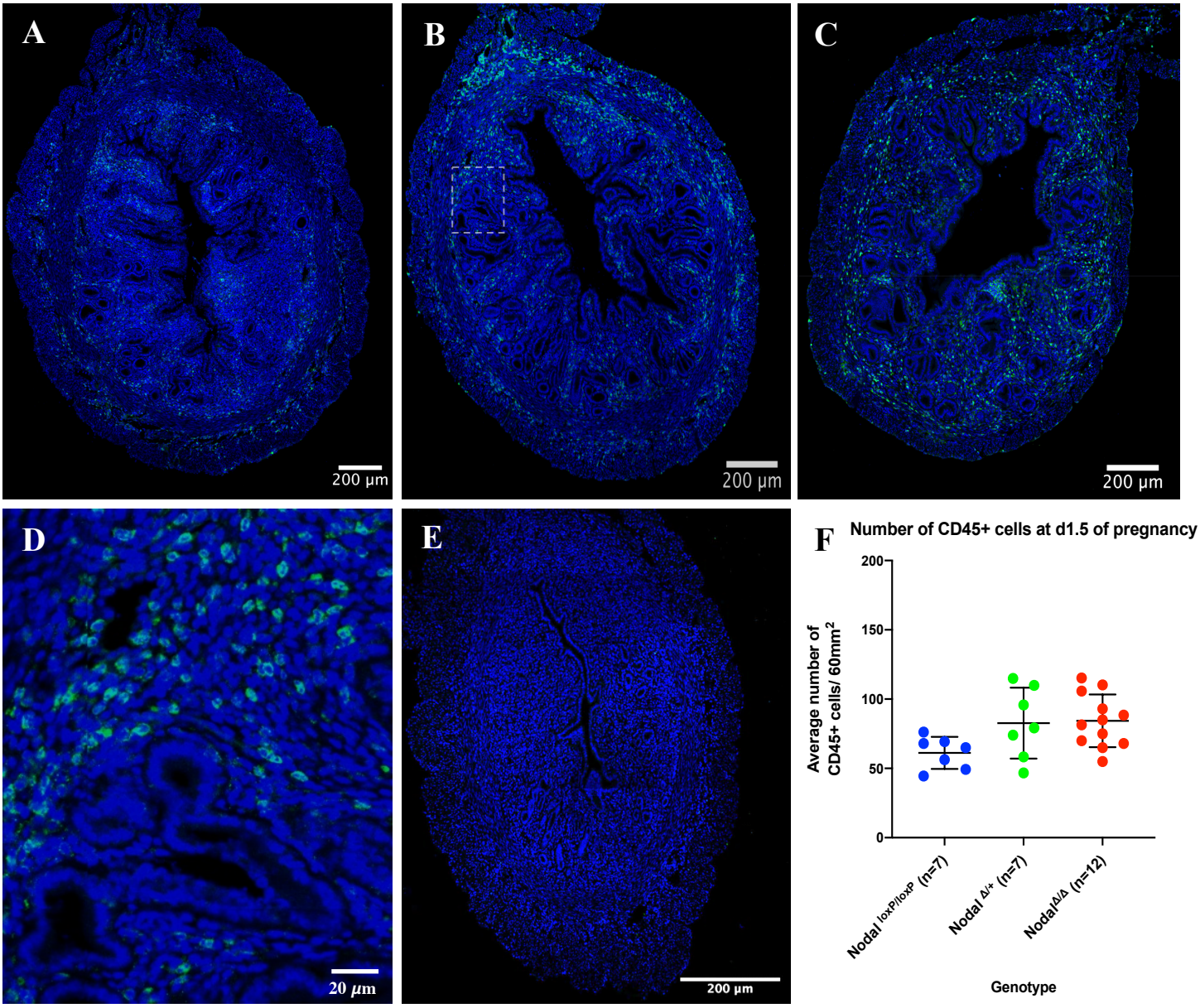

Figure 7 – Immunofluorescent staining for CD45 (green) and DAPI (blue) at day 1.5 of pregnancy. (A) Uterine cross section from Nodal$^{loxP/loxP}$ female. (B) Uterine cross section from Nodal$^{\Delta/+}$ female. (C) Uterine cross section from Nodal$^{\Delta/\Delta}$ female. (D) Magnification of selected area in panel B showing leukocytes, nucleated cells surrounded by a positive CD45 stain. (E) No primary antibody control showing no signal. (F) Semi-quantification of CD45+ cells from immunofluorescent results showing average number of CD45+ cells at day 1.5 of pregnancy in Nodal^{loxPloxP} (N=7), Nodal$^{\Delta/+}$ (N=7) and Nodal$^{\Delta/\Delta}$ (N=12).

A similar pattern of distribution was seen at day 2.5 of pregnancy where leukocytes were largely localized in proximity to, or in the myometrium, in Nodal$^{loxP/loxP}$ mice (Fig. 8 A). However, in Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ mice, a large infiltration of immune cells was seen throughout the endometrium, where cells were present close to the uterine lumen (Fig. 8 B-C).

Semi-quantification of leukocytes in the uterine tissue at day 2.5 of pregnancy showed a significant increase in the mean average number of CD45+ cells in both the Nodal$^{\Delta/\Delta}$ and Nodal$^{\Delta/+}$ group in comparison to the controls (Fig. 8 E).

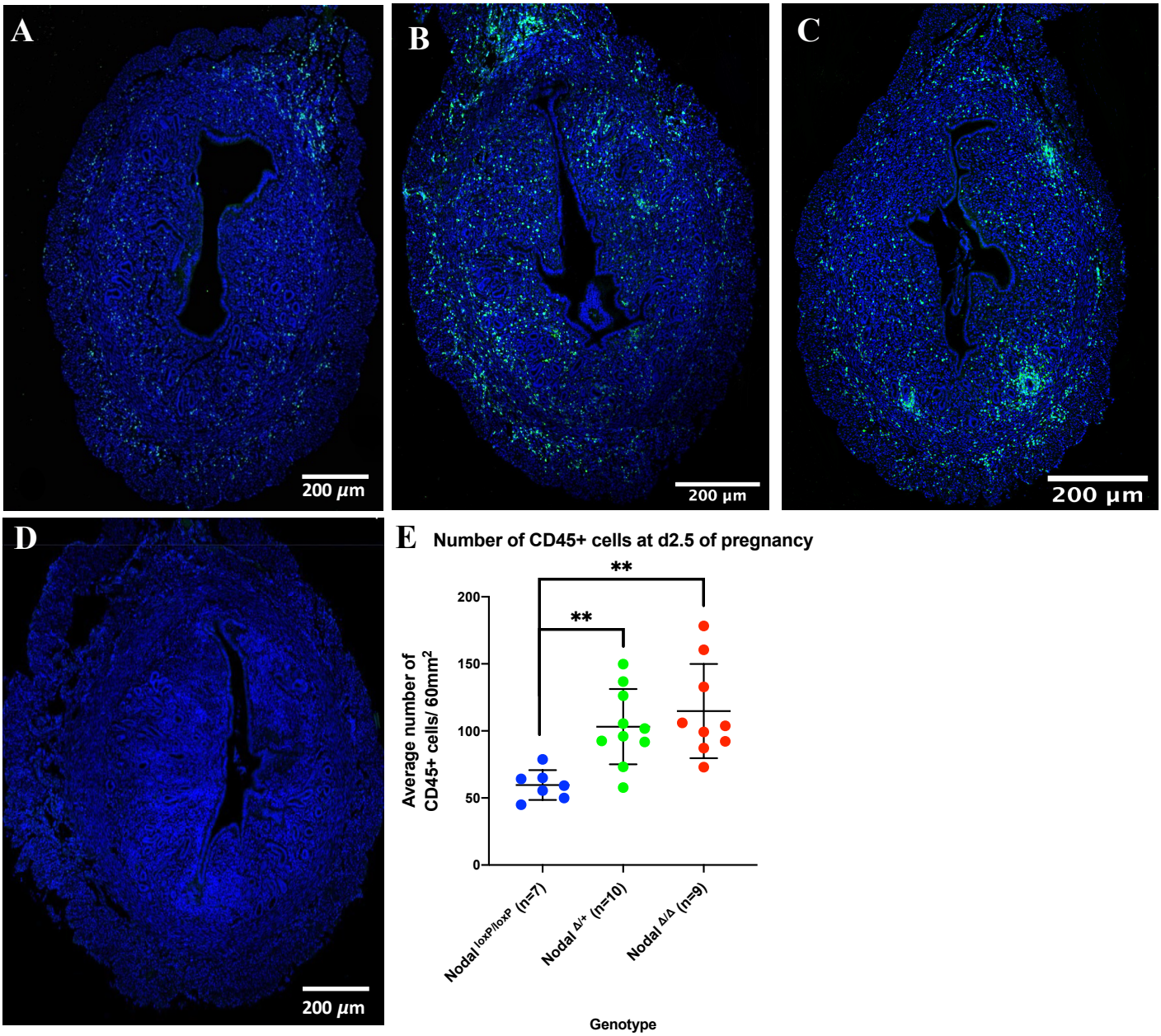

Figure 8 – Immunofluorescent staining for CD45 (green) and DAPI (blue) at day 2.5 of pregnancy. (A) Uterine cross section from Nodal$^{loxP/loxP}$ female. (B) Uterine cross section from Nodal$^{\Delta/+}$ female. (C) Uterine cross section from Nodal$^{\Delta/\Delta}$ female. (D) No primary antibody control showing no signal. (E) Semi-quantification of CD45+ cells from immunofluorescent results showing average number of CD45+ cells at day 2.5 of pregnancy in Nodal^{loxPloxP} (N=7), Nodal$^{\Delta/+}$ (N=10) and Nodal$^{\Delta/\Delta}$ (N=9).

At day 3.5 of pregnancy, there was an increase in the endometrial infiltration of leukocytes in Nodal$^{loxP/loxP}$ mice, reaching a similar abundance of leukocytes seen in the Nodal deficient mice in the earlier days of pregnancy (Fig. 9 A). Leukocytes were now localized throughout the uterine tissues, with a larger number of immune cells located close to the uterine lumen. Compared to controls, Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ samples had elevated levels of CD45+ cells throughout the uterine tissue (Fig. 9 B-C). Semi-quantification at day 3.5 of pregnancy showed a significant increase in the mean average number of CD45+ cells in both the Nodal$^{\Delta/\Delta}$ and Nodal$^{\Delta/+}$ group in comparison to the controls (Fig. 9 E).

Finally, when comparing the mean average number of CD45+ cells over the first three days of pregnancy, Nodal$^{loxP/loxP}$ samples had constant levels of leukocytes present in the uterus in the first two days with a slight non-significant increase on day 3.5 (Fig. 10). However, in both the Nodal$^{\Delta/\Delta}$ and Nodal$^{\Delta/+}$ group, which already had elevated levels at day 1.5, there was a further non-significant increase in the average number of immune cells over the two consecutive days. A significant difference in the average number of leukocytes present in the uterine tissue was observed in the Nodal$^{\Delta/\Delta}$ at day 3.5 of pregnancy (Fig. 10). These results indicate that there is an impaired modulation of the maternal immune system in Nodal deficient mice causing an earlier and larger infiltration of leukocytes into the uterine endometrium during early pregnancy.

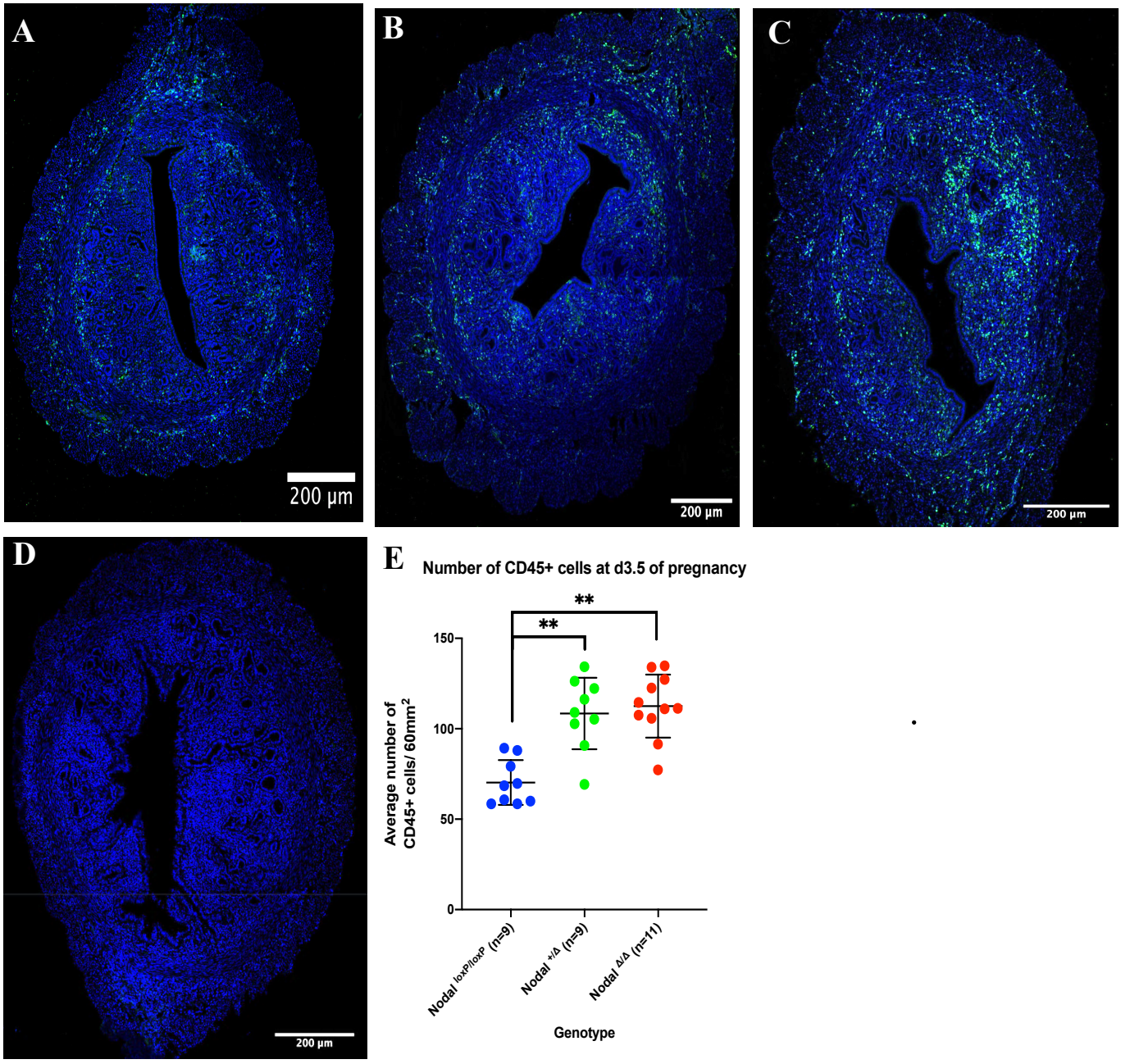

Figure 9 – Immunofluorescent staining for CD45 (green) and DAPI (blue) at day 3.5 of pregnancy. (A) Uterine cross section from Nodal$^{loxP/loxP}$ female. (B) Uterine cross section from Nodal$^{\Delta/+}$ female. (C) Uterine cross section from Nodal$^{\Delta/\Delta}$ female. (D) No primary antibody control showing no signal. (E) Semi-quantification of CD45+ cells from immunofluorescent results showing average number of CD45+ cells at day 3.5 of pregnancy in Nodal^{loxPloxP} (N=9), Nodal$^{\Delta/+}$ (N=9) and Nodal$^{\Delta/\Delta}$ (N=11).

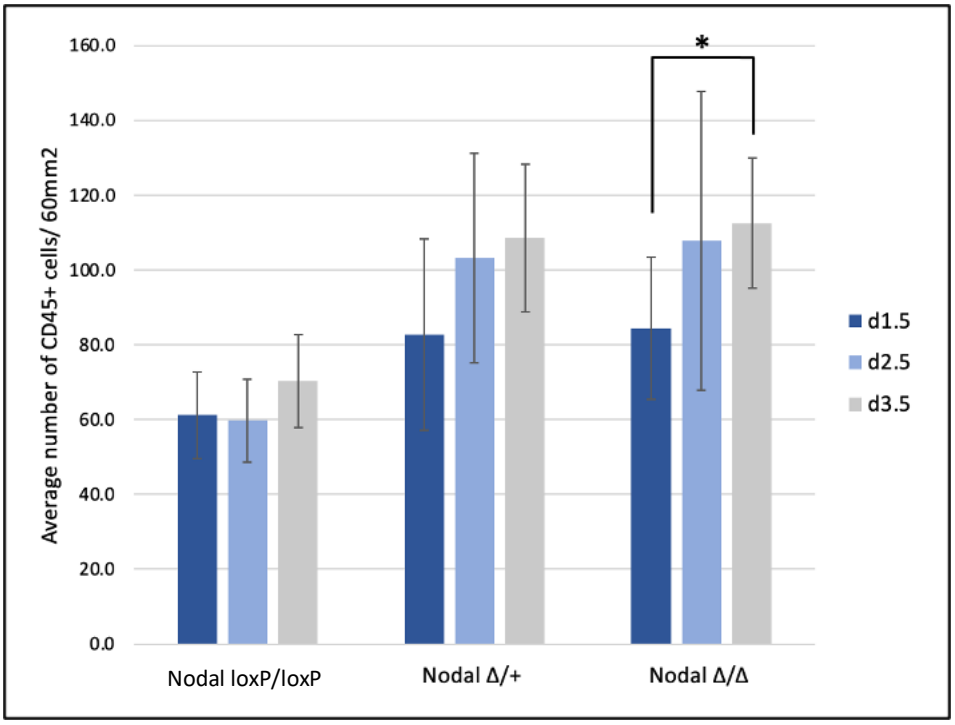

Figure 10 – Average number of CD45+ cells in during peri-implantation. (A) Average number of CD45+ cells at day 1.5 of pregnancy in Nodal$^{loxP/loxP}$ (N=7), Nodal$^{\Delta/+}$ (N=7) and Nodal$^{\Delta/\Delta}$ (N=12). (B) Average number of CD45+ cells at day 2.5 of pregnancy in Nodal$^{loxP/loxP}$ (N=7), Nodal$^{\Delta/+}$ (N= 10) and Nodal$^{\Delta/\Delta}$ (N=10). (C) Average number of CD45+ cells at day 3.5 of pregnancy in Nodal$^{loxP/loxP}$ (N=9), Nodal$^{\Delta/+}$ (N= 9) and Nodal$^{\Delta/\Delta}$ (N=11). * P-value < 0.05.

3.1.2 Nodal deficient females have an altered immune cell composition at day 3.5 of pregnancy

Studies have shown that various immune cells are important during the peri-implantation period and play a critical role in immune tolerance, tissue remodeling, and vascularization. By means of flow cytometry, we investigated whether the composition of immune cells in the uterine tissue was normal at early pregnancy in Nodal deficient females. Using specific markers for various different immune cell types and gating strategies, described in Figure 11, populations of B cells, T cells, helper T (Th) cells, cytotoxic T (Tc) cells, , DCs, eosinophils, monocytes, macrophages, neutrophils, and NK cells were identified in $Nodal^{loxP/loxP}$, $Nodal^{\Delta/+}$, and $Nodal^{\Delta/\Delta}$ uterine samples at day 3.5 of pregnancy.

No significant difference was observed between $Nodal^{\Delta/+}$/$Nodal^{\Delta/\Delta}$ and $Nodal^{loxP/loxP}$ in the abundance of most immune cell types including: B cells, T cells, Tc cells, DCs, neutrophils, and monocytes (Fig. 12 A, B, D, E, F, I). However, $Nodal^{\Delta/\Delta}$ females had significantly lower levels of macrophages and NK cells compared to both $Nodal^{loxP/loxP}$ and $Nodal^{\Delta/+}$(Fig. 12 G, J). Interestingly, $Nodal^{\Delta/\Delta}$ females had significantly higher levels of eosinophils compared to $Nodal^{loxP/loxP}$ (Fig. 12 H). Lastly, the abundance of Th cells was significantly higher in $Nodal^{\Delta/+}$ compared to controls (Fig. 12 C). These results indicate that Nodal has a role in regulating the types of immune cells present in the uterine tissue and their respective quantities during the peri-implantation period.

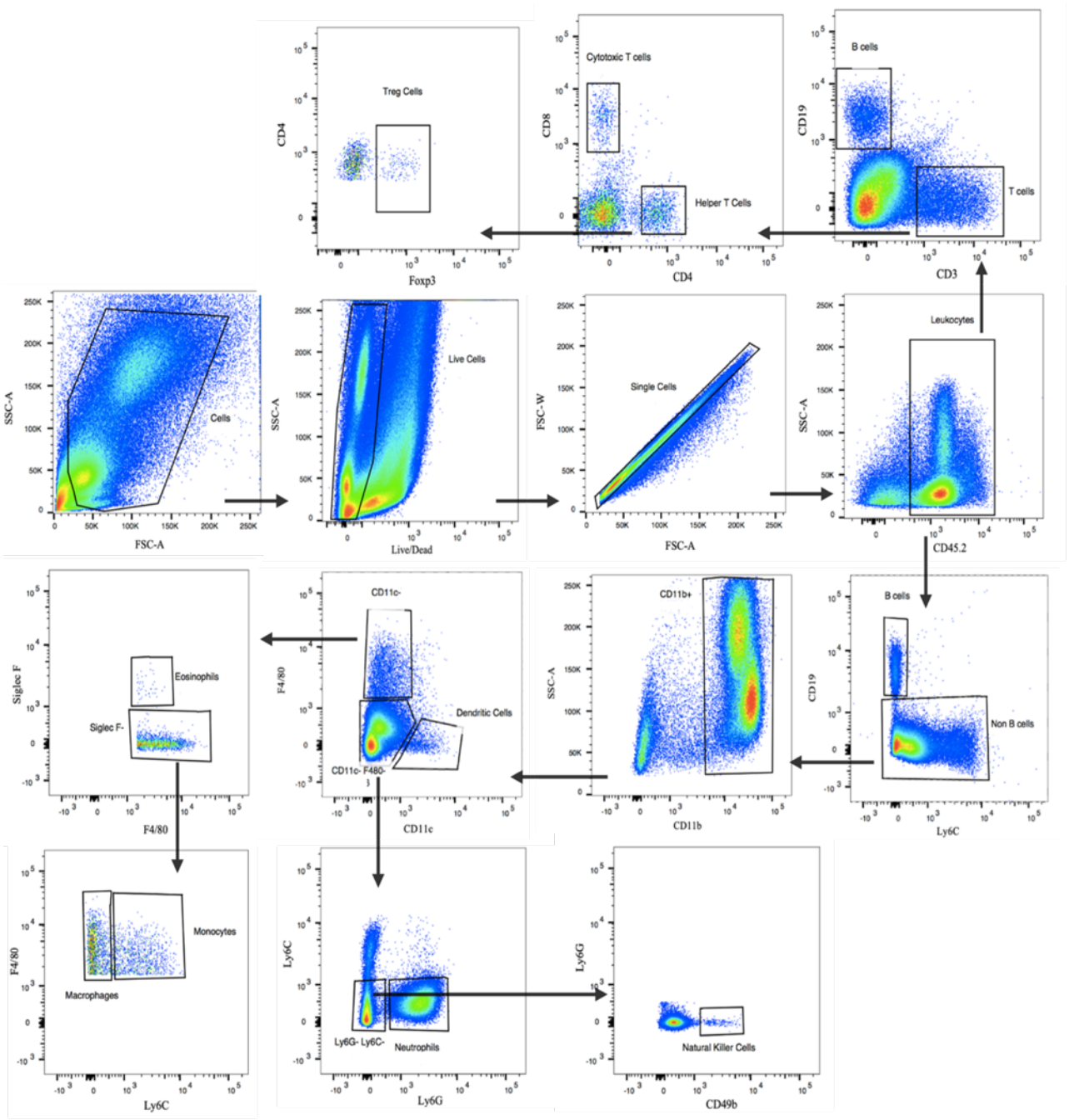

Figure 11 – Flow cytometry analysis of leukocytes in day 3.5 pregnant uterine tissue. Gating scheme to identify several immune cell types present at day 3.5 of pregnancy in uterine tissue. Cells were chosen apart from debris and particles within the sample using gating for forward scatter area (FSC-A, representing the size) and side scatter area (SSC-A, representing the complexity). A fixable viability dye was used to mark the dead cells and live single cells were selected using FSC-A and FSC-W (width). Live single cells were gated for CD45 to define the all leukocytes. Other immune cell types were defined as follows: **B cells**: CD45+CD19+, **T cells:** CD45+CD3+, **Cytotoxic T cells:** CD45+CD3+CD8+, **Helper T cells:** CD45+CD3+CD4+, **Regulatory T cells (Treg):** CD45+CD3+CD4+Foxp3+, **Conventional Dendritic cells (DC):** CD45+CD11b+F4/80-CD11c+, **Eosinophils:** CD45+CD11b+f4/80Mid,SiglecF+, **Monocytes:** CD45+CD11b+SiglecF-F4/80Mid,Ly6CMid-Hi, **Macrophages:** CD45+CD11b+F4/80Hi,SiglecF Ly6C, **Neutrophils:** CD45+CD11b+F4/80-CD11c-Ly6G+, **Natural Killer cells (NK):** CD45+CD11b+F4/80-Ly6C-Ly6G-CD49b+

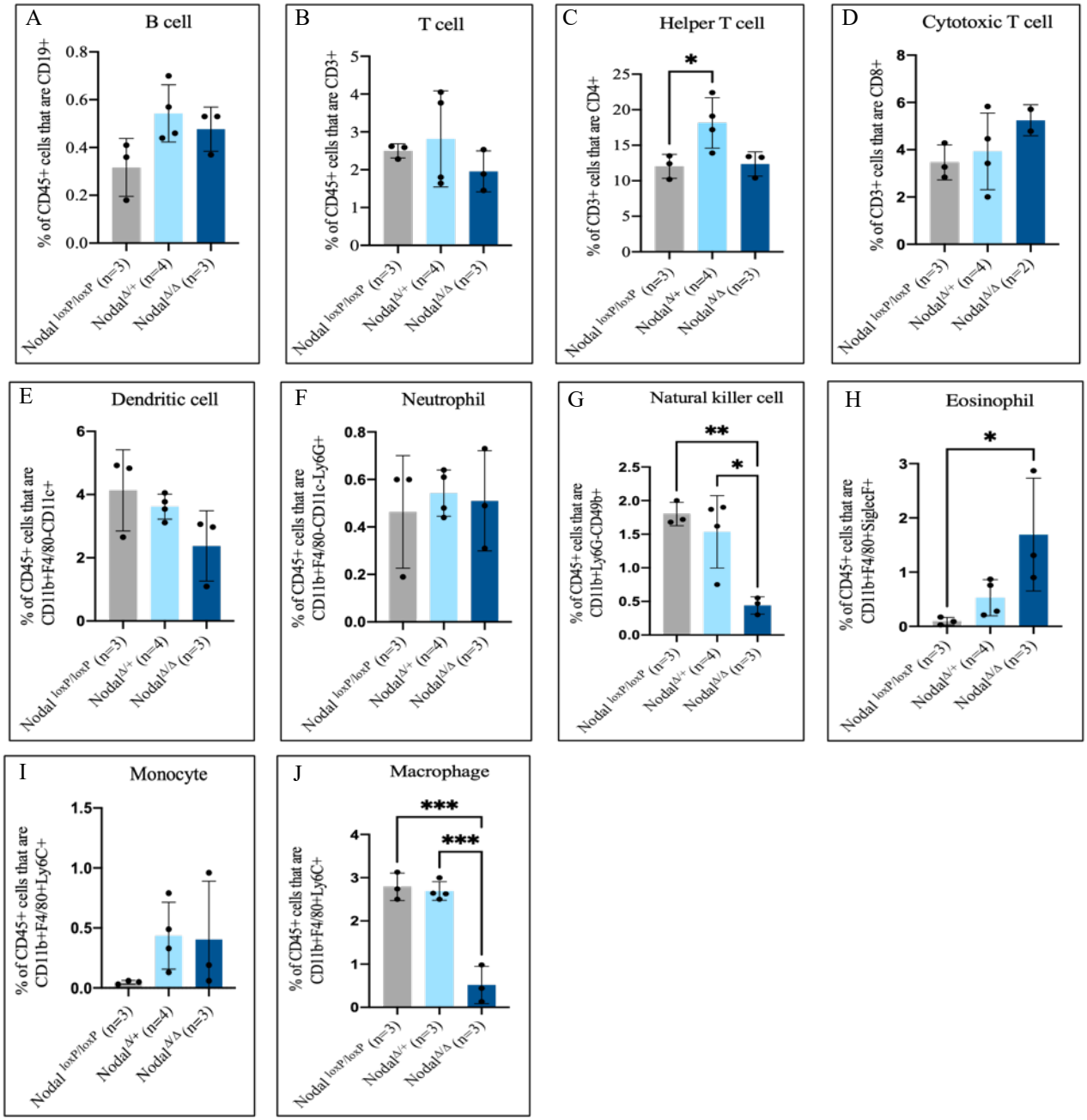

Figure 12 - Frequency of different leukocyte populations at day 3.5 of pregnancy in Nodal$^{loxP/loxP}$, Nodal$^{\Delta/+}$, and Nodal$^{\Delta/\Delta}$ uterine tissue by flow cytometric analysis. Frequency of B cells (A), T cells (B), Th cells (C), Tc cells (D), DCs (E), neutrophils (F), NK cells (G), eosinophils (H), monocytes (I), and macrophages (J) in Nodal$^{loxP/loxP}$ (N=3), Nodal$^{\Delta/+}$(N=4 or 3), and Nodal$^{\Delta/\Delta}$ (N=3 or 2) day 3.5 pregnant uterine samples. *P-value <0.05, ** P-value <0.02, ***P-value <0.01.

3.2 Determine the chemokine and cytokine levels during the peri-implantation period

3.2.1 Nodal deficient females have altered cytokine and chemokine gene expression at day 2.5 of pregnancy

To further characterize the state of the immune system during the peri-implantation period in mice devoid of uterine Nodal, the gene expression of various cytokines and chemokines was assessed. 84 genes commonly involved in innate and adaptive immunity was examined using an RT^2 Profiler PCR Array (Table. S1). From this, 6 genes were found to be significantly differentially expressed in the Nodal deficient females. Chemokine receptors genes Ccr5 and Ccr8 were significantly over-expressed in $Nodal^{\Delta/+}$ compared to $Nodal^{loxP/loxP}$ (Fig. 13 A,B). In the $Nodal^{\Delta/\Delta}$ females however, only Ccr8 was significantly over-expressed compared to $Nodal^{loxP/loxP}$. While the fold change in gene expression of Ccr5 was higher in the $Nodal^{\Delta/\Delta}$ females compared to $Nodal^{loxP/loxP}$, it remained non-significant. Moreover, the histocompatibility antigen gene H2-q10 was significantly up-regulated in the $Nodal^{\Delta/\Delta}$ females compared to controls (Fig. 13 C).

The expression of cytokine genes such as Il-1$\boldsymbol{\beta}$ and Il-6 had altered expression in the Nodal deficient females compared to Nodal control. Il-1$\boldsymbol{\beta}$ expression was significantly up-regulated in Nodal heterozygous females in comparison to Nodal controls (Fig. 13 D). Contrastingly, the expression of Il-6 was significantly down-regulated in both $Nodal^{\Delta/+}$ and $Nodal^{\Delta/\Delta}$ females compared to $Nodall^{oxP/loxP}$ (Fig. 13 E). Finally, the gene for the adapter protein Myd88 was significantly under-expressed in $Nodal^{\Delta/+}$ females compared to $Nodall^{oxP/loxP}$ (Fig. 13 F). These results indicate that the immunological profile of the uterine tissue during the peri-implantation period in Nodal deficient females is further altered due to the differences in gene expression of several immune related genes.

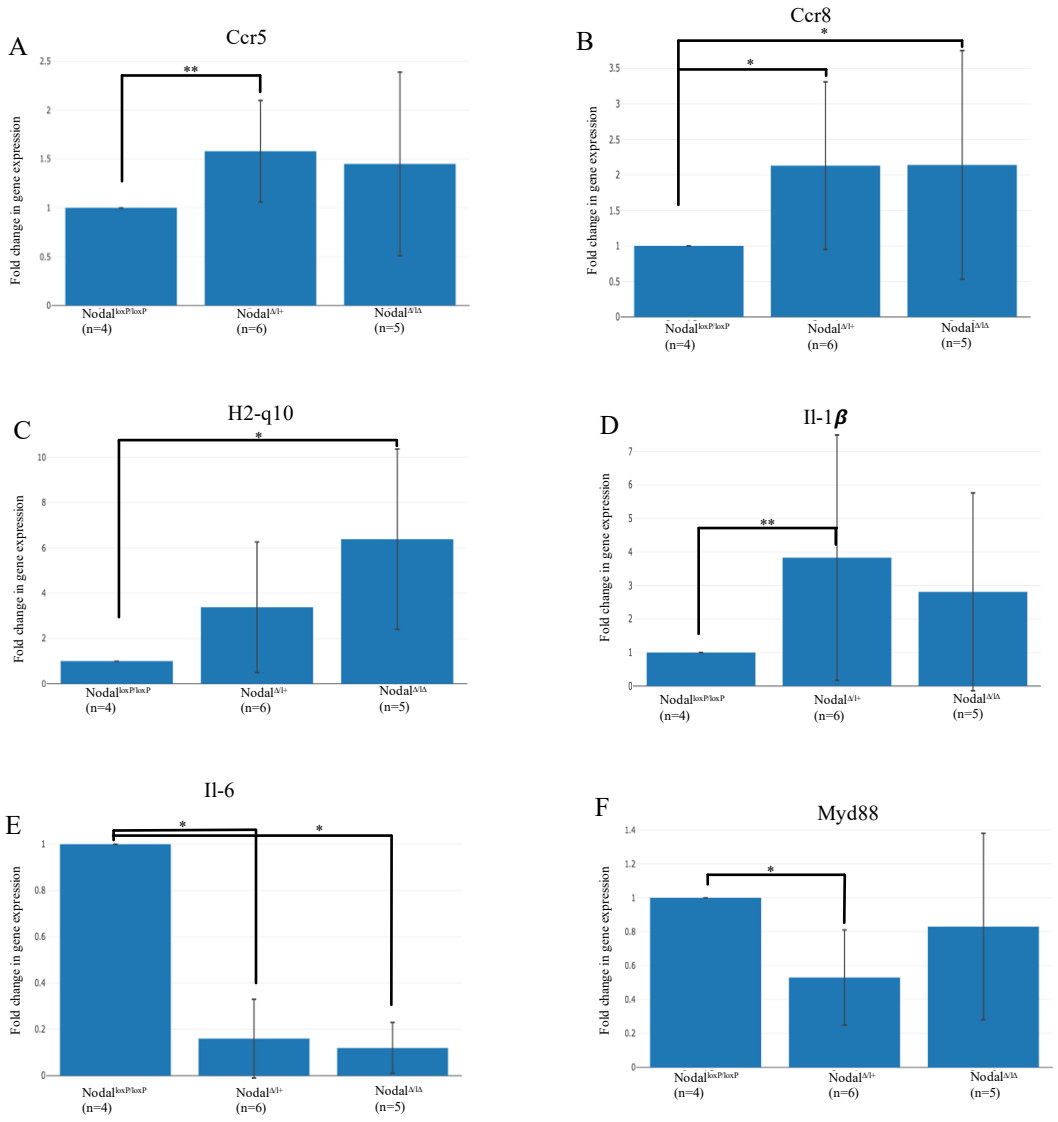

Figure 13 – Significant fold change in gene expression of immune related genes at day 2.5 of pregnancy in Nodal$^{loxP/loxP}$ (N=4), Nodal$^{\Delta/+}$ (N=6), and Nodal$^{\Delta/\Delta}$ (N=5) by RT PCR analysis. (A) CCR5, (B) CCR8, (C) H2-Q10, (D) IL-1β, (E) IL-6, (F) MYD88. ** P-value <0.02, * P-value <0.05.

4 Discussion

This thesis aimed to investigate the role of the morphogen Nodal in the modulation of the maternal immune system during the peri-implantation period. Prior investigations into the effects of Nodal in early pregnancy revealed the necessary action of this secreted factor. A conditional deletion of Nodal in the mouse uterus resulted in a dramatic reduction in fertility of Nodal conditional homozygous knock-out ($Nodal^{\Delta/\Delta}$) and conditional heterozygous females ($Nodal^{\Delta//+}$) before day 5.5 of pregnancy (Park et al., 2012). This decrease in fertility was attributed to a reduction in the quality of embryos starting at day 2.5 of pregnancy and a worsening in this phenotype by day 3.5 of pregnancy. Moreover, Nodal mutant mice demonstrated reduced rates of implantation in comparison to the $Nodal^{loxP/loxP}$ controls (Park et al., 2012). Lastly, in late pregnancy, Nodal deficient females had elevated basal pro-inflammatory cytokine levels and immune cell infiltration at the maternal-fetal interface (Ayash et al., 2020). These results led us to investigate the state of the maternal immune system in early pregnancy to potentially explain the phenotype observed during the peri-implantation period.

Immunofluorescent results revealed altered localization of immune cells within the uterine tissue in $Nodal^{\Delta/\Delta}$ and $Nodal^{\Delta//+}$ during the peri-implantation period. Increased infiltration of leukocytes within the uterine endometrium localizing close to the uterine lumen was observed in Nodal deficient mice. Whereas, $Nodal^{loxP/loxP}$ had immune cells localized mainly in proximity to or in the myometrium during this time. These results suggest that Nodal plays a role in regulating the distribution of immune cells throughout the uterine tissue. Nodal expression increases over the first three days of pregnancy and is localized in the epithelium of glands throughout the endometrium. This could suggest that Nodal negatively regulates the infiltration of immune cells into areas of high Nodal expression causing the cells to localize further from the endometrium. However, in the Nodal deficient mice where Nodal expression is reduced or absent, leukocytes can infiltrate the endometrium and come in close proximity to the uterine lumen. The physiological impact of this alteration could involve higher concentrations of pro-inflammatory factors, released by the leukocytes, to reach the uterine lumen and impact the survival of the blastocyst. Moreover, considering leukocytes respond differently depending on the local microenvironment they are found in, this disruption in localization could affect the response of each immune cell type.

The inhibitory effect of Nodal on the distribution of uterine leukocytes also seems to affect their quantity. Semi-quantification of the immunofluorescent results indicates higher levels of uterine leukocytes in $Nodal^{\Delta/\Delta}$ and $Nodal^{\Delta/+}$ compared to $Nodal^{loxP/loxP}$ controls at day 2.5 and 3.5 of pregnancy. This increase in leukocyte number is significantly higher in the $Nodal^{\Delta/\Delta}$ females over the first three days of pregnancy. In contrast, the population of leukocytes found in the $Nodal^{loxP/loxP}$ controls remains constant over this period. These results indicate that as Nodal expression increases over the first three days of pregnancy, this keeps immune cell numbers constant in the uterine tissue. However, this regulatory effect is diminished in $Nodal^{\Delta/+}$ and is absent in $Nodal^{\Delta/\Delta}$.

Preliminary investigations into the immune cell composition found in the uterine tissue during the peri-implantation period showed altered levels of various immune cells in the Nodal deficient females compared to controls. Significantly lower numbers of NKs were found within the uterine tissue of $Nodal^{\Delta/\Delta}$ compared to $Nodal^{loxP/loxP}$ controls at day 3.5 of pregnancy. uNKs are required for adequate trophoblast invasion into the uterine endometrium and maternal vasculature remodeling during the process of implantation. Therefore, the implantation defects observed in the Nodal deficient mice could result from diminished uNKs numbers. Moreover, $Nodal^{\Delta/\Delta}$ mice also had significantly fewer macrophages in the uterine tissue at day 3.5 compared to $Nodal^{loxP/loxP}$ controls. This suggests that the epithelial remodeling and vasculature establishment at the site of embryo implantation is disturbed, further explaining the implantation defects in the Nodal deficient females.

Interestingly, the number of eosinophils present in the uterine tissue in $Nodal^{\Delta/\Delta}$ was significantly higher than in $Nodal^{loxP/loxP}$ controls. Normally, as seen in the $Nodal^{loxP/loxP}$ control females, eosinophils are very low in pregnancy and do not play an important role. For this reason, limited research has been done evaluating the impacts of eosinophils in early pregnancy. While the increase in eosinophils in the Nodal knock-out females indicates that the immunological response is different from that in Nodal controls, the physiological impact of this difference needs to be investigated.

The immunofluorescence results obtained indicated that there were higher levels of leukocytes in the uterine tissues of $Nodal^{\Delta/\Delta}$ and $Nodal^{\Delta/+}$ compared to $Nodal^{loxP/loxP}$ controls.

However, the results obtained with flow cytometry revealed that there were significantly less macrophages and NK cells. There are a several proposed explanations for this discrepancy. First, the significantly higher levels of eosinophils and non-significantly higher levels of monocytes, neutrophils, cytotoxic T cells, and B cells account for the significant increase in leukocytes seen in the Nodal mutant mice. Second, the flow cytometry panel designed in these experiments could be missing some sub-populations of leukocytes that could be altered in the Nodal mutant mice. Finally, limitations in the quantification of leukocytes from the immunofluorescence results using a semi-quantitative method could be a cause in this discrepancy.

The types of immune cells present in the uterine tissue during the peri-implantation period and their respective quantities will affect the immunological landscape of the tissue. However, the gene expression of these cells will also delineate the maternal immune response. Therefore, to further characterize the state of the immune system in Nodal deficient mice during the peri-implantation period, 84 genes commonly involved in innate and adaptive immunity were examined on day 2.5 of pregnancy. From this, six genes were significantly altered in the Nodal deficient females compared to the controls.

The C-C chemokine receptor 5 (Ccr5) gene was significantly over-expressed in the uterine tissue of $Nodal^{\Delta/+}$ females compared to controls. The Ccr5 gene encodes a member of the beta chemokine receptor family commonly expressed by T cells and macrophages and regulates their trafficking to normal and inflamed tissues (Barmania & Pepper., 2013). Moreover, the CCR5 receptor also plays a role in granulocyte lineage proliferation and differentiation (Tuttle et al., 1998) Similarly, the C-C chemokine receptor 8 (Ccr8) gene was significantly over-expressed in the uterine tissue of both Nodal deficient groups compared to Nodal controls. The Ccr8 gene also encodes a member of the beta chemokine receptor family involved in the migration of various cell types into inflammatory sites. More specifically, this receptor plays a role in regulating monocyte chemotaxis and proper positioning of activated T cells within the antigenic challenge sites (Kang et al., 2021). Thus, the increased expression of these two chemokine receptors in the Nodal deficient females could explain the increased infiltration of leukocytes within the uterine tissues during the peri-implantation period. Moreover, since CCR5 plays an important role in granulocyte lineage proliferation and differentiation, over-expression of this gene could explain the increased population of eosinophils and decreased population of macrophages in the uterine tissue.

The H-2 class I histocompatibility antigen, q10 alpha chain (H2-q10) gene encoding a non-classical MHC molecule belonging to the MHC class I family was also significantly over-expressed in Nodal$^{\Delta/\Delta}$ compared to Nodal$^{loxP/loxP}$ controls. This family of molecules plays an important role in presenting foreign antigens to the adaptive branch of the immune system. MHC class I peptides are presented on nucleated cells and are used to differentiate healthy host cells from non-host or infected cells (Wieczorek et al., 2017). These markers will present either self or non-self antigens to cytotoxic CD8+ T cells (Rossjohn et al., 2015). In the case that non-self antigens are presented, the cytotoxic T cells will bind to these molecules and initiate an immune reaction to destroy the cell (Rossjohn et al., 2015; Sullivan et al., 2016). Over-expression of this gene in Nodal deficient females suggests that the leukocyte response is altered in these mice, giving rise to an increase in antigen presentation by nucleated cells, potentially increasing the cytotoxic T cell response.

The gene for the potent pro-inflammatory cytokine interleukin-1 beta (Il-1β) was also over-expressed in Nodal$^{\Delta/+}$ compared to Nodal controls. This cytokine is produced in response to inflammatory agents and is expressed by many leukocytes, including macrophages, NK cells, monocytes, and neutrophils (Dinarello, 2018; Kaneko et al., 2019). Moreover, this factor will bind to and activate the Toll-like/IL-1 receptor signaling pathways, further amplifying the local inflammatory microenvironment. Considering that the levels of cells that express IL-1β during the peri-implantation period in Nodal deficient mice is similar or lower to that of Nodal controls, this indicates that these cells are responding to an inflammatory event, further exacerbating the pro-inflammatory environment in the uterine tissue. Consequently, this will have an effect on the differentiation, activation, and response of leukocytes within the uterine tissue. This increased pro-inflammatory response was also observed in late pregnancy, where Nodal deficient females had higher levels of several pro-inflammatory cytokines, including IL-1β, in the maternal decidua (Ayash et al., 2020). This further suggests that Nodal functions as an anti-inflammatory factor regulating the maternal response in pregnancy.

Conversely, the gene for the cytokine interleukin-6 (Il-6) was significantly downregulated in Nodal$^{\Delta/+}$ and Nodal$^{\Delta/\Delta}$ compared to Nodal$^{loxP/loxP}$ controls. IL-6 acts as either a pro-inflammatory

cytokine or an anti-inflammatory cytokine depending on the receptor it binds to and its downstream signaling pathway. Multiple immune cell types, including mast cells, macrophages, dendritic cells, and T and B cells, are associated with the production of this cytokine (Luo & Zheng, 2016). IL-6 plays an important role in the differentiation of monocytes into macrophages, negative regulation of dendritic cell maturation, as well as the promotion of a pro-inflammatory cytotoxic T cell response (Dienz & Rincon, 2009; Velazquez-Salinas et al., 2019). Moreover, studies suggest that IL-6 is important in the differentiation of neutrophils and monocytes from myeloblast (Mehta et al., 2015). However, IL-6 is not involved in the differentiation of eosinophil and basophil from this common myeloblast progenitor. Therefore, the decrease in macrophages and increase in eosinophils found within the maternal uterine environment in Nodal deficient mice at day 3.5 of pregnancy could result from decreased Il-6 gene expression at day 2.5 of pregnancy. Moreover, the significant down-regulation of Il-6 further indicates an altered immune response in the Nodal deficient females.

Finally, the Myd88 gene was significantly under-expressed in only the $Nodal^{\Delta/+}$ compared to $Nodal^{loxP/loxP}$ control females. Myd88 encodes an adapter protein involved in the Toll-like receptor and IL-1 receptor signaling pathways expressed in most leukocytes. Activation of this pathway leads to NF-kappa-B activation and the production of pro-inflammatory factors that induce an inflammatory response (Kawai et al., 2004; Ohnishi et al., 2009). Since the state of the immune system in $Nodal^{\Delta/+}$ closely resembles that of Nodal controls, having on average the same proportion of different leukocytes, one could suspect that a decrease in expression of Myd88 could act as a compensatory mechanism limiting the action of IL-1 and other pro-inflammatory cytokines. Interestingly, studies into the role of the TGF-β superfamily revealed that MyD88 acts as a specific target of TGF-β factors, inhibiting TLR signaling (Naiki et al., 2005). Therefore, our results concur with was has been previously reported about the regulatory action of factors in the TGF-β superfamily.

In summary, mice that lack one or both alleles of Nodal in reproductive tissues have altered expression of genes relating to immune cell migration, differentiation, and response to inflammation. This results in an earlier and larger infiltration of leukocytes into the endometrium localizing close to the uterine lumen. In homozygous knock-out females, the leukocyte population found in the uterine tissue is also altered, resulting in high levels of eosinophils and low amounts

of uNKs and macrophages. These populations of immune cells are normal in heterozygote knock-out females, suggesting that the altered immunological response is less severe in these mice. Decreased expression of the Myd88 gene in these mice could indicate that there are compensatory mechanisms initiated in the presence of low levels of Nodal signaling. Overall, these results suggest that Nodal plays an important role in modulating the maternal immune response in early pregnancy and, in its absence, leads to an altered response, disrupting implantation and harming the blastocyst.

It is worth noting that further characterization of the maternal immune response in the absence of Nodal needs to be obtained. First, the proportion of immune cells characterized using flow cytometry was low, suggesting that some sub-populations of leukocytes were not being detected or that the leukocyte isolation protocol was not optimal. Future flow cytometry experiments will be further optimized and will include more leukocyte types. Moreover, there is increasing evidence suggesting that leukocytes segregate and cluster at the sites of implantation. Since Nodal expression adopts a banded-like pattern in the uterine tissue at the time of implantation, it would be interesting to characterize the effects of Nodal on immune clustering along the uterine tissue. Furthermore, a great in-depth analysis of the different sub-populations of leukocytes and their distribution in the different uterine layers should be performed.

A first look at the expression of immune-related genes in Nodal deficient mice revealed six significantly over/under-expressed genes. A greater analysis of the gene expression profile in these mice using RNA sequencing would need to be evaluated. Importantly, these changes in gene expression should be confirmed at the protein level to obtained an in-depth analysis of the state of the immune system. Finally, this study has shown that Nodal modulates the maternal immune response during early pregnancy; however, the mechanisms through which this is achieved have yet to be elucidated. Identifying which cells respond to Nodal through the expression of its co-receptor Cripto is required to understand its modulatory role fully.

Nevertheless, this study successfully characterized the maternal immune response in early pregnancy in Nodal deficient females compared to Nodal controls. In the in vivo experiments, confounding factors and biases were reduced by having the same individual perform all the dissections and experiments in the same environment, and by having control, heterozygous

mutants, and homozygous mutant mice during each session, as to reduce variability between the different mouse types. Observer bias was also reduced by assigning numbers to the control and mutant mice and performing blinded analyses. Furthermore, the sample size for each experiment was large enough to minimize variability and allow for a statistically significant analysis. Together, these measures support the validity of the claims made in this manuscript.

5 Conclusion

In conclusion, Nodal signaling, emanating from a maternal source in the uterus, is critical for the establishment of a healthy pregnancy. In addition to affecting early embryo development and viability, Nodal deletion from the maternal reproductive tract impairs implantation, leading to severe sub-fertility. This thesis investigating the causality behind this phenotype revealed that the Nodal signaling pathway is involved in regulating the maternal immune response to pregnancy. We have shown that the expression of genes relating to immune cell migration, differentiation, and response to inflammation are altered in Nodal conditional knock-out females. Moreover, these females have low levels of uterine macrophages and NK cells, and high levels of eosinophils. Lastly, Nodal deficient females have an increased infiltration of uterine leukocytes with cells localized in close proximity to the uterine lumen. These results indicate that the altered maternal immunological response to early pregnancy in Nodal deficient females is the underlying cause for their subfertility. Although we have not shown precisely the mechanistic role of Nodal in its modulatory effect on the immune system, we suggest that the signaling pathway exerts an inhibitory role on leukocytes, limiting their infiltration, differentiation, and pro-inflammatory response. This novel function of Nodal helps to better understand the role of the maternal immune system and its modulation in early pregnancy and can allow for the development of new therapeutic targets to decrease the incidence of RIF and infertility.

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SUPPLEMENTAL MATERIAL

	Fold Change (comparing to Nodal loxP/loxP)			
	Nodal Δ/+ (n=6)		Nodal Δ/Δ (n=5)	
Gene	Fold Change	p-value	Fold Change	p-value
Apcs	0.57	0.251705	0.56	0.257303
C3	**2.84**	**0.072038**	1.55	0.351448
C5ar1	1.63	0.233594	1.89	0.266683
Casp1	1.26	0.260561	1.13	0.368705
Ccl12	1.40	0.341826	1.33	0.364617
Ccl5	1.14	0.940752	0.84	0.823815
Ccr4	1.32	0.779029	0.78	0.393683
Ccr5	1.58	0.013592	1.45	0.259306
Ccr6	1.27	0.708382	**0.50**	0.209282
Ccr8	**2.13**	**0.026143**	**2.14**	0.056418
Cd14	1.16	0.492683	0.85	0.598538
Cd4	1.14	0.551282	1.28	0.320386
Cd40	1.25	0.259138	1.11	0.607840
Cd40lg	1.03	0.785101	0.71	0.309688
Cd80	1.65	0.120838	1.42	0.270296
Cd86	1.34	0.240078	1.10	0.757410
Cd8a	1.19	0.641082	0.61	0.321883
Crp	**0.47**	**0.134929**	0.62	0.583172
Csf2	0.63	0.547786	1.16	0.893718
Cxcl10	0.91	0.782057	1.05	0.522957
Cxcr3	1.16	0.786619	1.12	0.704020
Ddx58	0.91	0.520623	1.09	0.525955
Fasl	1.04	0.990098	1.29	0.500282
Foxp3	1.18	0.355018	1.13	0.454938
Gata3	1.39	0.177868	1.20	0.552520
H2-Q10	**3.38**	**0.075995**	**6.38**	**0.023259**
H2-T23	1.46	0.090797	1.19	0.423732
Icam1	1.38	0.023051	1.16	0.115949
Ifna2	0.89	0.747446	1.03	0.817923
Ifnar1	1.05	0.572697	1.00	0.911733
Ifnb1	0.61	0.135695	0.98	0.860272
Ifng	0.69	0.166719	0.54	0.616634
Ifngr1	1.31	0.165928	1.36	0.026547
Il10	1.56	0.218997	1.42	0.250854
Il13	0.80	0.286821	0.91	0.719063
Il17a	1.23	0.401248	0.68	0.046827
Il18	1.14	0.578425	1.06	0.943279
Il1a	**2.59**	**0.314023**	1.84	0.331919
Il1b	**3.83**	**0.023028**	**2.81**	0.100791
Il1r1	1.06	0.582479	1.03	0.757674
Il2	0.62	0.282558	0.60	0.284274
Il23a	1.20	0.480446	0.94	0.731608
Il4	1.90	0.099151	**2.04**	0.123394
Il5	0.94	0.853182	1.08	0.820802
Il6	**0.16**	**0.026137**	**0.12**	**0.033044**

Irak1	0.78	0.188471	0.82	0.124243
Irf3	0.80	0.385423	0.95	0.481530
Irf7	1.70	0.130875	1.49	0.258391
Itgam	1.73	0.189541	1.45	0.331899
Jak2	0.90	0.555217	0.78	0.033314
Ly96	1.22	0.304374	1.13	0.456130
Lyz2	1.06	0.954863	0.97	0.830841
Mapk1	0.88	0.453298	0.87	0.076517
Mapk8	0.61	0.184039	0.63	0.236852
Mbl2	0.53	0.226780	0.54	0.254614
Mpo	1.15	0.546597	0.87	0.355090
Mx1	1.45	0.193525	1.65	0.226818
Myd88	**0.53**	**0.026243**	0.83	0.626076
Nfkb1	1.03	0.655835	0.98	0.653493
Nfkbia	0.99	0.907127	1.06	0.565904
Nlrp3	1.67	0.064331	1.48	0.320229
Nod1	1.19	0.201816	1.15	0.158291
Nod2	1.60	0.093492	1.23	0.219410
Rag1	0.67	0.291100	0.79	0.427048
Rorc	1.44	0.138150	0.98	0.927636
Slc11a1	1.15	0.437166	1.05	0.739246
Stat1	1.18	0.296217	1.11	0.546963
Stat3	1.47	0.033491	1.20	0.096640
Stat4	1.14	0.850477	1.17	0.648225
Stat6	1.20	0.195784	1.05	0.650532
Tbx21	1.63	0.154437	1.56	0.376258
Ticam1	1.14	0.430913	0.92	0.466296
Tlr1	1.19	0.323373	1.30	0.138010
Tlr2	1.56	0.061628	1.19	0.371559
Tlr3	1.06	0.580032	1.03	0.708790
Tlr4	1.32	0.173589	1.24	0.255841
Tlr5	1.44	0.179411	1.27	0.252777
Tlr6	1.09	0.576267	0.99	0.950844
Tlr7	1.12	0.684418	1.21	0.541839
Tlr8	1.27	0.426407	1.13	0.750551
Tlr9	1.46	0.038749	1.27	0.425296
Tnf	**2.22**	**0.160963**	1.48	0.331203
Traf6	0.98	0.963236	1.03	0.753310
Tyk2	1.17	0.266125	1.09	0.483950

Supplementary Table 1- Expression of 84 genes in the RT^2 PCR Profile array in maternal uterine tissue at day 2.5 of pregnancy. *P*- value≤0.05 by 1-way ANOVA with Bonferroni's multiple comparisons test.