Nodal is Required to Maintain the Uterine Environment in an Anti-inflammatory State During Pregnancy

by
Taghreed Ayash

Division of Experimental Medicine

Department of Medicine

Faculty of Medicine

McGill University

Montreal, QC, Canada

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ABSTRACT

Preterm birth, defined as delivery before 37 weeks of gestation, is a global problem. It is a significant cause of neonatal morbidity and mortality where premature infants are at increased risk of blindness, respiratory defects and cerebral palsy. Preterm birth is a complicated phenotype resulting from interactions between genetic and environmental, social and health factors. Our laboratory has previously demonstrated that Nodal, a morphogen belonging to the TGF-ß superfamily, is involved in implantation, placentation, and most importantly, preterm birth (PTB). Interestingly, 80% of the pregnant Nodal conditional uterine-specific knockout females deliver two days prior to term which is a rare phenotype in mice. To determine the role of Nodal in normal pregnancy and to study the cause of preterm birth in Nodal conditional mutants, we have used Nodal conditional heterozygotes which do not have preterm births but may have increased susceptibility if challenged by other risk factors such as inflammation.

We have observed in our heterozygous Nodal mouse model that fertility is decreased to 50% compared to control mice. Furthermore, these mice are sensitive to a low dose of inflammatory mediator LPS, which leads to 50% of PTB 12h post-injection but did not affect control mice. We demonstrated that Nodal heterozygous mice have a higher basal level of proinflammatory cytokines in the maternal decidual tissue compared to control mice. We also demonstrated that Nodal reduces the expression of pro-inflammatory cytokines in macrophages that are challenged with the inflammatory agent LPS. Using the RAW264.7 macrophage cell line, we demonstrated that the reduction of proinflammatory cytokine expression by Nodal is by preventing phosphorylation of nuclear NFκB p65 as well as p38MAPK in response to LPS. Finally, we have addressed whether Nodal plays a role in human pregnancy. We showed that single nucleotide polymorphisms (SNPs) in NODAL, which affect expression or activity, were not

directly associated with preterm birth in humans. However, by subdividing our population according to different risk conditions, we were able to demonstrate that certain Nodal SNPs correlated with preterm birth in women who had an infection or fetal membrane inflammation.

Our results uncover a novel role of Nodal in maintaining the uterine environment in an anti-inflammatory state during mid-pregnancy and show that a deletion or mutation of one allele increases the susceptibility to give birth preterm if challenged with an inflammatory agent. Our work helps to better understand the role of Nodal during pregnancy and raises the possibility that Nodal may also be involved in other inflammatory diseases.

RÉSUMÉ

L'accouchement prématuré est défini comme l'accouchement avant 37 semaines de gestation et constitue un problème global mondialement. Cela présente une cause de morbidité et mortalité néonatales mondialement où les nourrissons prématurés ont un risque plus élevé de cécité, de malformations respiratoires et de paralysie cérébrale. L'accouchement prématuré est un phénotype compliqué qui résulte des interactions entre des facteurs génétiques, environnementaux, sociaux et des facteurs de santé. Notre laboratoire a déjà démontré que Nodal, un morphogène appartenant à la famille TGF-ß, est impliqué dans l'implantation, la placentation et surtout dans l'accouchement prématuré. En effet, 80% des souris femelles avec une inactivation conditionnelle de Nodal enceintes accouchent deux jours avant terme, ce qui est un phénotype très rare chez les souris. Pour déterminer le rôle de Nodal dans la grossesse normale et pour étudier la cause de l'accouchement prématuré chez les mutantes conditionnelles Nodal, nous avons utilisé des souris hétérozygotes pour l'inactivation conditionnelle de Nodal qui n'ont pas d'accouchement prématuré mais qui ont une plus grande susceptibilité à d'autres risques facteurs comme l'inflammation.

Chez nos souris hétérozygotes pour Nodal, nous avons observé que le taux de fertilité est diminué à 50% comparé aux souris contrôles. De plus, ces souris sont sensibles à une basse dose de LPS, un médiateur inflammatoire, ce qui résulte en un taux de 50% de naissances prématurés 12 heurs après l'injection, mais n'affecte pas les souris contrôles. Nous avons démontré que les souris hétérozygotes pour Nodal ont un niveau basal de cytokines pro-inflammatoires plus élevé dans le tissu maternelle déciduale comparé aux souris contrôles. Nous avons aussi démontré que Nodal réduit l'expression de cytokine pro-inflammatoires dans les macrophages qui sont attaqués par l'agent inflammatoire LPS. En utilisant un assai *in vitro* sur la lignée de cellules RAW264.7,

nous avons démontré que la réduction de l'expression de cytokines pro-inflammatoires par Nodal empêche la phosphorylation du facteur nucléaire NFκB p65 et de p38MAPK en réponse au LPS. Enfin, nous avons adressé la question du rôle joué par Nodal dans la grossesse humaine. Nous avons montré qu'un polymorphisme de nucléotide simple (PNS) dans NODAL, qui affecte son expression ou l'activité, n'était pas directement associé avec l'accouchement prématuré chez l'humain. Cependant, en subdivisant notre population d'après différentes conditions de risque, nous avons démontré que certains PNSs de Nodal sont corrélés avec l'accouchement prématuré chez les femmes qui ont eu une infection ou une inflammation de la membrane fœtale.

Nos résultats dévoilent un nouveau rôle de Nodal dans la maintenance de l'environnement utérin dans un état anti-inflammatoire durant la mid-gestation, et la délétion ou mutation d'un allèle augmente la susceptibilité d'accoucher prématurément s'il y a une attaque par un agent inflammatoire. Notre travail aide à approfondir nos connaissances sur le rôle de Nodal durant la grossesse et évoque la possibilité que Nodal pourrait aussi être impliqué dans d'autres maladies inflammatoires.

Acknowledgments

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I would also like to express my gratitude to Dr. Alaa Moamer who has been there for all the seasons of life whether it is the ups, the downs, the happy or the sad. Thank you for being my sister, thank you for loving me and giving me support no matter what I am going through and for being happy when I'm happy. Thank you being in my life.

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Dedication

Doing my PhD at McGill University took all my time and kept me far from the lovely people in my life. This thesis is dedicated primarily to my parents. To my father Mr. Ali Ayash: you have been such a great man in my life, even when you are far your presence is felt. I am honored to be the daughter that you raised. To my mother, Mrs. Sahlah Al-Gari, who has provided me with unconditional love, encouragement and motivation. Both of you have taught me to never back down and to take the world by storm. I have only had the strength to grow and become more because I knew you were behind me every step of the way.

To my siblings: we have been through a lot together in our lives. Without each other, I would not have made it. I am lucky to have many brothers and sisters who are always behind me. Whenever I feel down, I turn to find different shapes and forms of love and support, some overprotective, some strict, others passive, frank, and cool. I could not be prouder to be part of such an amazing family.

Preface and Contribution of Authors

This thesis is presented in a manuscript-based format. This thesis is written by Taghreed Ayash and revised by my supervisor Dr. Daniel Dufort. It consists of five major chapters. Chapter 1 includes the introduction and a review of the related literature. Chapters 2 and 3 contain manuscripts under submission; Chapter 4 contains a published in manuscript in Journal of Perinatology in 2018.

Chapter 2 under submission. Taghreed Ayash, Dr. Lisa Starr and Dr. Dufort conceived and designed the study. Taghreed Ayash and Dr. Lisa Starr performed the experiments. Taghreed Ayash, Dr. Lisa Starr and Dr. Dufort wrote the manuscript.

Chapter 3 under submission. I wrote the manuscript under my supervisor's guidance. I designed the experiments described under his direction. Dr. Dufort contributed to the design of the experiments, supervised the project and helped draft the article

Chapter 4 constitutes a manuscript that was published in the Journal of *Perinatology Journal* in 2018. Dr. Starr, Taghreed Ayash, and Dr. Dufort conceived and designed the study. Dr. Starr and Taghreed Ayash performed the analyses experiments.

I have collaborated on, published, submitted and have currently under submission the following articles

As a first author:

1. Taghreed A. AYASH, Lisa M. STARR, Daniel DUFORT, Nodal Deletion Increases Susceptibility to LPS-Induced Preterm Birth in Mice (under submission)
2. Taghreed A. AYASH and Daniel DUFORT, NODAL Regulates LPS-Induced Inflammation Response by Macrophages through NFκB and MAPK Pathways. (under submission)

As second author:

3. Lisa M. STARR, Taghreed A. AYASH, Daniel DUFORT, Evidence of a gene-environment interaction of NODAL variants and inflammation in preterm birth. J Perinatol. 2018 May; Epub 2018 Feb 16

Contribution to knowledge

In this thesis, I investigated the role of Nodal in the immunological state during late pregnancy and determined that mutations in NODAL lead to an increase in the susceptibility of preterm birth incidence in mice and humans.

- In chapter 2, we uncovered that, in mice, missing one allele of NODAL in the uterus results in a 50% reduction in the Nodal heterozygous mice fertility.
- Also, in chapter 2 we showed that Nodal heterozygous mice have a higher basal level of chemokines and cytokines as well as in higher immune cell infiltration in the maternal decidua tissue.
- In this chapter, our data highlighted that NODAL is required for maintaining the uterine immunological environment in an anti-inflammatory state during late pregnancy.

- In chapter 3, our data showed that Nodal is decreasing cytokine expression in macrophages in response to LPS.
- In this chapter, our data revealed that Nodal significantly decreases global protein phosphorylation.
- We also highlighted that NODAL attenuates the LPS-induced inflammation, throughout NFkB and MAPK pathways.

- In chapter 4, in a secondary analysis of a human population, our data highlighted that NODAL SNPs were not directly associated with an increase in the incidence of preterm birth.

- In this chapter, our data highlighted that women with certain NODAL SNPs had increase the susceptibility for PTB in the presence of infection/inflammation, which showed the genetic-environment interaction between NODAL SNPs and infection/inflammation in our population.

Abbreviations

ActRII	activin receptor, typeII
ALK	activin receptor-like kinase
BSA	bovine serum albumin
BV	bacterial vaginosis
BMP	bone morphogenetic protein
CCL	chemokine c-c motif ligand
CD	cluster of differentiation
CI	confidence interval
χ^2	Chi-square
COX	cyclooxygenase
DAB	dolichos biflorus agglutinin
DCs	dendritic cells
EBAF	endometrial bleeding associated factors
EGE-CFC	epidermal growth factor-cripto, cryptic
ERK	extracellular signal-regulated kinase
$\mathbf{F_{ST}}$	fixation index
GPI	glycosylphosphatidylinositol
GDF	growth determining factor
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HWE	Hardy-Weinberg equilibrium
IUGR	intrauterine growth restriction

ICM	inner cell mass
IL	interleukin
IFN-γ	interferon-gamma
LPS	lipopolysaccharides
MAPK	mitogen activated protein kinase
MCP-1	macrophage chemoattractant protein-1
MMP	matrix metalloproteinase
mPGES1	membrane-associated PGE synthase1
MPS	Montreal Prematurity Study
NFkB	nuclear factor kappa
NO	nitric oxide
NS	non-significant
OR	odds ratio
P_4	progesterone
PBS	phosphate buffered saline
PBT	phosphate buffered saline,0.1% Tween-20
PCR	polymerase chain reaction
P-SMAD	phosphorylated-SMAD
PFA	paraformaldehyde
PG($F_{2\alpha}$, E_2, I_2, H_2)	prostaglandin(F,E,I,H)
PGFS/PGIS	prostaglandin (F, I) synthase
PI	placental inflammation
PR	progesterone receptor

PTB	preterm birth
PVDF	polyvinylidene fluoride
RT-PCR	real time polymerase chain reaction
s.e.m.	standard error of the mean
SP	surfactant proteins
SEPS1	Selenoprotein S
SNP	single nucleotide polymorphisms
TNF-α	tumor necrosis factor- alpha
TGF-β	transforming growth factor-beta
TGC	trophoblast giant cells
NK	natural killer (cells)
pNK	peripheral natural killer (cells)
uNK	uterine natural killer (cells)
VAF	variant allele frequency
100G	1000 Genomes Project

Chapter1: Background

1 Introduction

1.1 Pregnancy

Reproduction is a fundamental process for producing offspring. It is essential for the survival and persistence of a species. Different species use diverse strategies, including sexual and asexual processes, which result in the creation of new individuals. In mammals, the reproductive process is accomplished by the fusion of haploid gametes, namely, the female gamete (oocyte) and the male gamete (spermatozoa). This fusion results in the formation of diploid offspring.

The pregnancy process normally starts when spermatozoa travel into the female reproductive tract through the cervix, and into the uterine cavity. In the fallopian tubes, where fertilization takes place, the fertilized egg (embryo) travels to the uterine cavity for implantation in the endometrial wall of the uterus, and eventually, the placenta starts to facilitate the exchange of nutrition, gas and waste between the fetal and maternal blood circulation throughout the pregnancy ((UK). 2013, Georgiades et al 2002). In addition, the placenta provides immunological protection to the embryo *in utero*, and it works as an endocrine organ that has been implicated in the activation of the labor cascade (Georgiades et al 2002).

Pregnancy is a complicated process that includes different events such as implantation, decidualization, placentation, and finally, the activation of the parturition cascade, which leads to delivery. A defect in any of these events may lead to adverse pregnancy outcomes, including infertility and preterm delivery (Dey et al 2004, Wang & Dey 2006).

Pregnancy is controlled by different factors. Some of the factors are the timing and the incidence of sexual intercourse, the quality of both the sperm and the ova, the frequency and the length of the menstrual cycle and ovulation ((UK). 2013). In addition, other environmental factors affect pregnancy, such as alcohol consumption, smoking and body weight (obesity and low body weight). All these factors affect the pregnancy and pregnancy outcomes, including the timing of the delivery and pregnancy related complications such as preeclampsia (UK 2013). Moreover, pregnancy is controlled via a variety of complex molecules, including cytokines, hormones and growth factors. The dynamic role of these molecules controls the molecular and cellular actions that lead to implantation and pregnancy (Singh et al 2011, Wang et al 1994).

Infertility is a common clinical phenomenon, affecting approximately 7-10% of couples worldwide. Despite extensive research on the clinical causes of infertility, approximately 15-30% of cases are still unexplained (Quaas & Dokras 2008). Additionally, most infertility cases result from defects during preimplantation or implantation, with defects including ovarian deficiency, insufficient male/female gametes, or hormonal and genetic disorders, which result in implantation failure and miscarriage (Dey et al 2004, Park & Dufort 2013).

Following an effective implantation and the establishment of a successful pregnancy, some pregnancy complications can occur during the middle to late phases of the reproductive process that might increase the risks to fetal and maternal health. A deficient placenta can cause late pregnancy disorders including intrauterine growth restriction (IUGR) and fetal loss (miscarriage) due to a lack of nutrient exchange. Abnormally deep placentation known as placenta accreta can lead to hemorrhaging in the maternal uterine arteries, which

causes placental abruption. In addition, abnormal placental positioning over the cervix (placenta previa) results in vaginal bleeding and fetal malnourishment (Oyelese & Smulian 2006). The placenta might be implicated in some pregnancy complications, including preterm birth and pre-eclampsia. In the case of pre-eclampsia, insufficient placental development might cause placental hypoxia, which initiates a maternal immune response causing a high risk to both maternal and fetal life (Soleymanlou et al 2005). Preterm birth (PTB) is a leading cause of neonatal mortality and morbidity around the world (Purisch & Gyamfi-Bannerman 2017). PTB is a complex symptom resulting from multiple factors, and poor placentation has been implicated in causing preterm birth. In addition, other clinical complications are associated with preterm birth, such as vaginal bleeding, and maternal infection. (Goldenberg et al 2008, Romero et al 2007). Preterm birth is also affected by the interaction between genetics and other environmental factors such as stress, infection, smoking, etc. However, the causes of preterm birth are still being investigated (Faye-Petersen 2008, Goldenberg et al 2008, Romero et al 2007).

1.2 Implantation

Implantation is an essential stage for a successful pregnancy and is required for proper embryonic development to term. Implantation involves the attachment of the embryo, which is known as a blastocyst at this stage, to the endometrium of the uterine wall (Figure 1.1) (Kim & Kim 2017). Embryo implantation is under the control of ovarian steroid hormones, including progesterone and estrogen (Kim & Kim 2017, Lee et al 2007). Uterine receptivity is an essential prerequisite to implantation. Having a successful implantation depends on both a receptive uterus and a competent embryo (Figure 1.1) (Wang & Dey

2006). In addition, there is a period when the molecular crosstalk between the uterus, embryo and ovary is important for implantation. This time span during which both the blastocyst is competent and the uterus is receptive is known as the window of implantation (Wang & Dey 2006).

It has been shown that both the human and the mouse uterus undergo different stages of receptivity, from nonreceptive to prereceptive and finally to a refractive state (Dey et al 2004). In mice, early pregnancy is divided into three periods, namely, a prereceptive period (gestational day 1-4), a receptive period (gestational day 4-5) and finally a period that lasts for the remainder of the pregnancy (Dey et al 2004). On day 4 post coitum, the uterus becomes receptive to implantation. At this time, which lasts approximately 24 hours, the uterus becomes ready to receive an embryo. On day 5, if implantation has not occurred, the uterus shifts from the receptive to the nonreceptive stage and becomes hostile to blastocyst implantation and survival (Figure 1.1) (Cha et al 2012, Dey et al 2004).

Similarly, in humans, the histological and functional terms for the uterus are divided into proliferative (follicular) and secretory (luteal) stages during the 28-30-day menstrual cycle. The cycle begins with the proliferative stage where stromal and epithelial cells proliferate. After ovulation, the early secretory stage begins and lasts from 1- 7 days and is similar to the prereceptive stage in mice. During the mid-secretory stage (7-10 days after ovulation), the uterus becomes receptive. If implantation has not occurred, the endometrial tissues will be shed, in the process termed menses.

Gonadal hormones, estrogen (E2) and progesterone (P4) are known to direct the receptivity and overall pregnancy outcome in both mice and humans (Cha et al 2012). While progesterone is required for embryo implantation in all studied mammals, maternal estrogen is only needed for

implantation in only a subset (Dey et al 2004). In mice, on gestational day 4 during the preimplantation period, a peak is observed in estrogen level right before ovulation, but this is not the case with humans (Ma et al 2003). A proper level of estrogen during this period is thought to be crucial for maintaining the uterus in a receptive state (Ma et al 2003). However, the human embryo can synthesize estrogen, whereas the mouse embryo cannot (Blackburn 2007). Progesterone is often referred to as the pregnancy hormone. The level of P4 is gradually elevated during implantation time, and it remains high until the parturition, at which point it declines only in mice but not in humans (Ratajczak & Muglia 2008).

1.3 Decidualization

During the final stage of implantation, the stroma cells undergo significant morphological changes by differentiating into decidual cells, which is known as decidualization. Decidual cells provide nutrients for the implanted embryo as well as mediating the growing conceptus prior to the formation of the placenta (Figure 1.1)(Ramathal et al 2010).

In humans, the decidual event is coupled with each menstrual cycle during the late secretory stage (Popovici et al 2000). Each decidual cell is enclosed individually in a pericellular membrane and all the decidual cells together form a barrier that restricts the level of impending extraembryonic invasion. The decidual event is only completed at implantation, even though the decidualization event occurs independently each cycle(Cunningham FG 2010).

In mice, decidualization occurs after the implantation period. It is first initiated in the stromal region adjoining the implanting embryo in the anti- mesometrial region of the uterus and then extends towards the outer myometrium before extending towards the

mesometrium (Abrahamsohn & Zorn 1993). Many decidual cells undergo multiple rounds of DNA replication in the absence of division and this process is known as endoreduplication (Tan et al 2002). This process is essential for the formation and function of decidual cells. In addition, it increases protein synthesis and gene transcription (Mori et al 2011). In both humans and mice, the activity of progesterone followed by estrogen priming appears to be the primary mediator of decidualization (Ramathal et al 2010).

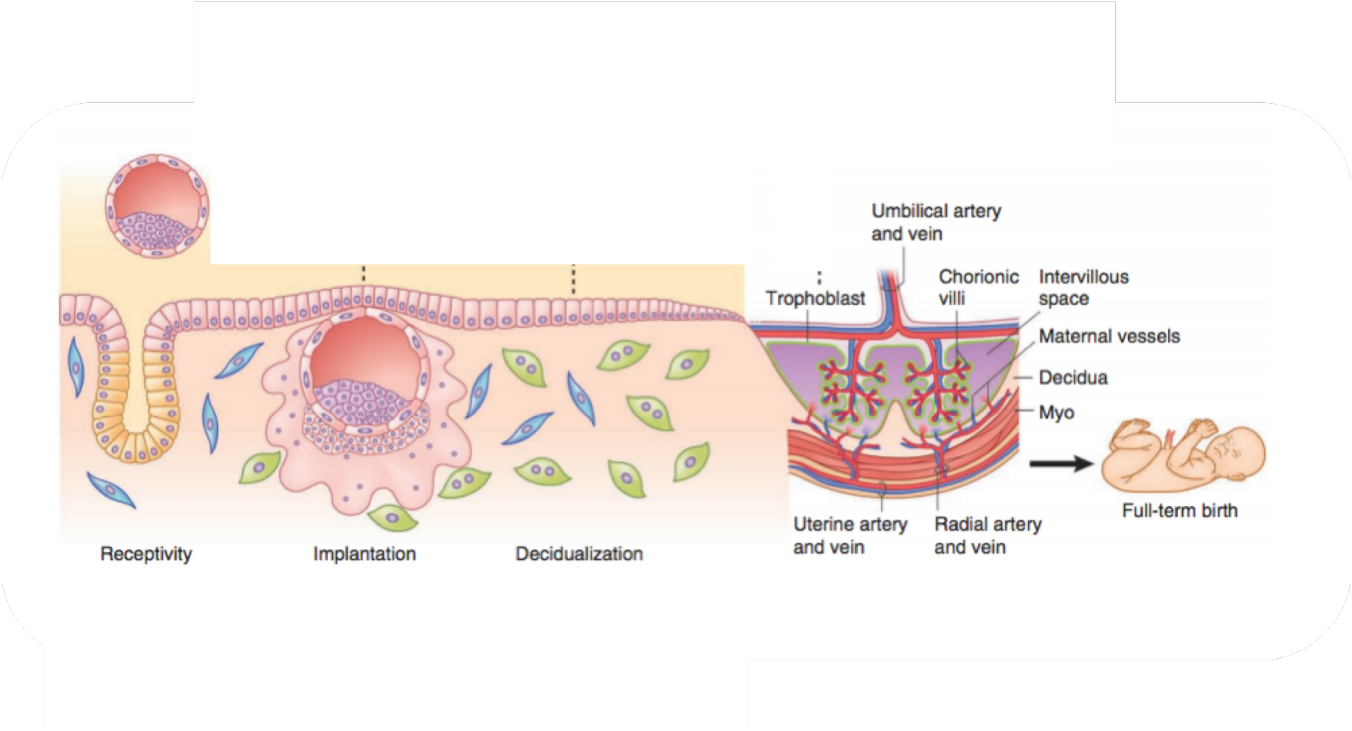

Figure 1.1A diagram showing the different stages of mouse pregnancy including uterus receptivity, implantation, decidualization, placentation and finally the time of parturition. Figure modified from (Cha et al 2012).

1.4 Placentation

The placenta is an important organ that facilitates pregnancy and plays a vital role in linking the maternal and fetal environment, which is essential for embryo survival (Rossant & Cross 2001). The placenta is needed for many functions during pregnancy, which includes providing adequate nourishment, such as nutrients and gases as well as waste removal via blood circulation between the mother and the fetus (Cross 2006). Furthermore, an endocrinological function of the placenta has been revealed; it secretes hormones and growth factors that are involved in fetal growth and facilitates the initiation of the parturition cascade (Rossant & Cross 2001). Moreover, the placenta acts as a barrier that protects the embryo from the maternal immune system (Georgiades et al 2002, Rawn & Cross 2008).

1.4.1 Human and mouse placentation

In humans, placentation initiates post embryo penetration, and the trophectoderm layer of the blastocyst will eventually differentiate into two layers, an outer layer called the syncytiotrophoblast and an inner layer known as the cytotrophoblast (Aplin 2010). Furthermore, the trophoblast differentiates into the villous trophoblast, which gives rise to the chorionic villi, as well as the extravillous trophoblast, which migrates and invades the maternal decidua and vasculature (Figure 1.2) (Lunghi et al 2007, Rossant & Cross 2001). The chorionic villi develop into a large, tree-like pattern that fills the intervillous space. After that, the connection between the chorionic villi and embryonic blood vessels is formed, and it facilitates contact between the maternal and fetal circulation (Figure 1.2) (Georgiades et al 2002).

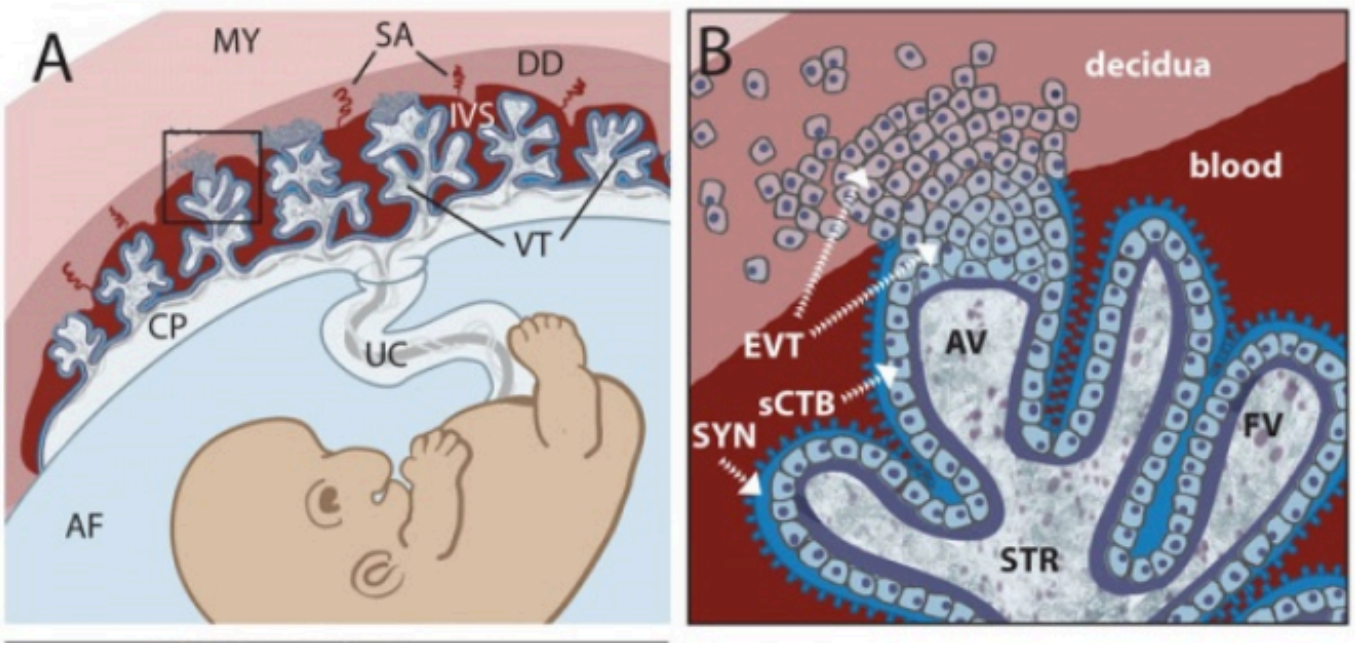

Figure 1.2Human placental structure

(A): showed structure of human placenta at 6 weeks of gestation. MY: myometrium, SA spiral arteries, DD: decidua, IVS: intervillous space, VT: villous tree, CP: chorionic plate, UC: umbilical cord, AF: amniotic fluid. (B): Enlargement of highlighted area in panel(A) The maternal blood surrounds the villous tree comprised of anchoring (AV) Villi and floating villi (FV) that are covered by a syncytiotrophoblast (SYN) layer, which the subsyncytial cytotrophoblast(sCTB)layer. (STR) stroma,(Robbins et al 2010)

Early on embryonic day 3.5 of mouse pregnancy, the blastocyst contains two distinct cell lineages, namely the inner cell mass (ICM) and the trophectoderm. The ICM gives rise to the entire embryo and some of the extraembryonic membranes. The outer trophectoderm layer of the blastocyst forms the fetal component of the placenta (Wang & Dey 2006). At embryonic day 4.5, which is when implantation occurs, the trophectoderm differentiates into two diploid cell types, the extraembryonic ectoderm and the ectoplacental cone. The remaining cells of the trophectoderm undergo endoreduplication to become trophoblast giant cells (TGCs) (Rossant & Cross 2001). The extraembryonic ectoderm grows to form the chorionic epithelium, and at embryonic day 8.5, the allantois attaches to the chorion to form the chorioallantoic membrane. Shortly after that, the primary villi begin to grow through the chorionic surface (Cross et al 2003, Woods et al 2018). The fetoplacental blood vessels then undergo extensive development and branching to form the labyrinth layer, which is the structure that forms the mature and functional placenta. The labyrinth is the primary site of interaction between the maternal and fetal circulation (Rossant & Cross 2001, Watson & Cross 2005). At embryonic day 10.5, the complete placenta has formed (Adamson et al 2002, Rossant & Cross 2001). As the labyrinth layer forms, the spongiotrophoblast also develops from the ectoplacental cone. This layer includes both the spongiotrophoblast and the trophoblast glycogen cells. A thin layer termed the parietal TGC layer is formed by the TGCs and functions as a median between maternal and fetal-derived tissues. The maternal compartment includes the decidua basalis, decidua parietalis and uterine wall (Figure 1.3)(Adamson et al 2002, Coan et al 2004).

There are some differences between the human and mouse placentas that affect the use of the mouse placenta as a model for human placentation. The small size of the mouse

placenta is one of these disadvantages; however, this issue is being addressed using advanced techniques, including imaging(Adamson et al 2002, Mu & Adamson 2006). Despite the differences between human and mouse placentas, their similar structure and organization still makes the mouse placenta a suitable model for studying human placentation(Carter 2007).

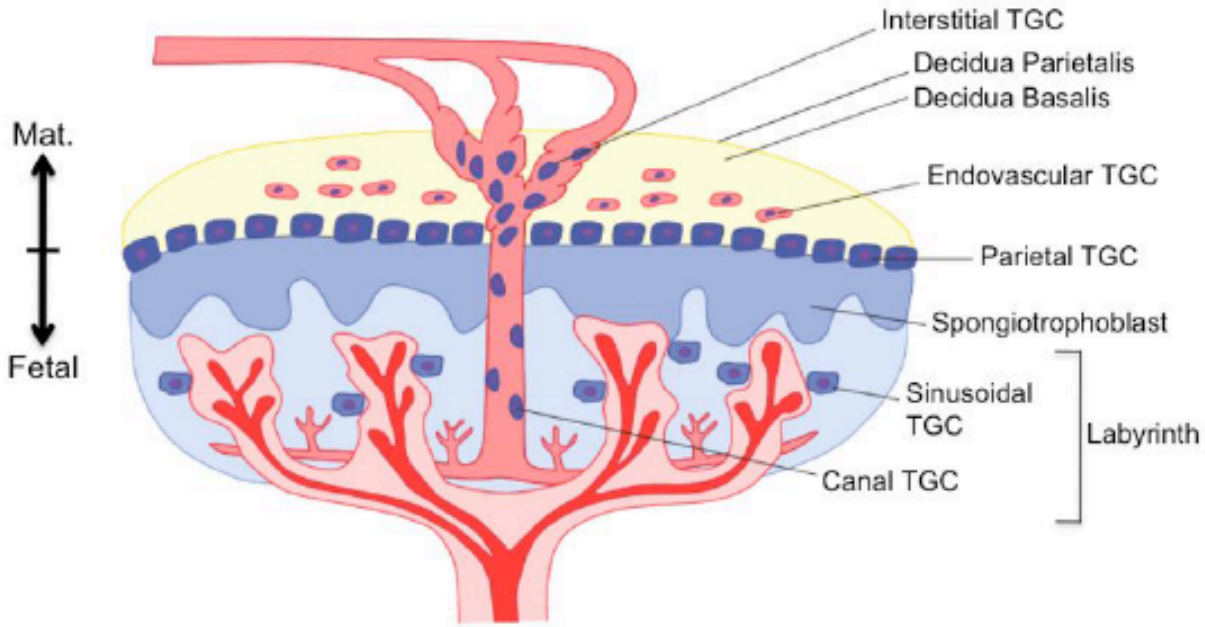

Figure 1.3 Structure of the mouse placenta

Structure of the mouse placenta at day 12.5 that shows the maternal compartment (top) and fetal compartment (bottom), as separated by trophoblast giant cells (TGC). From (Rawn & Cross 2008).

1.5 Parturition

Parturition is the process of expelling the fetus, placenta and membranes from the uterus through the birth canal at approximately the 40th week of human pregnancy. However, despite decades of research, the mechanisms involved in the initiation of labor remain unclear (Goldenberg et al 2008, Ravanos et al 2015). Parturition begins at the placental interface with the production of prostaglandins derived from the COX/PGFS/$PGF_{2\alpha}$ signaling axis (Figure 1.4). In brief, the increased COX1/2 converts arachidonic acid to PGH_2 and initiates labor. PGH_2 is converted into $PGF_{2\alpha}$, PGE_2, and PGI_2 by the enzymes PGFS, mpges1, and PGIS, respectively. Eventually, the production of $PGF_{2\alpha}$ results in the degradation of the corpus luteum, which causes a significant decline in the progesterone concentration in the maternal blood (Ratajczak & Muglia 2008). This process is known as "progesterone withdrawal" and it leads to the further activation of the labor pathway by stimulating the ripening and dilation of the cervix, followed by uterine contractions via myometrial contraction-associated proteins (Figure 1.4) (Condon et al 2004, Migale et al 2016, Muglia 2000, Ratajczak & Muglia 2008, Sugimoto et al 1997).

Despite extensive research on the parturition pathway, its underlying mechanism remains unclear. Animal models have been used extensively to understand the molecular mechanisms involved in the regulation and initiation of birth (Cappelletti et al 2016, Mitchell & Taggart 2009a). The low cost of mice and their short gestational time make them a good model for studying human parturition. Additionally, the pathways that regulate the immune response are conserved between mice and humans. This conservation indicates that using mice to study the role of

infection/inflammation in stimulating PTB makes them a useful animal model (Cappelletti et al 2016, Mesiano et al 2002).

Accumulating evidence supports the idea that, despite the different etiologies that cause spontaneous PTB and term birth, they all share a common final cascade leading to the activation of the labor pathway at different gestational time points: this involves inflammatory processes and the activation of uterine contractions and ripening and leads to the initiation of the labor pathway (Ravanos et al 2015).

Parturition is initiated when the immunological state of the uterus changes from an anti-inflammatory to a pro-inflammatory state. This specific switch results in the production of many cytokines and chemokines involved in the activation of the inflammatory cascade, which leads to the initiation of the labor cascade either as a term delivery or as PTB. This activation results in the induction of inflammatory-associated genes, including those that encode the transcription factor known as nuclear factor kappa light chain enhancer of activated B cells (NFkB) (Condon et al 2004, Migale et al 2016, Sugimoto et al 1997).

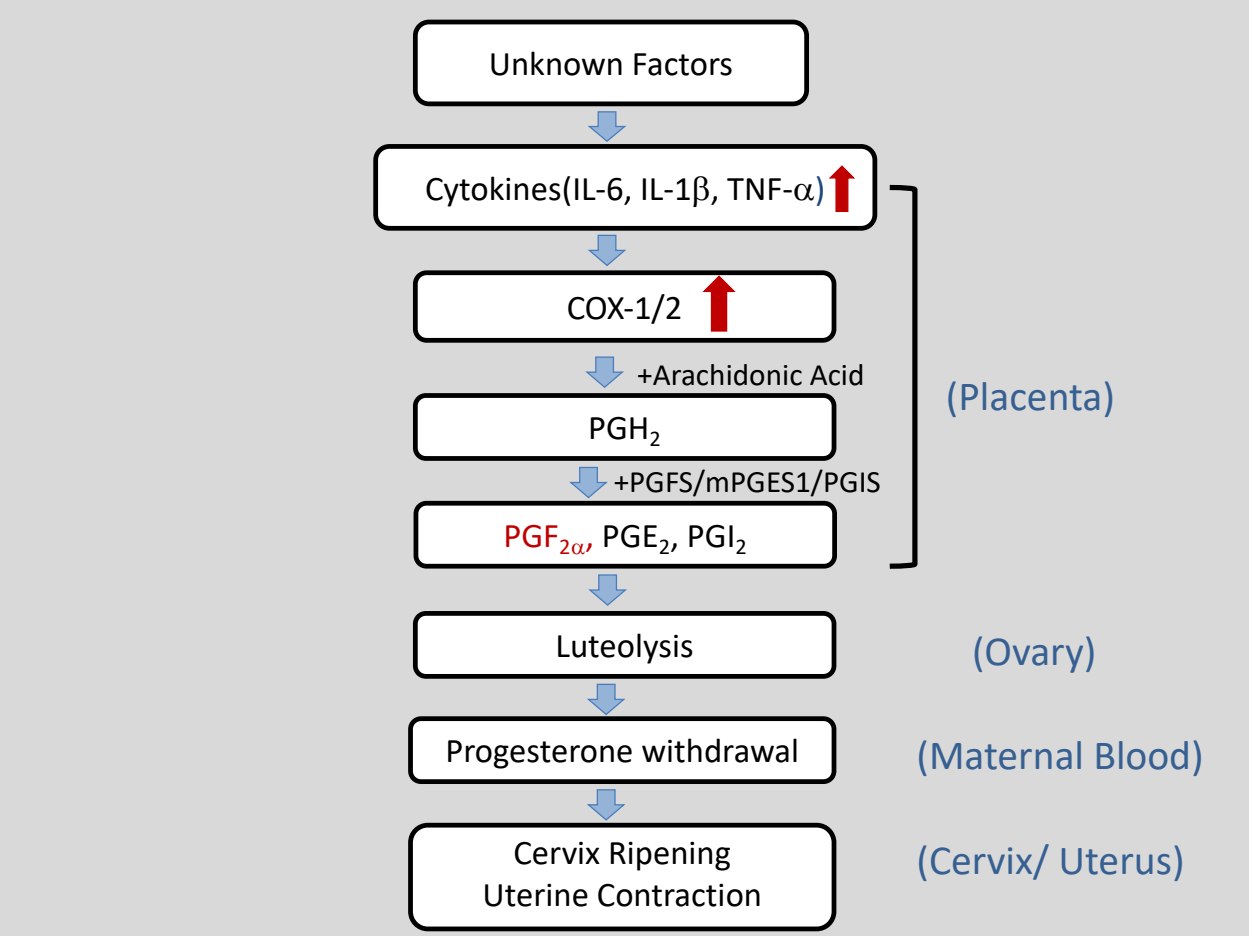

Figure 1.4 Parturition cascade

Unknown factors cause an increase in pro-inflammatory cytokines, which leads to increased COX1/2 in the placental tissue. The increased COX1/2 converts arachidonic acid to PGH_2 and initiates labor. PGH_2 is converted into $PGF_{2\alpha}$, PGE_2, and PGI_2 by the enzymes PGFS, mpges1, and PGIS, respectively. The production of $PGF_{2\alpha}$ results in the degradation of the corpus luteus, which causes a significant decline in the progesterone concentration in the maternal blood. Ultimately, progesterone withdrawal results in cervix ripening and uterine contractions to facilitate the delivery process.

1.6 Infection cascade during PTB

Infection/inflammation are major causes of PTB. Studies have shown that the infection of the genital tract is associated with approximately 25-40% of PTB cases (Nadeau et al 2016); this variation depends on the different methodologies used in infection detection as well as where the infection occurs in the female tract, placenta, amniotic fluid, umbilical cord, or fetus, which all affect the timing and the outcome of PTB (Goldenberg et al 2000b). There are many ways to distinguish and confirm the presence of infection, including clinical signs and symptoms (fever, vaginal discharge, or abnormal odors) and laboratory tests (histopathological exam and microbiological criteria diagnosis or blood test) (Cappelletti et al 2016, Goldenberg et al 2000a, Mitchell & Taggart 2009a, Nadeau et al 2016, Romero et al 2014, Tita & Andrews 2010).

In mammalian species, including rodents, the infection cascade and normal parturition pathway have different initiation factors and mediators. However, they share important features that lead to the same final stages of labor, including the increased production of pro-inflammatory cytokines coupled with the production of prostaglandin, chemokines and metalloproteases that stimulate uterine contractions, cervical dilation, and cervical ripening (Figure 1.5) (Cappelletti et al 2016, Keelan et al 2003, Timmons et al 2014, van Engelen et al 2009).

During a healthy term delivery, the presence of an inflammatory cascade is a normal part of the labor cascade. However, during preterm delivery, microorganisms can cause maternal infection and/or inflammation that prematurely triggers the parturition cascade (Cappelletti et al 2016, Goldenberg et al 2000b, Mitchell & Taggart 2009a, Romero et al 2014).

Both infection and infection-driven inflammation are well known risk factors of PTB (Cappelletti et al 2016, Simmons et al 2010). Additionally, systemic infections such as influenza, pneumonia, or sepsis and intrauterine infections including bacterial vaginosis, placental inflammation or viral infections play a significant role in preterm delivery (Newton et al 1997).

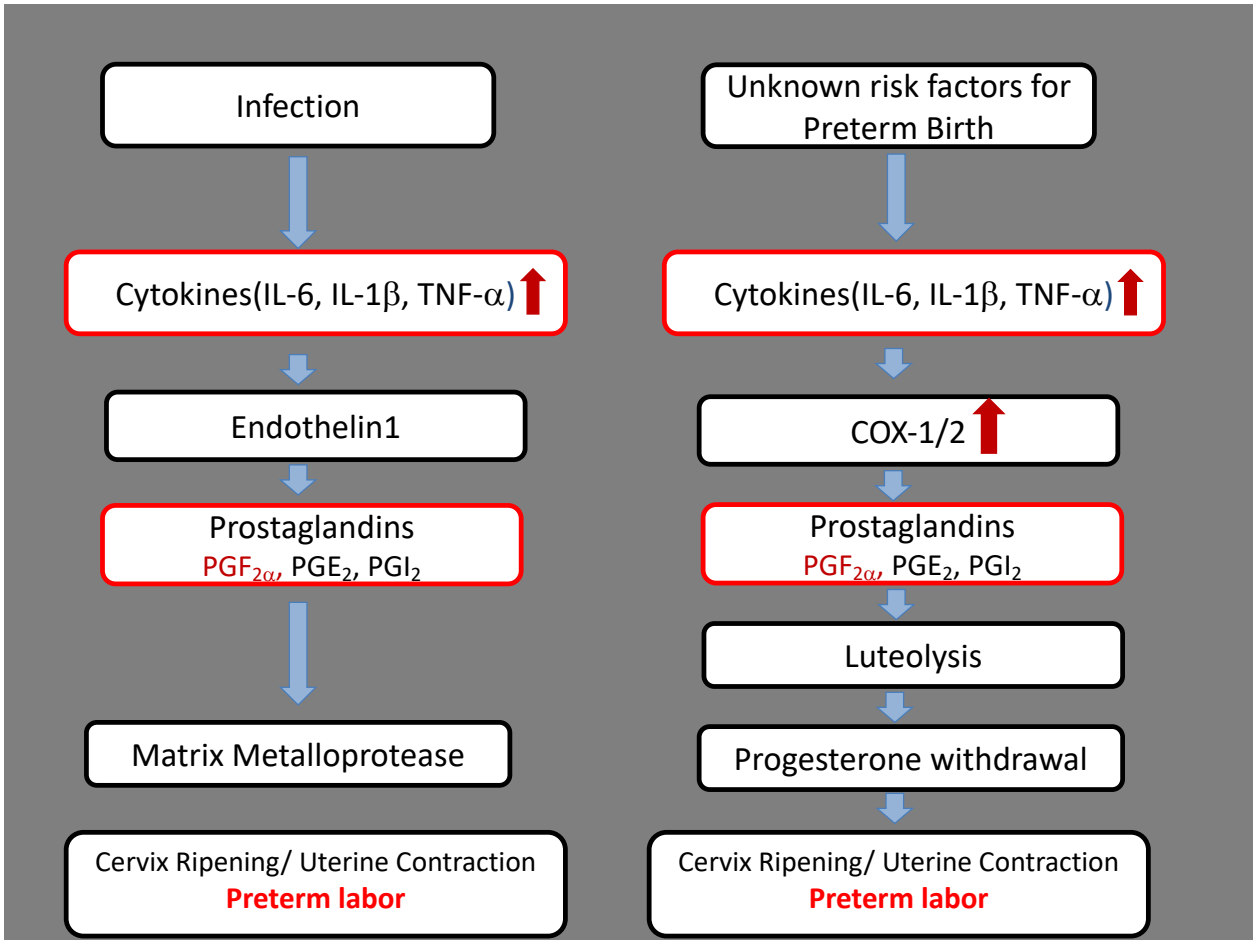

Figure 1.5 Similarities between PTB and infection pathway

1.7 Preterm birth

1.7.1 Epidemiology and definitions

PTB is defined as the delivery of a viable fetus prior to 37 weeks of gestation; it is a major health complication and the single leading cause of perinatal mortality and morbidity worldwide as well as the second-highest direct cause of death in children under 5 years of age (Blencowe et al 2012, Romero et al 2014). PTB affects up to 12% of pregnancies in the USA, 18% in some African countries, and 5-9% in Europe and other developed countries. It accounts for approximately 15 million preterm neonates each year (Blencowe et al 2012, Goldenberg et al 2008, Voltolini et al 2013). In the USA, the burden of PTB exceeds $26 billion annually (Behrman 2007, Blencowe et al 2012).

The mortality and morbidity risk of PTB is different from case to case and depends on the gestational age at birth, which is the length of the pregnancy starting from the first day of the last menstrual period. The most severe morbidities are associated with preterm infants who are born very premature (<28 weeks), followed by preterm infants born at 28-31 weeks, with the lowest for preterm infants who are born late premature at 32-36 weeks (Menon 2012, Romero et al 1994, Smith 2007). Premature babies are more susceptible to chronic health problems and have a higher risk of developing neurodegenerative disorders, including cerebral palsy, brain hemorrhage, respiratory distress, impaired vision, hearing problems and learning disabilities as well as long-term complications such as social, emotional and behavioral problems (Moster et al 2008, Platt 2014).

1.7.2 Etiology

Accumulating evidence supports the idea that PTB is a pathological condition caused by multiple factors, including socioeconomic factors, obesity, smoking, stress, poor nutrition, multiple gestations and infections as well as genetic factors (Figure 1.6). Interestingly, women who were born preterm themselves or women who have had clinical obstetric complications such as cervical disease, decidual senescence, placental abruption, hemorrhage and others are also at increased risk (Behrman 2007, Simhan & Caritis 2007). Of these maternal health conditions, multiple gestations are the most predominant cause of PTB (10.4%), followed by extrauterine infection (7.7%), intrauterine infection (7.6%) and mid/late pregnancy bleeding (6.2%)(Frey & Klebanoff 2016). However, in the case of preterm infants born at 28-31 weeks, the major cause (35.4%) was found to be intra-amniotic infection (Kim et al 2015). Thus, spontaneous preterm labor or severe pregnancy-associated clinical complications during labor seem to result from the interaction of multiple factors including fetal and maternal health complications, genetics, and socioeconomic factors (Goldenberg et al 2008, Ravanos et al 2015, Romero et al 2007, Romero et al 2006). Nevertheless, despite the prevalence and severity of PTB, the role of interactions between genetic susceptibilities and environmental factors remains unclear (Goldenberg et al 2008).

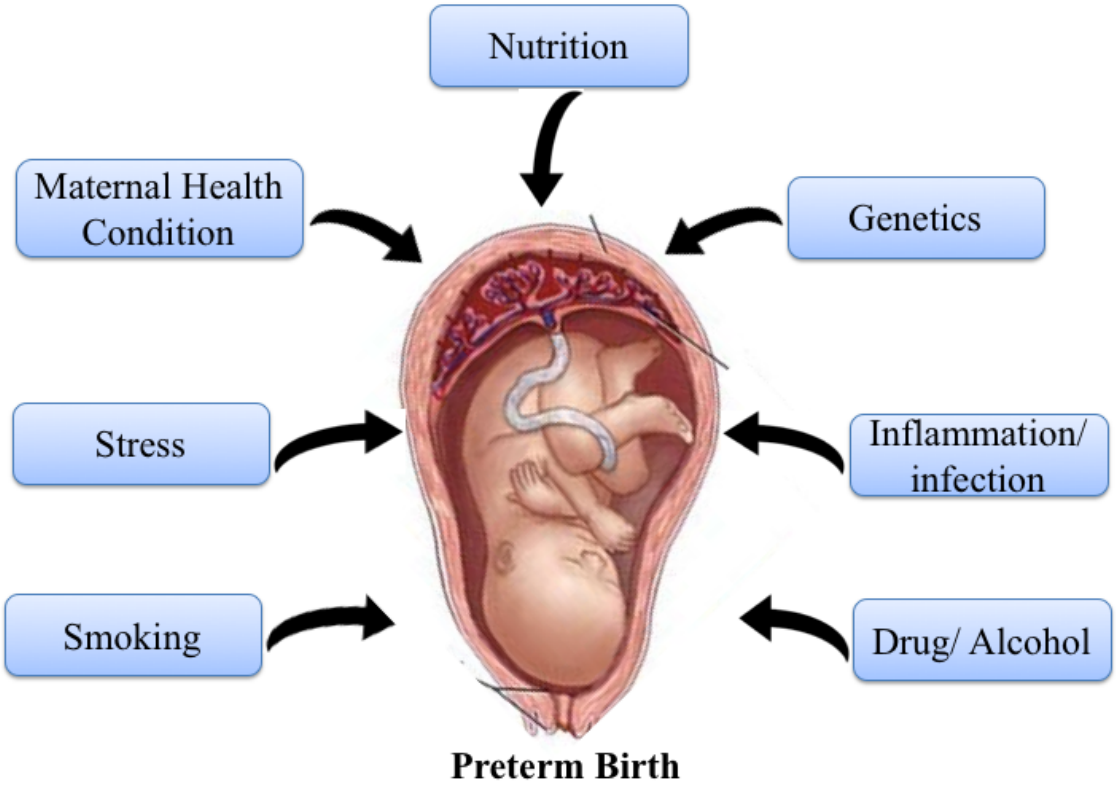

Figure 1.6 Proposed risk factors involved in PTB delivery

Genetic and environmental factors may interact to increase risk.

1.8 Immune system

The immune system protects and defends against infection by microorganisms. Classically, the immune system is divided into innate and adaptive immunity. Innate immunity provides a fast response against foreign microbes and pathogens, whereas adaptive immunity is an advanced response to specific pathogens (Abul K. Abbas 2012, informedhealth.org 2006,).

1.8.1 Innate immune system

The innate immune system is comprised of different cell types including macrophages, dendritic cells (DC), mast cells, and natural killer (NK) cells. The innate immune system plays a crucial role in recruiting immune cells to the site of infection, and this recruitment occurs through the secretion of cytokines and chemokines, small proteins that are important for cell-cell communication. (Warrington et al 2011).

1.8.2 Adaptive immune system

The adaptive immune system consists of T and B lymphocyte cells as well as antibodies, which are soluble proteins in the blood. All the components of adaptive immunity react in distinctive ways depending on where the pathogen is located in the body (Abul K. Abbas 2012, informedhealth.org 2006,).

T cells are one of the white blood cells that are generated in bone marrow. They mature in the thymus gland and have a specific structure compared to other lymphocytes, including a T cell receptor on the surface. T cells are responsible for identifying and destroying infected cells and removing them from the body (Charles A Janeway 2001,

Vantourout & Hayday 2013). B cells are lymphocytes that are key players in adaptive immunity that produce antibodies in the blood plasma circulation and lymph. B cells also secrete cytokines and present antigens. B cells have their receptor on their surface, bind directly to a particular antigen, and initiate an antibody response (informedhealth.org 2006,, Murphy 2012).

1.9 Bacterial infection

Bacterial infections within the uterus occur when an infection appears in one or more parts of the maternal tissue, between the maternal and fetal membranes, within the fetal membranes, or within the placenta, umbilical cord, or amniotic fluid (Figure 1.7). Bacterial infections reach the uterus in different ways, through the cervix from the vagina or through the fallopian tubes from the abdominal cavity.

Bacterial vaginosis (BV) is an imbalance of the vaginal flora (Brabant 2016). It is one of the most common infections associated with several reproductive complications, including PTB. BV is the most common vaginal disorder and is considered a risk factor for intrauterine infections (Newton et al 1997) and has been shown to be a risk factor for PTB (Cappelletti et al 2016, Nadeau et al 2016, Starr et al 2018b). The abundant presence of certain types of bacterial species in the vagina increases the production of pro-inflammatory cytokines, including IL-6 and IL-1β, which are involved in the parturition cascade (Imseis et al 1997, Mitchell & Marrazzo 2014, Stallmach et al 1995, Starr et al 2018a).

Notably, there is a high rate of variation among populations that have BV infections and have PTB (Goffinet et al 2003). This variation might be related to genetic predispositions. Interestingly, single nucleotide polymorphisms (SNPs) that amplify inflammatory responses

have been associated with PTB, including the gene Selenoprotein S (*SEPS1*), which has been reported by Wang et al. In a Chinese population, the SEPS1 polymorphism was implicated in mediating the inflammatory response and thus this SNP is associated with a risk of PTB delivery in this population. This SNP was shown to increase the expression level of different cytokines, which were shown to be associated with PTB in this population (Wang et al 2013). Another group also reported that a SNP in the tumor necrosis factor alpha (TNFA) gene was associated with the presence of genital tract infections that increase the risk of PTB (Roberts et al 1999).

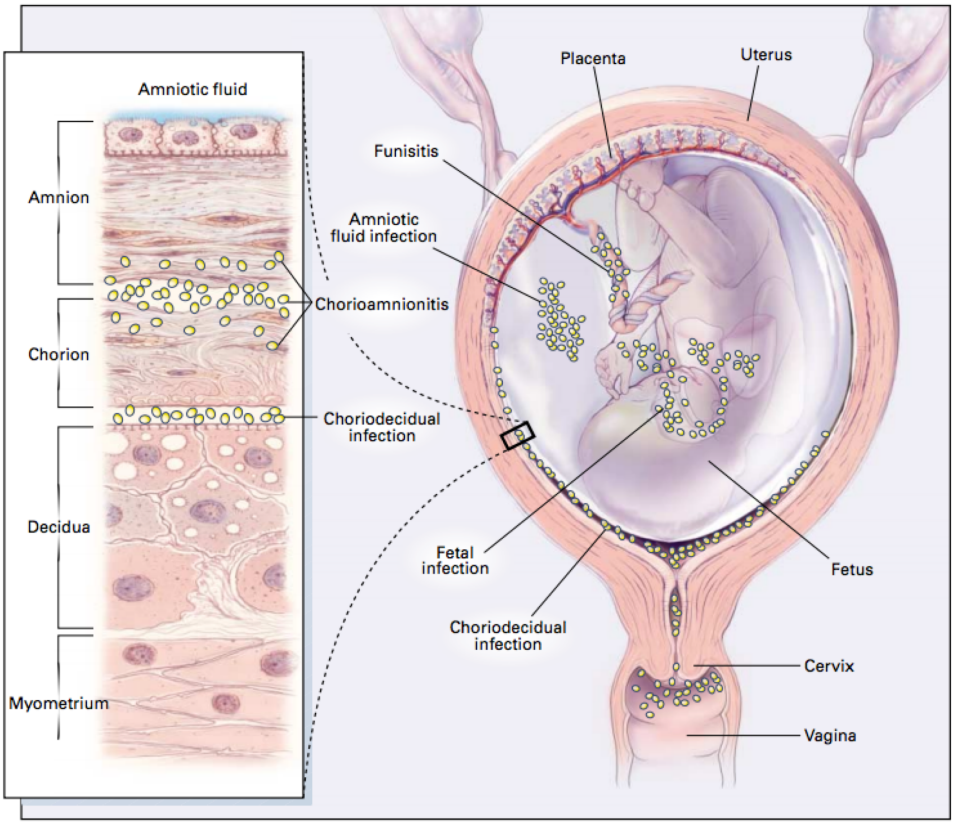

Figure 1.7 Bacterial infection within the uterus (Goldenberg et al 2000a).

1.10 Viral infection

Viral infections pose higher risks to pregnant women than nonpregnant women. The outcomes of maternal viral infection affect both the mother as well as the infant, leading to PTB and spontaneous abortion in the mother and adverse fetal outcomes such as blindness, hearing loss or visual impairment resulting from transmission from mother to infant (Silasi et al 2015). Influenza is a viral infection that has very high risk in pregnant women compared to the general population. In Minnesota, USA, influenza was a major cause of death in pregnant women that was associated with pregnancy. During this pandemic period, influenza was the leading cause of death, with approximately 20% of deaths associated with pregnancy (Silasi et al 2015). In mice, prenatal influenza infection leads to abnormal brain development (Fatemi et al 2002). In humans, influenza infection has been shown to increase the risk of abnormal behavior, including autism and schizophrenia in infants (Shi et al 2003).

1.11 Immune changes during pregnancy

During the course of pregnancy, from its establishment until the day of parturition, the immune status of the female reproductive tract undergoes dynamic changes. During peri-implantation and implantation, activated macrophages and other immune cells release pro-inflammatory cytokines, including IL-6, IL-1β, and TNF-α. During placental development and pregnancy maintenance, the immune status shifts to become anti-inflammatory by releasing anti-inflammatory mediators such as IL-10 and TGF-β. To initiate parturition, an important shift occurs in the immunological state of the female reproductive tract from an anti-inflammatory to a pro-inflammatory state through the release of the pro-inflammatory

cytokines mentioned above, which activates the labor cascade (Figure 1.8) (Bonney 2013, Zhang et al 2017).

Several studies in humans and mice have revealed the requirement for innate and adaptive immune cells during early pregnancy, late pregnancy, labor at term and in PTB (Bollapragada et al 2009, Osman et al 2003), which play an important role in the maintenance of fetomaternal tolerance during pregnancy (Cappelletti et al 2016, Negishi et al 2018).

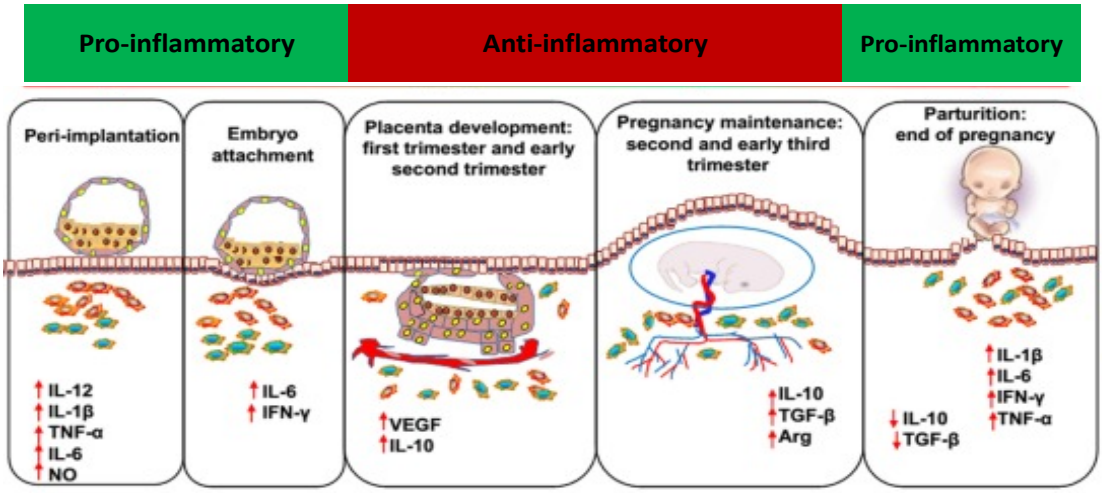

Figure 1.8 Dynamics between the pro-inflammatory and anti-inflammatory statuses during pregnancy

During the course of a pregnancy, the immune status of the female reproductive tract is dynamically regulated. During peri-implantation and implantation, the immune state is pro-inflammatory, and many pro-inflammatory cytokines are secreted, including IL-6, IL-1β, and TNF-α. During pregnancy maintenance, the immune status shifts to anti-inflammatory by producing anti-inflammatory cytokines such as TGF-β and IL-10. Through the secretion of many pro-inflammatory cytokines, the immune status shifts again to pro-inflammatory, which activates the parturition cascade. This figure is modified from (Zhang et al 2017).

1.12 Immune cells throughout pregnancy

During human pregnancy, leukocytes are a crucial component from the time of implantation to early pregnancy. During the first trimester, approximately 30-40% of endometrial stromal cells are leukocytes (Bulmer et al 2010). At this time, pregnancy macrophages, uterine natural killer (uNK) cells and T lymphocytes are the three primary leukocyte populations. DCs (Gardner & Moffett 2003), NK cells (Tsuda et al 2001) and regulatory T cells (Heikkinen et al 2004) are also functionally essential but less abundant leukocyte populations (Bulmer et al 2010). Growing evidence shows that both innate and adaptive immune cells play a critical role during implantation and early pregnancy, during which there are vital morphological and functional changes in the immune cells, such as increases in the number of uNK cells (Guimond et al 1998, Hsu & Nanan 2014, Lee et al 2011). During implantation in mice, studies have shown that immune cells are involved in promoting appropriate vascularization, which affects placental development and size, thus affecting embryo survival; the impairment of both NK cells and T cells results in placental growth restriction and major fetal loss (Christiansen 2013, Guimond et al 1998, Lee et al 2011).

Notably, labor is an inflammatory event (Bollapragada et al 2009). During late pregnancy, innate immune cells, including macrophages and DCs, are involved in the activation of the parturition cascade (Cappelletti et al 2016). Nevertheless, the specific molecular pathways and mechanisms underlying the stimulation of immune pathways linked to the induction of parturition are still elusive (Cappelletti et al 2016).

Decidual macrophages in mice have been shown to be significantly elevated at day 18.5 compared to day 15.5 of pregnancy (Shynlova et al 2013). In addition, a significant decrease in macrophages in human decidual tissue was found in women who had undergone

cesarean sections compared to those who had experienced labor at term (Hamilton et al 2012). Taken together, these findings show that immune cells are implicated in many processes during PTB and labor, and yet the precise roles of macrophages are still disputed (Cappelletti et al 2016, Gomez-Lopez et al 2014).

1.12.1 Macrophages

Macrophages play an essential role during early pregnancy, particularly during the peri-implantation and implantation phases. During a normal pregnancy, the female reproductive tract needs to be in a pro-inflammatory state to accomplish implantation. Macrophages secrete many proinflammatory cytokines, including IL-1β, IL-6, and TNF-α, which provide the appropriate microenvironment for implantation, and the depletion of macrophages impairs implantation (Care et al 2013, Van der Hoek et al 2000, Wu et al 2004). Care et al 2013 and others have demonstrated the indispensable role of macrophages in implantation by using *Cd11b-Dtr* conditional macrophage deletion mice. In these mice, the macrophages are deleted in the uterus and ovaries, resulting in failed embryo implantation due to a lack of ovarian progesterone production, which leads to an insufficient capillary network, which in turn plays a key role in the maturation of the corpus luteum (Care et al 2013).

Macrophages are also involved in activating the parturition cascade for term and preterm birth. In humans and mice, numerous studies have shown that macrophages play a crucial role during late pregnancy due to their secretion of TNF-α, IL-1β, IL-6, matrix metalloproteinases (MMP) and nitric oxide (NO)(Gomez-Lopez et al 2014, Huang et al 2012). Additionally, activated macrophages secrete prostaglandins, including PGE2, which plays a

primary role in activating the parturition cascade in both normal pregnancy as well as PTB by increasing uterine contractility (Vos et al 2000). Macrophages release both MMP-1 and MMP-9, high levels of which are also linked to PTB(Athayde et al 1999, Maymon et al 2000).

1.12.2 Natural killer cells (NK)

NK cells are identified by the presence of the surface marker CD56 and the absence of CD3 (Acar et al 2011, Robertson & Ritz 1990). There are two types of NK cells, uterine NK (uNK) cells and peripheral NK (pNK) cells which circulate in the blood and lymphatic circulation. uNK cells are involved in placental development and embryonic growth. In contrast, pNK cells primarily protect the body from viral infections or from developing cancer cells (Bulmer et al 2010). uNK cells have an important physiological role during pregnancy and have been identified as the primary cause of reduced implantation. They are also involved in regulating trophoblast invasion and spiral artery remodeling by secreting various cytokines, including TNFa, IL-8, and interferon-inducible protein-10 chemokines (Acar et al 2011).

In humans, NK cells are located in the endometrium of nonpregnant women, and their expression dramatically increases post-ovulation (Bulmer et al 2010, Hunt et al 2000). During the first trimester, lymphoid cells make up 40% of the immune cells and uNK cells represent 50-90% of these lymphoid cells (Acar et al 2011, Bulmer et al 2010, Gomaa et al 2017, Robertson & Ritz 1990, Williams et al 2009). However, uNK expression drops as pregnancy progresses. Studies have shown that uNK cells are linked to extravillous trophoblasts (EVTs) and spiral arteries. Therefore, uNK cells play a crucial role in implantation, placental development and trophoblast invasion (Acar et al 2011).

In mice, at day 5 of gestation during decidualization, uNK differentiation is triggered and uNK cells are abundant in the mesometrial decidua, peaking at approximately days 10-12 of gestation. The number of uNK cells in the decidua then declines until the time of parturition (Acar et al 2011, Bilinski et al 2008). Additionally, an increased number of NK cells is associated with different clinical conditions, including endometriosis, preeclampsia, and idiopathic PTB (Murphy et al 2009). uNK cells secrete numerous chemokines, cytokines and angiogenic factors, including IL-1, IL-10, IL-12, IL-15, IL-18 and CCL5, and cell-mediated cytokines, including IFN-γ and granulocyte-macrophage colony stimulating factor (GM-CSMF)(Gaynor & Colucci 2017).

1.13 Nodal

Nodal is a morphogen belonging to the transforming growth factor-beta (TGF-β) superfamily, Nodal is a member of the TGF-β superfamily together with the activin, inhibin, bone morphogenetic protein (BMP) and growth-determining factor (GDF) families (Piek et al 1999).

Nodal was originally discovered by Conlon *et al* in transgenic mice through a retroviral-induced recessive lethal embryonic mutation termed 413.d, which caused severe gastrulation defects at embryonic day 7.5 (E7.5) (Conlon et al 1991, Iannaccone et al 1992). Subsequent work showed that this insertional mutation, which mapped alongside 413.d, encoded a novel member of the TGF-β superfamily that was named *Nodal* due to its expression in the mature node at E7.5 (Zhou et al 1993). In fact, this mutation results in failed mesoderm induction, which leads to embryonic lethality at E10.5 (Iannaccone et al 1992, Zhou et al 1993). Together, these results highlight the essential role of Nodal during mesoderm induction. Nodal has been identified as a

key 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 but are not found in *Drosophila* or *Caenorhabditis elegans* (Schier & Shen 2000). Interestingly, the Nodal gene was identified as a single protein in some species, including humans, mice and chicks; however, many other species contain multiple Nodal proteins, such as the zebrafish (Cyclops, Squint, Southpaw, Xenopus [Xnr1, -2, -4, -5, -6] and Amphioxus [AmphiNodal]) (Feldman et al 1998, Takahashi et al 2000).

1.14 Nodal signaling pathway

The TGF-β pathway governs cellular functions such as migration, differentiation, apoptosis, proliferation and secretion (Gray & Vale 2012). Nodal is secreted as a 38 kD precursor protein that is posttranslationally modified to 42 kD by glycosylation before exocytosis (Blanchet et al 2008). To activate the Nodal signaling pathway and mediate embryogenesis processes, including mesoderm induction, the secreted Nodal precursor protein must be cleaved to release its mature 12 kD form (Blanchet et al 2008, Brennan et al 2002, Le Good et al 2005). Near the target cell surface, the precursor protein dimerizes and its pro-domain is proteolytically cleaved by the convertases SPC-1 and SPC-4 (known as Furin and Pace4, respectively)(Beck et al 2002, Le Good et al 2005, Ross & Hill 2008) (Figure 1.9).

To activate the Nodal signaling pathway, mature Nodal signals through a heterodimeric receptor complex that binds to type I (ALK4/ALK7) and type II activin (ActRIIA/ActRIIB) serine/threonine kinase receptors (Reissmann et al 2001, Schier 2009). Like other members of the TGF-β super family, Nodal needs serine-threonine kinase transmembrane receptors; however, Nodal activity also requires the glycosylphosphatidylinositol

(GPI)-linked coreceptor EGF-CFC (Cripto/Cryptic) to establish signaling transduction (Schier 2009, Strizzi et al 2008). The coreceptor EGF-CFC (Cripto/Cryptic) plays an obligatory coreceptor role at the cell surface for Nodal by binding the convertases Furin and PACE4 to the receptor complex and allowing Nodal to bind to the Type I receptor ALK4/7 (Blanchet et al 2008, Shen 2007). The type I receptor recruits and phosphorylates SMAD2 and SMAD3. The SMAD2/3 complex recruits and phosphorylates a common SMAD4, which then translocates to the nucleus to regulate the target genes through different transcription factors, including P53, MIXER and FOXH1 (Figure 1.9) (Cruz et al 2015, Kumar et al 2001, Shen 2007).

Nodal is a morphogen that has the ability to diffuse over a long range within tissues and to act directly on distant cells in a concentration-dependent manner (Chen & Schier 2001). One of the remarkable events of Nodal signaling is the induction of Nodal expression as well as its inhibitor, Lefty. Lefty is an extracellular inhibitor that blocks Nodal activity by binding to Nodal and to the EGF-CFC coreceptor. Thus, Lefty limits Nodal activity due to its potent inhibition and longer range of diffusion, and the loss of Lefty causes ectopic mesoderm formation as well as abnormal left-right patterning (Chen & Shen 2004, Chen & Schier 2001, Chen & Schier 2002). Cerberus is another extracellular molecule that binds directly to Nodal and inhibits its binding to the receptor to regulate embryonic patterning (Chen & Schier 2001, Tavares et al 2007, Yamamoto et al 2004).

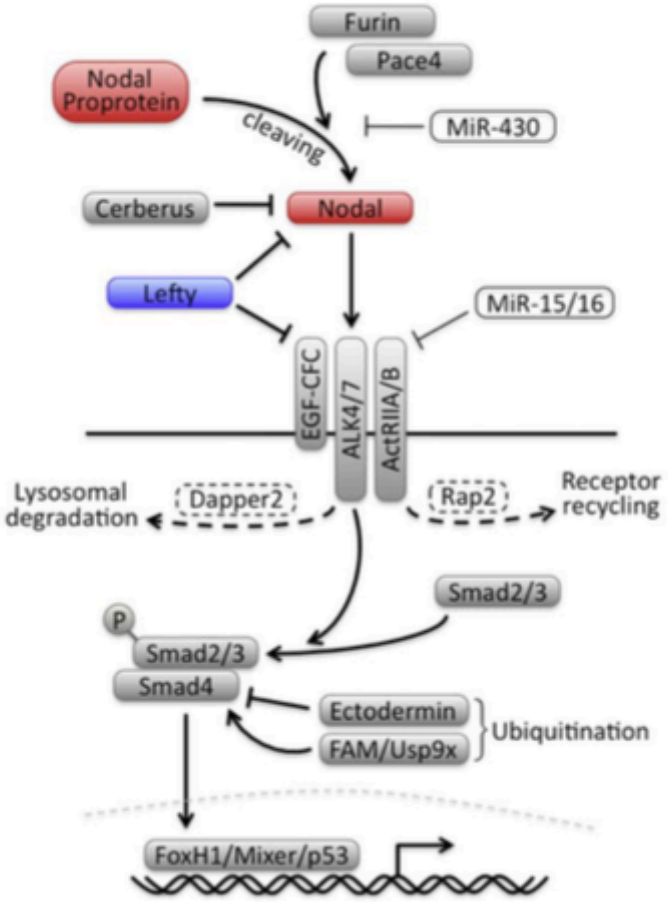

Figure 1.9 Nodal canonical signaling pathway

Nodal pro-protein is cleaved by the convertases Furin and Pace4. The Nodal ligand, along with the receptors ALK4/7 and ActIIA/B and the coreceptor EGF-CFC, create 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. Notably, the target genes of Nodal signaling include *Nodal* itself and its inhibitor *Lefty.* The Nodal signaling pathway is regulated at different levels. The extracellular inhibitors Lefty and Cerberus negatively regulate Nodal signaling by binding directly to Nodal and/or the coreceptor EGF-CFC. However, at the receptor level, Nodal is positively and negatively regulated by Rap2 and Dapper2. Smad4 is also regulated by FAM/Uspx and Ectodermin. Moreover, several microRNAs, including miR-15/16 and miR430, inhibit Nodal signaling by reducing translation of Nodal and Lefty mRNA and ActIIA/B mRNA, respectively. The figure is modified from (Park & Dufort 2011, Ross & Hill 2008).

1.15 Roles of Nodal signaling components during pregnancy

1.15.1 Nodal signaling components during peri-implantation

Nodal and its components have been detected in the uterus at different stages during different mammalian reproduction events, including implantation, placentation and the timing of parturition (Ma et al 2001, Papageorgiou et al 2009). In humans, Nodal expression begins during the early proliferative stage and increases during the late proliferative stage to reach its peak during the early secretory stage, and then it significantly declines at the mid-secretory to menstrual stages. The Nodal inhibitor Lefty was shown to have the opposite expression pattern; it is not expressed during the proliferative or early to mid-secretory stages but increases during the late secretory stage (Papageorgiou et al 2009). In addition, Nodal signaling components are present throughout the menstrual cycle during the secretory stage(high mRNA levels of Cripto, ALK4 and ActRIIB). (Papageorgiou et al 2009)

In terms of histology, Nodal and its coreceptor Cripto are detectable through the uterine stroma, luminal epithelium and glandular epithelial cells at all phases of the menstrual cycle. In addition, Nodal is detectable in the uterine fluid, showing that Nodal is secreted from the glandular epithelium into the lumen. Lefty is also detectable in the glandular epithelium stromal cells (Papageorgiou et al 2009). Additionally, some Nodal signaling could be implicated in the causes of infertility in humans. Lefty levels decline in the uterine fluid of fertile women during receptivity, but in subfertile women, high levels of Lefty are detectable in uterine fluid (Tabibzadeh et al 2000). In addition, Nodal protein was found in the endometrial fluid of fertile women. Collectively, these findings imply that Nodal and its components maybe involved in regulating tissue proliferation and remodeling during the

menstrual cycle, and that the dysregulation of some Nodal signaling components might be implicated in infertility in humans. However, additional experimentation is required to investigate the potential role of Nodal components during preimplantation (Papageorgiou et al 2009, Tabibzadeh et al 2000).

To study *Nodal* expression in the mouse uterus, a Nodal LacZ reporter mouse strain was used, showing that Nodal is not expressed during the estrus cycle of a nonpregnant mouse uterus (Figure 1.10 a-c)(Park & Dufort 2011). However, at the peri-implantation period from days 0.5 to 3.5, *Nodal* expression increases in the uterus, specifically in the glandular epithelium during the prereceptive stage (Figure 1.10 d-f)(Park & Dufort 2011). In contrast, *Lefty* expression is present in the endometrium at all stages of the estrus cycle in nonpregnant mice. During the estrus stage, *Lefty* expression declines compared to the diestrus stage (Park & Dufort 2013, Tang et al 2005). Additionally, other Nodal signaling components, including *Smad2* and *Smad4*, are expressed during the estrous cycle and early pregnancy, and they increase during implantation (Liu et al 2004). These findings suggest that Nodal and its signaling components play a role in uterine cycling.

1.15.2 Nodal in implantation

Recent studies have shown that Nodal and its signaling pathway components are implicated in embryo implantation. According to results in Nodal-LacZ reporter mice, *Nodal* expression was detectable in a banding pattern along the proximal-distal axis of the uterine horn. On day 4.5 of pregnancy, *Nodal* expression was restricted to the interimplantation sites (Figure 1.10 g-i)(Park & Dufort 2011). Remarkably, this expression pattern was found to be controlled by the embryo, given that the expression was not detectable on day 4.5 in

pseudopregnant Nodal LacZ mice that were mated with vasectomized males (Park & Dufort 2011). To confirm the embryo-dependent Nodal expression at implantation on day 4.5 of pregnancy, an embryo transfer of healthy blastocysts into pseudopregnant Nodal-LacZ females was performed. These females were able to recover the banding pattern of *Nodal* expression, showing that the biological factors of the embryo are indispensable in facilitating *Nodal* expression (Park & Dufort 2011).

Furthermore, Park & Dufort 2011 generated a conditional uterine Nodal knockout mouse model using the Cre-LoxP system, because the global *Nodal* knockout is embryonically lethal to mice. Interestingly, the uterine Nodal knockout mice experience severe subfertility, and after mating with CD1 stud males, only 25% of Nodal knockout mice become pregnant (Park et al 2012). These mice have normal uterine morphology, normal cycling and normal ovulation but reduced implantation efficiency (Park et al 2012). Furthermore, transferring healthy wild type blastocysts to pseudopregnant Nodal conditional knockout mice on day 2.5 resulted in low pregnancy rates of approximately 33% (Park et al 2012). Taken together, these findings show that Nodal is a crucial component for effective implantation.

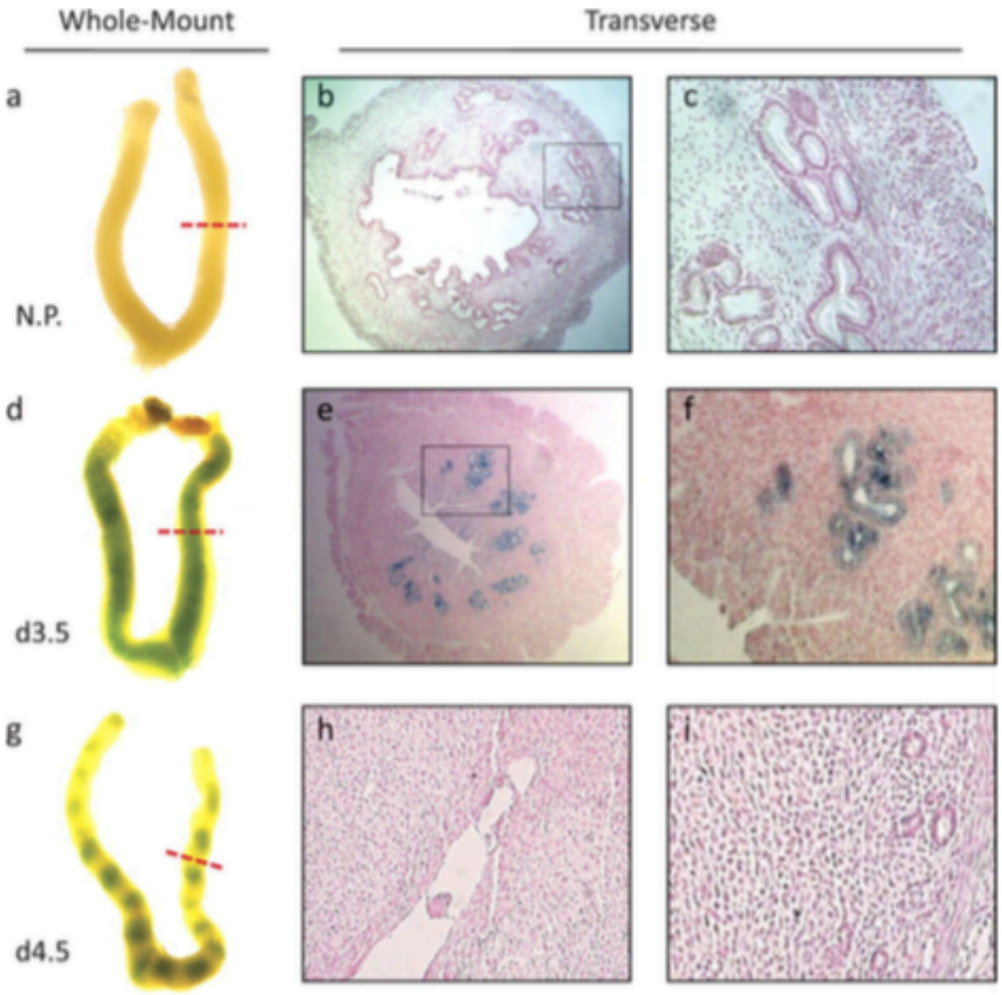

Figure 1.10 Nodal-LacZ expression during peri-implantation in a mouse uterus
(a) Whole mounted and (b-c) cross-sectioned nonpregnant mouse uterus showing no beta-galactosidase staining, representing no *Nodal* expression. (d) At day 3.5, *Nodal* expression appears throughout the entire uterine horn. (e-f) Transverse section staining showing that Nodal expression is restricted to the glandular epithelium. (g) At 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 interimplantation site (Park & Dufort 2011).

1.15.3 Nodal in placentation

Nodal is not only an embryonic-specific factor that facilitates development, but it has also been found to be involved in mouse placentation (Ma et al 2001). Nodal is expressed during mid gestation, early on day 10.5 of pregnancy, in the spongiotrophoblast layer. In addition to playing a crucial role during embryo development, Nodal has been shown to inhibit trophoblast differentiation into giant cells *in vitro*. Null Nodal mutant mice and hypomorphic Nodal mutants have an expanded trophoblast giant cell layer and reduced spongiotrophoblast and labyrinth layers (Lowe et al 2001, Ma et al 2001). Park & Dufort 2011 showed that Nodal on the maternal side is restricted to the maternal decidual parietalis layer during late pregnancy, and they demonstrated a role for Nodal in placentation using a Nodal conditional knockout mouse model. These mice experience severely abnormal trophoblast giant cell expansion and a significant loss of maternal decidua basalis, which results in reduced proliferation and increased apoptosis (Park & Dufort 2011). These findings indicate that Nodal is essential for proper placentation.

1.15.4 Nodal and preterm birth

In mice, the role of Nodal in the female reproduction tract of adult mice has recently been identified (Park & Dufort 2011). *Nodal* conditional uterine-specific knockout mice have several phenotypes as the result of the complete deletion of the Nodal gene. In terms of fertility, these mice were subfertile at percentages of up to 75% (Park et al 2012). In late pregnancy, the offspring of *Nodal* knockout mice have IUGR (Park et al 2012). In addition, the most significant phenotype was that 80% of the pregnant mice had PTB, and they delivered on day 17.5 instead of day 19.9 of their pregnancy (Park et al 2012). This

phenotype is rarely observed in rodents (Elovitz & Mrinalini 2004). In the current study, we are using *Nodal* heterozygous mice as a model to study PTB in humans. These mice showed a 50% reduction in their fertility; however, they do not experience preterm births (Park et al 2012).

The parturition cascade is regulated by the production of prostaglandins derived from the COX2/PGFS/$PGF_2\alpha$ signaling axes (Challis et al 1997). The precise molecular basis for the initiation of the natural parturition cascade is still poorly understood. However, many components of this cascade have been identified. In *Nodal* knockout mice, the protein level of COX2 enzyme, which plays an important role in the activation of the parturition cascade, was significantly increased within the maternal tissues of *Nodal* knockout mice at day 16.5 of pregnancy. In addition, the progesterone concentration in the maternal blood in these mice was significantly less at day 16.6 compared to the control mice (Park et al 2012). Eventually, having no Nodal in the uterus results in the disruption in the parturition cascade, leading to an early decline in progesterone, which results in preterm birth.

1.16 Nodal in human pregnancy

Several studies in mice and humans have demonstrated the involvement of Nodal in PTB. There is very little known about the role of Nodal in humans during development (Brennan et al 2002, Iannaccone et al 1992). However, multiple SNPs in the human NODAL gene have been identified, including SNPs that were found to decrease the NODAL activity (Roessler et al 2009). Further analysis by Roessler et al reported that SNPs in NODAL resulted in a significant reduction in gene activity among patients with heart defects and holoprosencephaly (Roessler et al 2009). These findings raised the possibility that a mutation in NODAL might also predispose patients to pregnancy-associated disorders. Moreover, a previous collaboration between our lab and the Dijk

lab (Thulluru et al 2013) to investigate a familial form of early-onset, IUGR-complicated pre-eclampsia examined the NODAL sequences in mothers who had IUGR-complicated pre-eclampsia; they showed that all the mothers had a *NODAL* H165R variant associated with a 50% reduction in *NODAL* activity (Thulluru et al 2013). This was the first evidence that a mutation in NODAL predisposed mothers to pregnancy-associated disorders.

1.17 Rationale

Pregnancy is a complex process that is comprised of several stages, including implantation, decidualization, placentation and finally parturation. Any abnormality in these stages can disrupt pregnancy and lead to complications such as infertility, miscarriage, IUGR, pre-eclampsia and preterm birth.

Preterm birth is a complex phenotype resulting from the interactions between genetics and environmental factors. It is a leading cause of neonatal morbidity and mortality worldwide. Recently, our lab has demonstrated that the Nodal gene is implicated in pregnancy events including implantation, placentation and timing of parturation. In mice, complete deletion of Nodal from the female reproductive tract results in impaired implantation, severe placental defects, intrauterine growth restrictions and, most importantly, preterm birth. Although Nodal has been shown to be involved in these various processes, the exact mechanism by which Nodal modulates these events is unknown. The aim of this thesis was therefore to decipher the mechanism by which Nodal modulates the timing of parturition. Furthermore, the second objective was to determine if Nodal also plays a similar role in human pregnancies. Based on that, we **hypothesized** that Nodal plays a crucial role in the timing of parturition and that mutations within the NODAL gene leads to increased susceptibility to preterm birth in mice and humans.

Chapter 2: Nodal Deletion Increases Susceptibility to Inflammation-Induced Preterm Birth

Nodal Deletion Increases Susceptibility to Inflammation-Induced Preterm Birth

Taghreed A. Ayash[1], Lisa M. Starr[2], Daniel Dufort[1,2]

[1]Division of Experimental Medicine, McGill University, Montreal, Quebec, Cananda.

[2]Department of Obstetrics and Gyneocology, McGill University, Montreal , Quebec, Canada.

Corresponding author: Daniel Dufort, Research Institute of the McGill University Health Centre, Glen Site, EM0.3211, Montreal, Quebec, Canada. Tel: 1-514-934-1934 x34743. E-mail: dainel.dufort@mcgill.ca.

Taghreed.heba@mail.mcgill.ca Lisa.starr@mail.mcgill.ca

Conflict of intrest: The authors have declared that no conflict of interest exists.

2.1 Preface

Preterm birth remains the major cause of perinatal mortality and morbidity worldwide, accounting for approximately 75% of all neonatal deaths. However, the mechanisms and causes that underlie it remain largely unknown. Previous studies in our lab have focused on the role of the Nodal signaling pathway during pregnancy. Nodal is a morphogen that belongs to the (TGF-ß) superfamily. We have demonstrated that Nodal is required for implantation and proper timing of parturition. We have used a conditional uterine-specific deletion to generate a Nodal heterozygous mouse model to evaluate the role of Nodal during late pregnancy. We revealed that Nodal knockout mice experience reduced fertility and, interestingly, 80% of the pregnant mice had preterm births, delivering on day 17.5 instead of 19.5.

In this chapter, we demonstrate that Nodal heterozygous mice also had reduced fertility and increased sensitivity to LPS. Interestingly, injection of a low dose of LPS caused 50% of pregnant Nodal heterozygous mice to give birth preterm whereas this dose did not affect controls. Subsequently, we determined the immunological state of the mice which showed an increase in basal levels of pro-inflammatory cytokines which also corresponded to an increase in the number of uterine macrophages in maternal decidual tissue. Finally, we revealed that pre-treatment of Bone Marrow-Derived Macrophages (BMDMs) with recombinant Nodal reduces pro-inflammatory gene expression when these cells are challenged with LPS using.

Our findings illustrate the inhibitory role of Nodal in maternal decidual tissue which can prevent pro-inflammatory cytokine expression. Moreover, they also highlight how absence of Nodal leads to an increase in pro-inflammatory cytokines which subsequently increases the risk of preterm birth.

2.2 Abstract

Preterm birth remains the major cause of perinatal mortality and morbidity worldwide, affecting up to 12% of pregnancies and accounting for approximately 75% of neonatal deaths. However, the mechanisms and causes that underlie it are still largely unknown. Our lab has been studying the role of the Nodal signaling pathway during pregnancy. Nodal is a morphogen that belongs to the (TGF-ß) superfamily. Nodal has been shown to play critical roles during embryonic development and our lab has shown that it is also required for implantation and proper timing of parturition. We have used a conditional uterine-specific deletion to generate a *Nodal* heterozygous mouse model to evaluate the role of Nodal during late pregnancy. We demonstrated that *Nodal* heterozygous mice have reduced fertility and an increased sensitivity to LPS. Injection of a low dose of LPS caused 50% of pregnant *Nodal* heterozygous mice to give birth preterm whereas this dose had no effect on controls. This increased sensitivity to LPS appears to be due to the increase in basal levels of proinflammatory cytokines IL-1β, IL-6, IL-12p, TNF-α, and IFN-γ which also corresponded to an increase in the number of uterine NK cells and macrophages in the maternal tissue in *Nodal* heterozygous mice. Finally, using Bone Marrow Derived Macrophages, we demonstrated that pretreatment with recombinant Nodal reduces proinflammatory gene expression when these cells are challenged with LPS. Our results demonstrate that Nodal expressed in maternal tissues prevents proinflammatory cytokine expression and its absence leads to a precocious increase of these factors leading to an increased risk for preterm birth.

2.3 Introduction

Preterm birth, defined as delivery prior to 37 weeks of gestation, is a serious health complication and the single leading cause of perinatal mortality and morbidity worldwide, affecting up to 12% of pregnancies and accounting for approximately 75% of neonatal deaths (Blencowe et al 2012, Elovitz & Mrinalini 2004, Goldenberg et al 2008). Preterm birth is a pathological condition caused by multiple environmental factors, including smoking, stress, and infection, that interact with genetic predispositions (Blencowe et al 2012, Romero et al 2014). However, despite the predominance and severity of preterm birth, the role of interactions between genetic susceptibilities and environmental factors remains unclear (2007, Blackburn 2007).

Maternal infection and inflammation are major causes of spontaneous preterm birth and it has been shown that infection is responsible for as many as 40% of all preterm births (Mitchell & Taggart 2009b, Romero et al 2014). In mammalian species, including rodents, the inflammatory cascade is similar to the normal parturition pathway and leads to the same final stages of labor, including uterine contractions, cervical dilation, and cervical ripening (Keelan et al 2003, Timmons et al 2014, van Engelen et al 2009). Normal parturition is initiated by the production of prostaglandin (PG) F2α derived from cyclooxygenase (COX). COX1 and COX2 convert arachidonic acid to PGH2, which is then converted to PGF2α and PGE2 by the PGF synthase enzyme (Challis et al 1997, Helliwell et al 2004). The presence of PGF2α results in degradation of the corpora lutea (luteolysis) and a decline in progesterone levels in the maternal blood circulation, which leads to the initiation of labor by stimulating cervical dilation and ripening (Ratajczak & Muglia 2008).

Nodal, a member of the transforming growth factor beta (TGF-β) superfamily, plays an integral role in many processes of vertebrate development. During embryogenesis, NODAL

activates a signaling pathway that is essential to primary germ layer induction and axis specification (Jones et al., 1995, Brennan et al., 2002). Insertional mutation of the *Nodal* gene in mice results in failed mesoderm formation, hyperplasia of the embryonic and extraembryonic ectoderm, and lethality on Day 10.5 (Iannaccone et al., 1992). Furthermore, Nodal acts to direct left-right development, and misexpression studies affect tissue and organ laterality (Lowe et al., 2001, Horne-Badovinac et al., 2003). In addition to these established roles, Nodal has also been implicated in neural patterning and anterior-posterior axis specification (Schier and Shen, 2000, Tian and Meng, 2006, Shen, 2007). Aside from axis specification, *Nodal* has been shown to be expressed in the mouse uterus during early pregnancy (Park & Dufort 2011). Expression begins shortly after mating and is initially expressed throughout the uterus. Prior to implantation, Nodal expression is repressed at the future site of embryo implantation but is maintained in inter-implantation sites. A tissue specific deletion of Nodal using the Progesterone receptor Cre line results in 75% reduction in fertility and 80% of the mice who do become pregnant give birth early on day 17.5 instead of day 19.5 of pregnancy (Park et al., 2012). The reasons for this infertility and preterm birth phenotype are currently unknown.

Components of the NODAL signaling pathway have been shown to be involved in regulation of the immune system (Wang et al 2014, Zhang et al 2016). NODAL polarizes tumor-associated macrophages to an alternatively activated phenotype and decreases their production of pro-inflammatory cytokines (Wang et al 2014). CRIPTO, the co-receptor of NODAL, upregulates the production of pro- and anti- inflammatory cytokines in macrophages (Zhang et al 2016). Furthermore, we have previously demonstrated that although there is no direct association between *NODAL* SNPs and preterm birth, when bacterial vaginosis (BV) or placental inflammation were included in our models we found that three SNPs had a differential effect on preterm birth risk

depending on the presence of infection or inflammation (Starr et al., 2018). These results suggest that NODAL may play a role in the regulation of immune cells during pregnancy, particularly in response to infection.

To address this possibility, we have made use of the heterozygous tissue specific Nodal female (Nodal $^{\Delta/+}$) and shown that these females have decreased fertility and an increased sensitivity to lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria with pro-inflammatory properties. Furthermore, the deciduas of day 17.5 Nodal $^{\Delta/+}$ have altered expression of immunity-related genes as well as an increase in proinflamatory cytokine levels. Finally, we demonstrate that macrophages have decreased levels of cytokine expression in response to LPS when exposed to rNodal. Our results demonstrate that Nodal is required to maintain the environment in an anti-inflammatory state and its absence results in an increase in proinflammatory cytokine expression leading to an increased suseptibility to giving birth prematurely.

2.4 Results

2.4.1 *Nodal* $^{\Delta/+}$ mice have decreased fertility

In order to evaluate the fertility of heterozygous mice in which one allele of the *Nodal* gene was conditionally deleted in the reproductive tract of adult females (*Nodal* $^{\Delta/+}$), 8-24 week *Nodal* $^{\Delta/+}$ and control (Nodal $^{loxP/loxP}$) females were mated with CD1 males overnight and the presence of a vaginal plug was confirmed the following morning (day 0.5). Pregnant mice were monitored from day 13.5 of pregnancy until delivery. As previously demonstrated, only 50% of the Nodal $^{\Delta/+}$ females became pregnant in comparison to 90% in *Nodal* control females (Figure 2.1A) (Park et al 2012). Thus, loss of one allele of Nodal results in a significant decrease in fertility. Additionally, we observed that *Nodal* $^{\Delta/+}$ mice had a significantly smaller number of pups per litter (8.6, n=172) compared to control mice (10.3, n=206) (Figure 2.1B).

2.4.2 *Nodal* $^{\Delta/+}$ mice have increased sensitivity to LPS induced inflammation

Inflammation is known to be a major cause of adverse pregnancy outcomes, including preterm birth (Klebanoff & Searle 2006, Nadeau-Vallée et al 2016). Furthermore, we have previously demonstrated a correlation between certain single nucleotide polymorphisms (SNPs) in the *Nodal* gene and an association with increased risk for preterm birth among women with bacterial vaginosis or placental inflammation (Starr et al., 2018). Since we had demonstrated a link between Nodal and inflammation in preterm birth in humans, we investigated whether our *Nodal* $^{\Delta/+}$ females had increased sensitivity to placental inflammation which would lead to preterm birth.

In order to investigate whether inflammation affects pregnancy outcomes in Nodal $^{\Delta/+}$ females, *Nodal* control and *Nodal* $^{\Delta/+}$ females were injected with different doses of bacterial lipopolysaccharide (LPS) on day 15.5 of pregnancy. LPS induces chorioamnionitis by eliciting a strong immune response in mice (Edey et al., 2016).

Injection of 0.57 mg/kg of LPS had no adverse effects on either *Nodal* control mice or *Nodal* $^{\Delta/+}$ females (Figure 2.1c). In contrast, injection of 1.4 mg/kg of LPS led to premature delivery 12–14 hours after injection in 50% of *Nodal* $^{\Delta/+}$ females. Conversely, this dose of LPS had no effect on *Nodal* control mice as these delivered on time at day 19.5 of pregnancy (Figure 2.1d). These results suggest that *Nodal* heterozygous mice have an increased sensitivity to LPS-induced preterm birth.

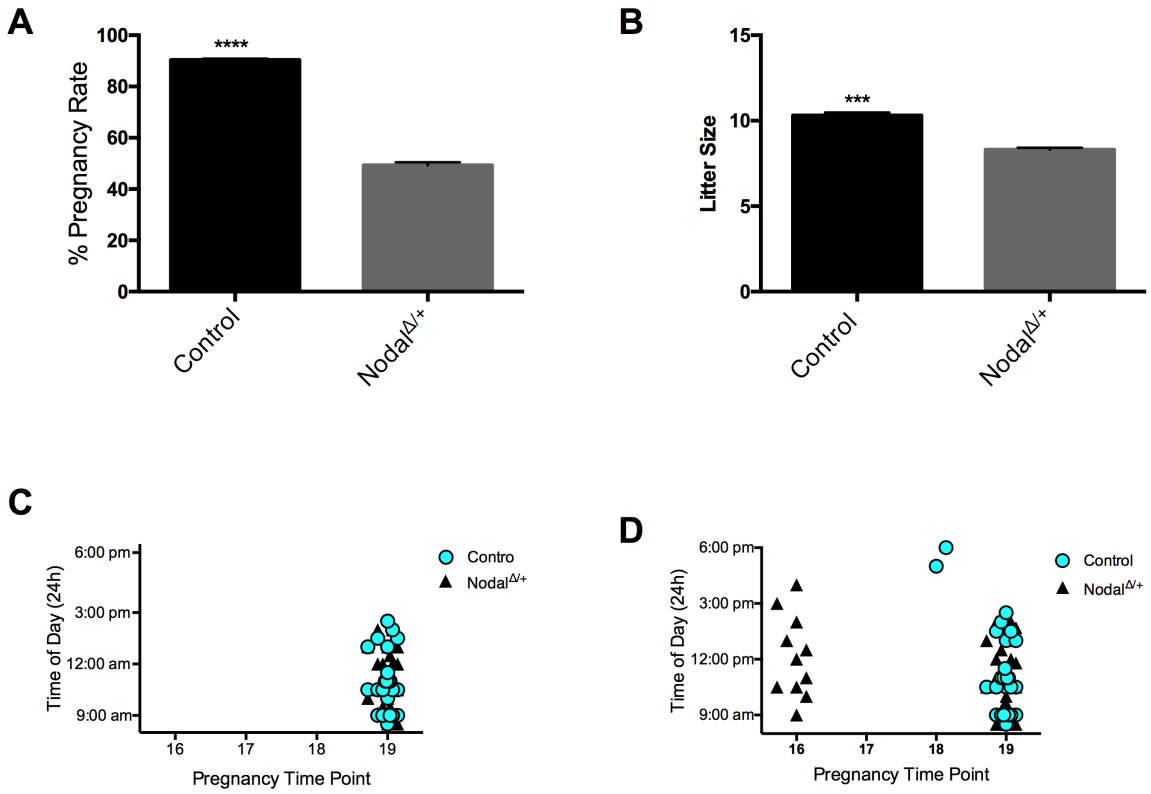

Figure 2.1 Nodal$^{\Delta/+}$ mice have decreased fertility and increased sensitivity to LPS
(A) *Nodal*$^{\Delta/+}$ mice mated with CD1 males had a reduced fertility rate (50%, *n*=40) compared to control mice (90%, *n*=40). **(B)** *Nodal*$^{\Delta/+}$ mice mated with CD1 males had a significantly smaller number of pups per litter (8.6, *n*=172) compared to control mice (10.3, *n*=206). **(C)** control (*n*=20) and *Nodal*$^{\Delta/+}$ (*n*=20) mice i.p. injected with 0.57 mg/kg of LPS on day 15.5 of pregnancy gave birth on time at day 19.5 of pregnancy. **(D)** Injection with 1.42 mg/kg of LPS caused 50% of *Nodal*$^{\Delta/+}$ mice (*n*=20) to give birth prematurely 12–14 h after injection, whereas all injected control mice (*n*=20) gave birth at term on day 19.5, or on day 18.75 which is also a term birth. Mice were used in 8–24-week-old. Data show mean ± SEM and were compared using an unpaired Student's t-test. ****P < 0.00001, ***P < 0.001.

2.4.3 Inflammatory-related genes are elevated in maternal decidual tissue of *Nodal* $^{\Delta/+}$ females

It has been shown that elevated concentrations of cytokines and chemokines in late pregnancy lead to activation of the parturition cascade through factors such as prostaglandins (PG) (2007, Blackburn 2007). Given that parturition is an inflammatory process, we evaluated the inflammatory factors and pathways affected by a *Nodal* deletion in the uterus during late pregnancy by examining the expression of innate and adaptive immunity-related genes in the maternal decidual on day 16.5 of *Nodal* $^{\Delta/+}$ and *Nodal* control mice. Using an RT^2 Profiler PCR Array of 84 genes, we found that 27 genes were significantly up-regulated, and 5 genes were significantly down-regulated in maternal decidual tissue of *Nodal* $^{\Delta/+}$ compared to *Nodal* controls (Table S2.1). Basal expression of several cytokine genes, including *Il1a, Il1b, Il6, Il18, Il10, Il4, Il23a,* and *Tnfa* in *Nodal* $^{\Delta/+}$ were significantly elevated compared to *Nodal* controls (Figure 2.2). *Nodal* $^{\Delta/+}$ females also lack one allele of the progesterone receptor caused by the insertion of the Cre gene ($Pgr^{Cre/+}$). We therefore tested whether this increase in basal cytokine expression was due to the loss one allele of progesterone receptor. Levels of *Il1b, Il6, Il10* and *Tnfa* in $Pgr^{Cre/+}$ were similar to Nodal controls demonstrating that the increase in basal cytokine levels was due to the loss of one allele of Nodal (Figure S2.1). Also elevated in *Nodal* $^{\Delta/+}$ were *Cxcl10*, *Ccl12*, and *Ccl5* chemokine genes as well as the receptor/co-receptor and transcription factor genes *Ifngr1*, *Ifnar1*, *Ccr5*, *C5r1*, *Cd14*, *CD40*, *Myd88* and *Gata3* (Table 2.1). Other important genes involved in innate and adaptive immunity, including *Ddx58*, *Casp1*, *Icam1* and *Jak2*, were significantly up-regulated in *Nodal* $^{\Delta/+}$ compared to *Nodal* control mice (Table 2.1). Among the toll-like receptor (TLR)

genes assessed, *Tlr2* and *Tlr4* were significantly up-regulated in *Nodal* $^{\Delta/+}$ (Table 2.1) whereas *Tlr1*, *Tlr3*, *Tlr5*, *Tlr9* and *Tlr6* were significantly down-regulated in *Nodal* $^{\Delta/+}$ compared to *Nodal* control mice (Table 2.2).

Although levels of many Inflammatory-related genes were evaluted in *Nodal* $^{\Delta/+}$ maternal decidual tissue, these mice did not deliver preterm. Since injection of 1.4 mg/kg of LPS led to premature delivery in *Nodal* $^{\Delta/+}$ females, we examined the expression of inflammatory-related genes in the LPS-injected mice. Interestingly, all genes with elevated expression in *Nodal* $^{\Delta/+}$ were further elevated as compared to LPS injected *Nodal* controls (Figure Supplementray 2.2 and Table Supplementray 2.2). These results demonstrate that the expression of several genes involved in innate and adaptive immunity is elevated in *Nodal* $^{\Delta/+}$ females and is further elevated in LPS-injected *Nodal* $^{\Delta/+}$ females, which may lead to premature activation of the labor cascade.

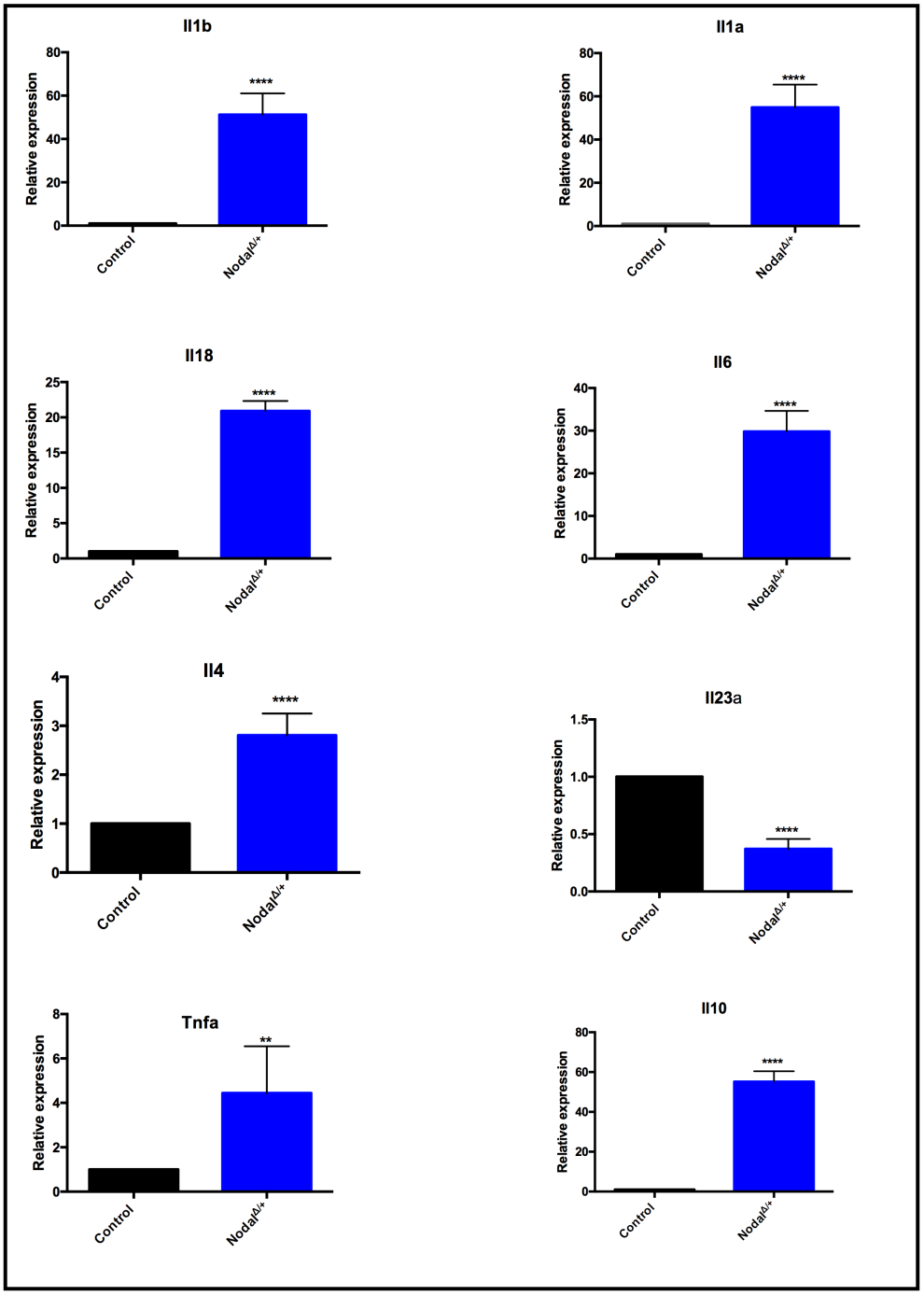

Figure 2.2 Inflammatory-related genes are elevated in maternal decidual tissue of Nodal$^{\Delta/+}$ mice

Basal levels of Il1b, Il1a, Il18, Il6, Il4, Tnfa, and Il10 were significantly higher in Nodal$^{\Delta/+}$ mice compared to control mice**.** Il23a was significantly increased in control than Nodal$^{\Delta/+}$ mice. N=4 animals each genotype (4 different tissues from each animals). Data were analyzed using an unpaired Student's t-test. $*P < 0.05$.

Table 2.1 Expression of genes in the RT2 PCR Profile array that were significantly upregulated in maternal decidual tissue of Nodal$^{\Delta/+}$ mice relative to control mice

Data were analyzed using an unpaired Student's t-test. $P < 0.05$.

Gene ID	Control	Nodal$^{\Delta/+}$	P value
Il1b	1	51.27	0.0001
Il1a	1	53.47	0.0001
Il6	1	29.83	0.0001
Il18	1	20.99	0.0001
Il10	1	55.25	0.0002
Il23a	1	0.36	0.0001
Il4	1	2.82	0.0002
Tnfa	1	4.5	0.008
Chemokines			
Cxcl10	1	35.37	0.001
Ccl5	1	2.1	0.0001
Ccl12	1	4.1	0.001
TLRs			
Tlr2	1	4.5	0.0001
Tlr4	1	6.09	0.0001
Receptor, Co-receptors and Transcription Factors			
Ifngr1	1	3.86	0.0003
Ifnar1	1	2.25	0.0001
Ccr5	1	8.3	0.0001
C5r1	1	0.11	0.0001
Cd14	1	5.11	0.0001
Cd40	1	3.5	0.01
Myd88	1	3.5	0.01
Others			
Ly96	1	4.2	0.01
Icam1	1	3.6	0.0001
Mapk8	1	1.3	0.0001
Irf7	1	1.6	0.02
Jak2	1	3.5	0.0001
Nfkb1	1	2.4	0.0001

Table 2.2 Expression of gens in the RT2 PCR Profile array were significantly down regulated in maternal decidual tissue of Nodal$^{\Delta/+}$ mice relative to control mice

Data were analyzed using an unpaired Student's t-test. *$P < 0.05$.

Gene ID	Control	Nodal$^{\Delta/+}$	P value
Chemokines			
Tlr1	1	0.08	0.02
Tlr3	1	0.06	0.02
Tlr5	1	0.06	0.001
Tlr9	1	0.1	0.01
Tlr6	1	0.3	0.01

2.4.4 Basal protein levels of pro-inflammatory cytokines are elevated in *Nodal* $^{\Delta/+}$

In order to confirm the results of the RT^2 Profiler PCR Array, we measured maternal decidual tissue protein levels of several pro-inflammatory cytokines that are important during parturition, including IL-1β, IL-6, IL-12p, TNF-α, and IFN-γ, and the anti-inflammatory cytokine IL-10 by multiplex ELISA. We observed that the basal concentration levels of the pro-inflammatory cytokines IL-1β, TNF-α, IL-6, IL-12p70, and IFN-γ were significantly higher in *Nodal* $^{\Delta/+}$ mice compared to *Nodal* control (Figure 2.3). Furthermore, the stimulated concentration levels of pro-inflammatory cytokines IL-6, IL-1β and IFN-γ were higher in LPS-injected *Nodal* $^{\Delta/+}$ compared to LPS-injected *Nodal* control mice (Figure S2.3). These results showed that *Nodal* $^{\Delta/+}$ have elevated basal levels of pro-inflammatory cytokines and an increased inflammatory response to LPS.

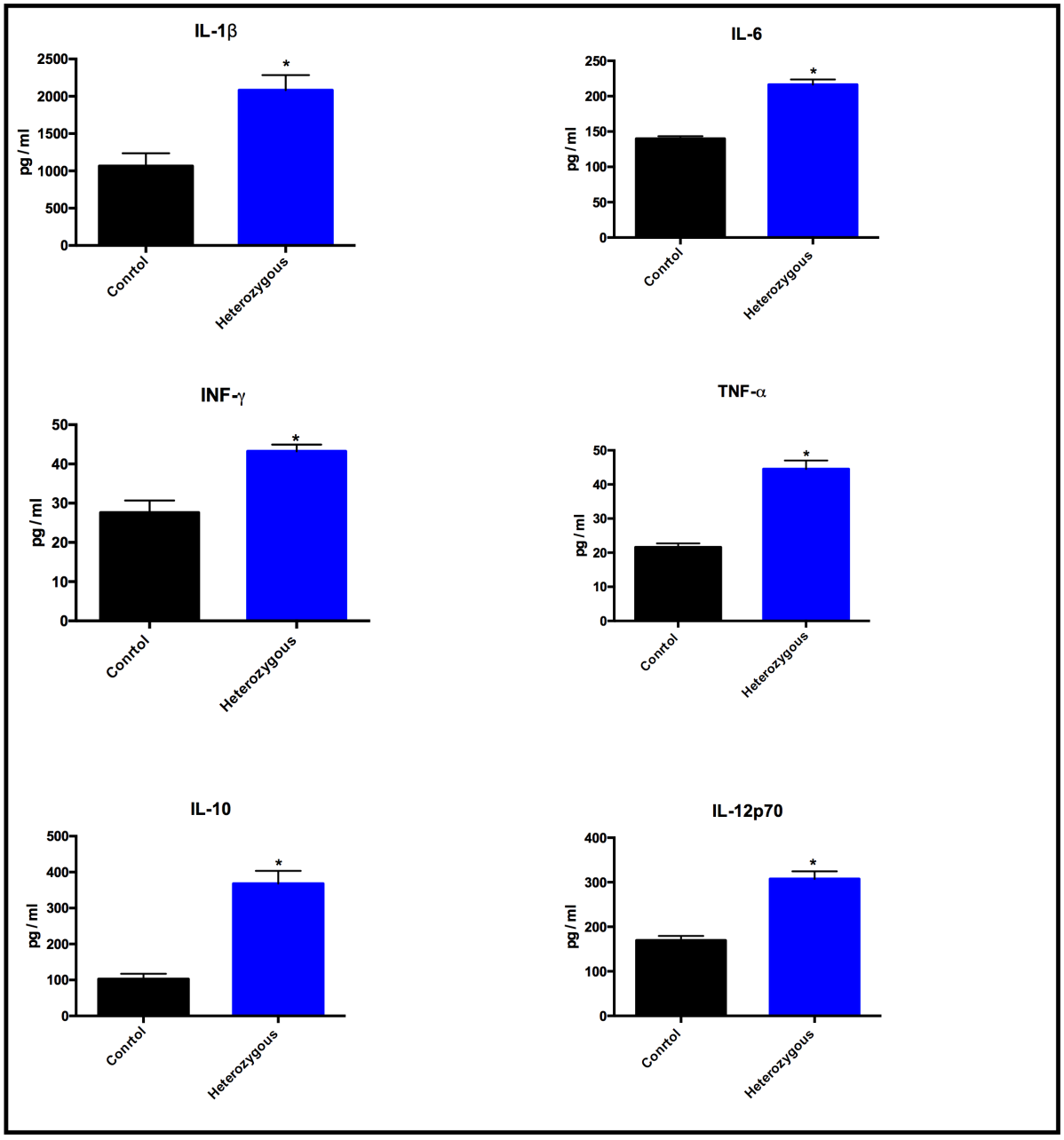

Figure 2.3 Basal protein levels of pro-inflammatory cytokines are elevated in maternal decidual tissue of Nodal $^{\Delta/+}$

Basal levels of IL-1β, TNF-α, IL-6, IL-12p70, and IFN-γ were significantly higher in Nodal$^{\Delta/+}$ mice compared to control mice**.** Anti-inflammatory IL-10 was significantly increased in Nodal$^{\Delta/+}$ mice compared to control mice. N=4 animals each genotype (4 different tissues from each animals). Data were analyzed by compared using an unpaired Student's t-test. $*P < 0.05$.

2.4.5 Levels of Cox-2 are elevated in maternal decidual tissue *Nodal* $^{\Delta/+}$

Elevated levels of cytokines at parturition results in and increased levels of COX-2 (Belt et al., 1999, Yan et al., 2002). Since cytokine levels were elevated in *Nodal* $^{\Delta/+}$, we measured protein levels of COX-2 in d16.5 placentas decidual tissue by western blotting. We observed that the basal protein level of COX-2 was significantly higher in non-injected *Nodal* $^{\Delta/+}$ mice compared to non-injected *Nodal* control mice (Figure 2.4). Additionally, protein levels of COX-2 was significantly higher in LPS- injected *Nodal* $^{\Delta/+}$ compared to LPS- injected *Nodal* controls (Figure 2.4). These results showed that *Nodal* heterozygous mice have elevated basal protein levels of COX-2 and a further increase upon LPS injection.

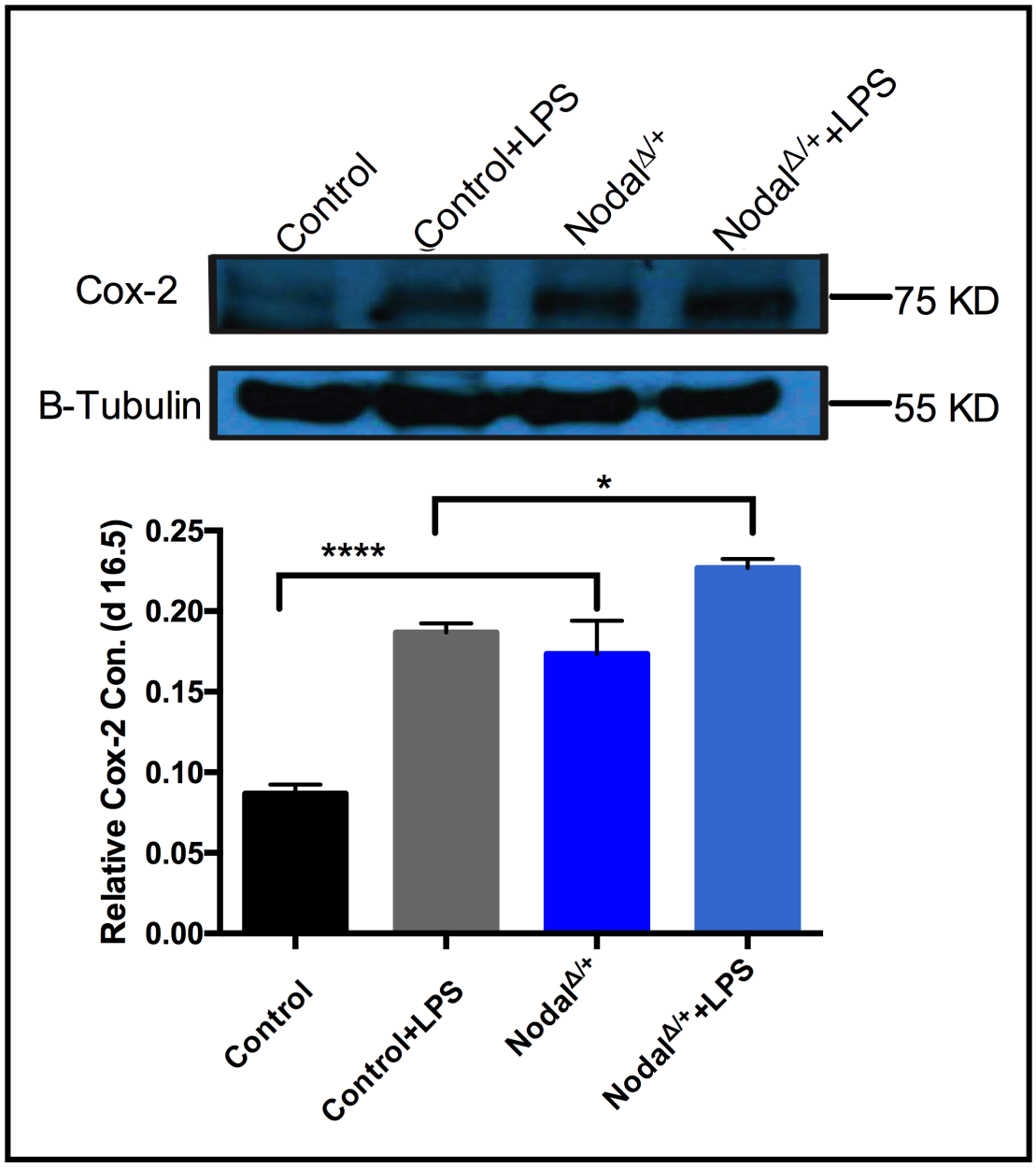

Figure 2.4 Levels of Cox-2 is elevated in maternal decidual tissue Nodal $^{\Delta/+}$ mice

In d16.5 maternal tissue COX-2 protein expression level was significantly higher in Nodal$^{\Delta/+}$ (N=3) and Nodal$^{\Delta/+}$+ LPS mice (N=3) compared to d16.5 maternal decidual tissue control (N=3), control +LPS mice(N=3). An antibody against Cox-2 was used to detect, the blot was stripped and reprobed with an internal antibody (anti Tubulin) to confirm equal protein loading. Three independent experiments.

2.4.6 Inflammation increases the infiltration of uNK and macrophages in the placenta decidual tissue of *Nodal*$^{\Delta/+}$

Since one of the hallmarks of a prturition is the infiltration of leukocytes, we performed immunofluorescence in control and *Nodal* $^{\Delta/+}$ maternal decidual tissue section on day 16.5 of pregnancy using markers for uterine NK (uNK) cells) and macrophages. We did not detect any uNK cells in *Nodal* controls and only a few uNK cells were detected in *Nodal* $^{\Delta/+}$ (Figure 2.5). Although injection of LPS did not result in an increase in uNK cells in *Nodal* controls, an increase in the number of uNK cells were observed in LPS injected *Nodal* $^{\Delta/+}$ mice (Figure 2.5). Only a few macrophages were observed in both *Nodal* controls and *Nodal* $^{\Delta/+}$ (Figure 2.5). Similar to uNK cells, injection of LPS did not result in an increase in macrophages in *Nodal* controls but a dramatic increase was observed in *Nodal* $^{\Delta/+}$ females (Figure 2.5). Thus, LPS injections result in a higher infiltration of uNK cells and macrophages in *Nodal* $^{\Delta/+}$ mice suggesting that the loss of one allele of *Nodal* increases the susceptibility to leukocyte infiltration due to mild inflammation

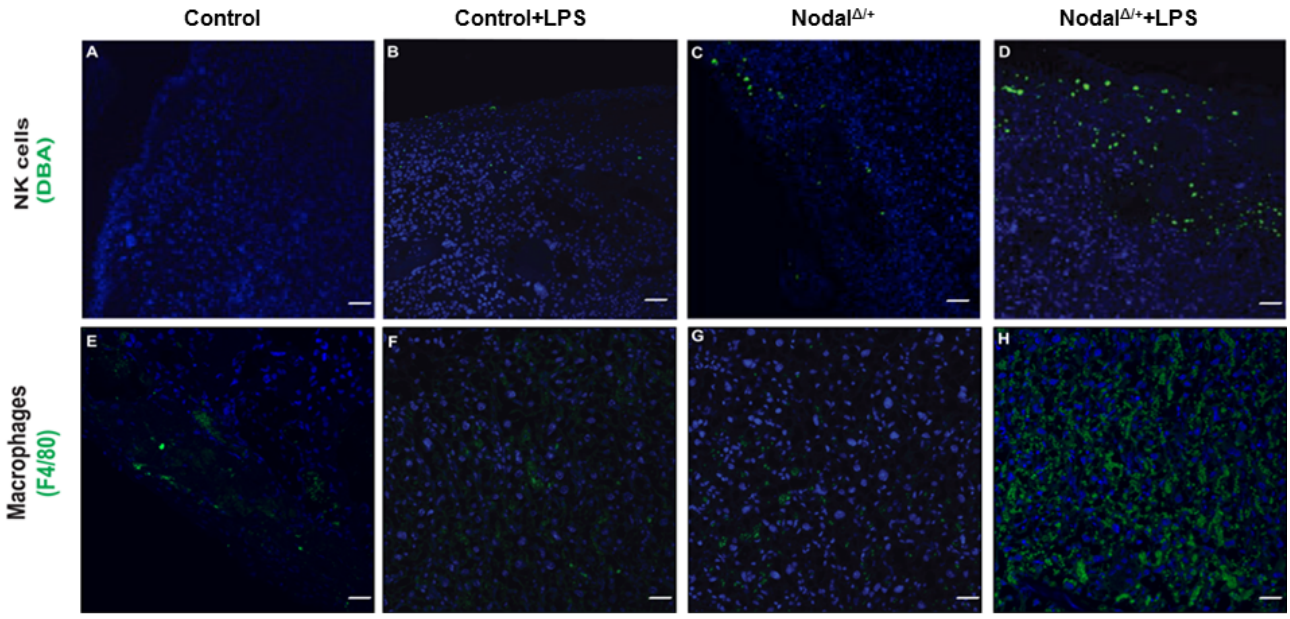

Figure 2.5 Localization of immune cells in maternal decidual tissue of Nodal$^{\Delta/+}$ mice

A confocal immunofluorescence representative images of maternal decidual tissue **(A-D)** Merged images showed staining for NK cells (DBA) is shown in green and nuclear staining for DNA (Dapi) shown in blue, (A) Control mice (N=3); (B) control+LPS (N=3); (C) Nodal$^{\Delta/+}$ (N=3); (D) (Nodal$^{\Delta/+}$+LPS) (N=3). **(E-H)** Merge images showed staining for macrophages (F4/80) is shown in green and Nuclear staining for DNA (Dapi) shown in blue: (E) control mice; (F) control+LPS; (J) Nodal$^{\Delta/+}$ (H) (Nodal$^{\Delta/+}$+LPS). Mice were injected with 1.42 mg/kg of LPS. Scale bar = 200µm. Three independent experiments.

2.4.7 Recombinant Nodal downregulates the expression of proinflammatory cytokines in primary bone-derived macrophages (BMDM)

Since *Nodal* $^{\Delta/+}$ females had higher basal levels of proinflammatory cytokines, including IL-1β, IL-6, IL-12p, TNF-α and IFN-γ, we further investigated how Nodal may regulate proinflammatory cytokine expression. One of the major leukocyte populations expressing these proinflammatory cytokine at parturition are macrophages. We thus isolated murine primary bone marrow derived macrophages (BMDM) and treated these cells with LPS which induces the expression of proinflammatory cytokines (Mantovani et al. 2004, Lawrence and Natoli, 2011). BMDM cells did not express Il1b, Il6, Tnfa or Il10 and addition of recombinant Nodal (rNodal) had no effect on cytokine expression (Figure 2.6). As expected, addition of LPS resulted in a significant increase in Il1b, Il6, Tnfa and Il10 expression. Interestingly, pre-treating BMDM cells with rNodal for 1h prior to the addition of LPS significantly reduced the expression of these cytokines (Figure 2.6). These results demonstrate that Nodal is able to repress proinflammatory cytokine expression in response to LPS.

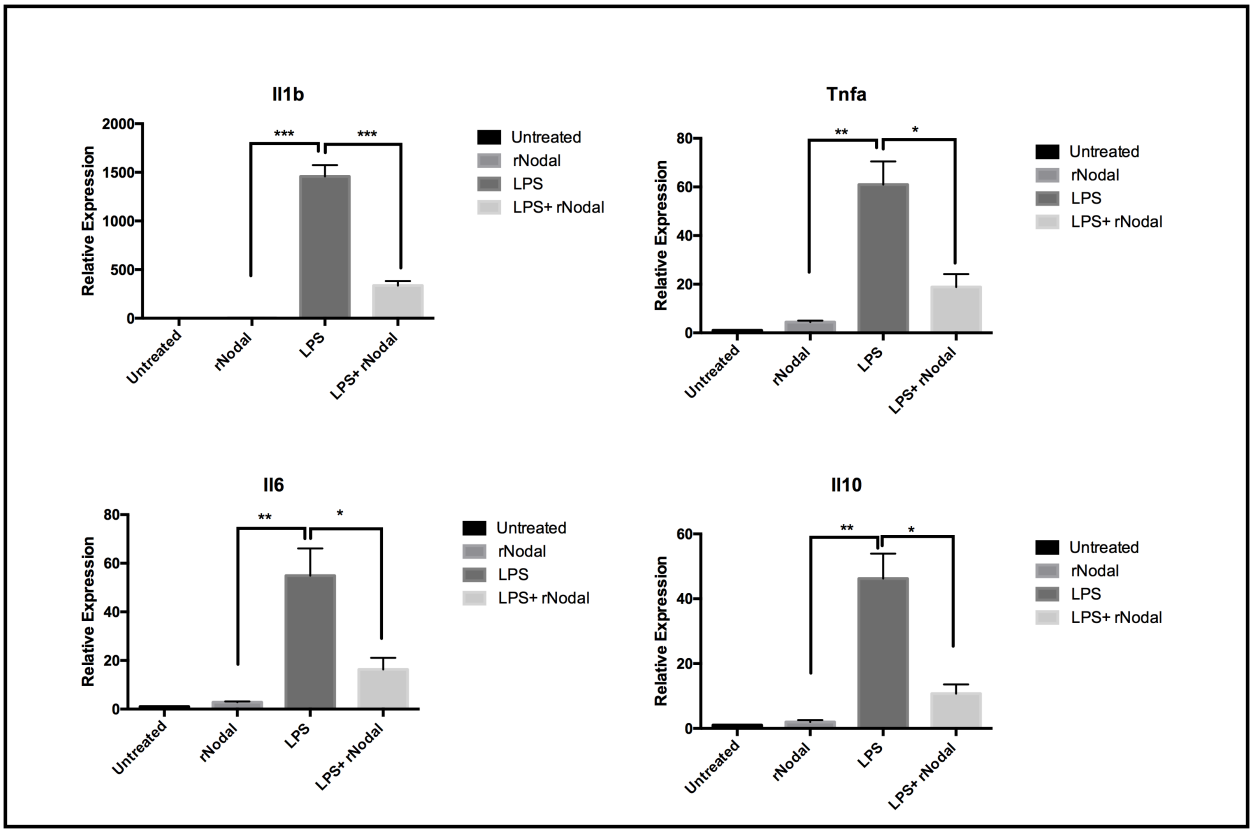

Figure 2.6 Recombinant Nodal downregulated the expression of proinflammatory cytokines in BMDM

mRNA levels of cytokines (Ilb1, Tnfa, Il6 and Il10) were low in both untreated cells and those treated with 300ng/ml of rNodal for 1h. mRNA levels of cytokines were significantly higher when cells were stimulated with 100ng/ml LPS for 6h. Cytokine levels were significantly lower when cells were stimulated for 1h with 300ng/ml of rNodal and then stimulated with 100ng/ml LPS for 6h. rNodal was able to suppress the expression of these cytokines. Data were analyzed and compared using an unpaired Student's t-test. $*P < 0.05$. Three independent experiments.

2.5 Discussion

Despite the prevalence and severity of premature delivery, all of the causes and molecular mechanisms that underlie the majority of spontaneous preterm births are still unknown. Part of the reason is a lack of suitable animal models available for preterm birth research. We have previously demonstrated that a tissue specific deletion of *Nodal* in tissues expressing the progesterone receptor led to a 75% decrease in fertility and, most importantly, to preterm birth by homozygous *Nodal* mutant females (*Nodal* $^{\Delta/\Delta}$) (Park et al., 2012). Since *Nodal* $^{\Delta/\Delta}$ had decidual and placental defects, we have concentrated our analysis of the function of Nodal in *Nodal* $^{\Delta/+}$ females which do not have any morphological defects. However, *Nodal* $^{\Delta/+}$ mice have reduced fertility with 50% of females becoming pregnant after mating which is less severe than the 25% observed in *Nodal* $^{\Delta/\Delta}$ mice (Park et al., 2012).

We have demonstrated that *Nodal* $^{\Delta/+}$ females have increased sensitivity to having preterm births when challenged with LPS which is known to induces inflammation. Injection of a low dose of LPS that did not affect control mice, resulted in 50% of *Nodal* $^{\Delta/+}$ females delivering within 24 hours after injection. To address this increased sensitivity, we examined the expression of innate and adaptive immunity-related genes in the placenta decidual tissue since LPS is known to induce increases in a pro-inflammatory reaction. Interestingly, we demonstrated that there was a significant increase in the basal levels of pro-inflammatory cytokines and chemokines in *Nodal* $^{\Delta/+}$ females at both the RNA and protein levels at the placenta decidual tissue. Thus, loss of one allele of Nodal in reproductive tissues resulted in a higher basal level of pro-inflammatory cytokines suggesting a role for Nodal in maintaining the uterine environment in an anti-inflammatory state. Since levels of proinflammatory cytokines are already elevated in *Nodal* $^{\Delta/+}$, these mice are more

suscceptible to having preterm births when challenged with LPS as compared to controls that have low basal levels of pro-inflammatory cytokines. Furthermore, the parturition cascade appears to be primed as Cox-2 levels are higher in *Nodal* $^{\Delta/+}$ than controls, possibly contributing to the increased sensitivity to have a preterm birth if challenged with LPS.

It has been well documented that parturition is initiated by an increase in both pro-inflammatory cytokines and an infiltration of leukocytes (Thomson et al., 1999, Osman et al., 2003, Hamilton et al., 2012). The main cellular sources of the cytokines, including IL-1β, IL-6, IL-12p70 and TNF, are macrophages and monocytes (Arango Duque & Descoteaux 2014, Bryant et al 2017). Macrophages also release other agents that lead to activation of the labor cascade, such as chemokines, including CXCL10, CCL5, and CCL12, prostaglandins, and complement proteins (Arango Duque & Descoteaux 2014). Since the basal levels of pro-inflammatory cytokines were elevated in *Nodal* $^{\Delta/+}$, we examined the infiltration of macrophages that are known to be present in high numbers at parturition (Hamilton et al., 2012) as well as uNK cells which are normally absent or at low numbers at this stage (Hofmann et al., 2014). Although macrophage levels were slightly higher in the *Nodal* $^{\Delta/+}$ than in controls, LPS induced a dramatic increase in *Nodal* $^{\Delta/+}$ placenta decidual tissue where only a modest increase was observed in controls. Thus, the uterine environment in *Nodal* $^{\Delta/+}$ mice appears to be primed for macrophage infiltration as compared to controls. Interestingly, only a slight increase in uNK cells was observed in LPS injected *Nodal* $^{\Delta/+}$ mice suggesting that there is a selectivity in the immune cells that can infiltrate the uterine environment of *Nodal* $^{\Delta/+}$ mice upon induction of inflammation by LPS. Further studies are required to elucidate the nature of this suscceptibility to leukocyte infiltration in response to inflammation in *Nodal* $^{\Delta/+}$ uteri.

Previous studies have linked the NODAL signaling pathway to regulation of the immune system. NODAL and its co-receptor CRIPTO polarize cultured macrophages towards an anti-inflammatory phenotype (Wang et al 2014, Zhang et al 2016). These results are in agreement with our present results in which the absence one allele of *Nodal* in mice results in increased proinflammatory cytokine expression.

We have previously demonstrated that women with certain single nucleotide polymorphisms (SNP) in the Nodal gene that decrease its activity or expression are more susceptible to have a preterm birth if they have an infection or placental inflammation (Starr et al., 2018). This is in agreement with our current studies demonstrating that *Nodal* $^{\Delta/+}$ mice are more sensitive to LPS than control mice. This suggests that Nodal function in human pregnancy is most likely conserved. This would suggest that there may be higher levels of proinflammatory cytokines in women that carry SNPs that affect Nodal expression or activity. It would be interesting to measure proinflammatory cytokine levels in these woman.

In conclusion, our study demonstrated that the absence of one allele of *Nodal* leads to increased susceptibility to LPS-induced preterm birth in mice. We also provide evidence that NODAL may regulate the uterine immune system during late pregnancy and labor. The *Nodal* heterozygous mouse is a suitable model to study gene-environment interactions that are important in human preterm birth.

2.6 Material and Methods

2.6.1 Maintenance of *Nodal* heterozygous females

Mice with loxP sites flanking exons 2 and 3 of the *Nodal* gene ($Nodal^{loxP/loxP}$) on a mixed background were previously generated and kindly donated 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 previously been reported to demonstrate normal fertility and $Pgr^{Cre/+}$ mice have become the standard tool to study uterine-specific gene function (Lee et al 2006, Mukherjee et al 2007). In this study, 8–24-week-old *Nodal* control ($Nodal^{flox/+}$) and *Nodal* heterozygous ($Nodal^{\Delta/+}$) females from $Nodal^{loxP/loxP}$ mice crossed with $Pgr^{Cre/+}$ mice were used.

2.6.2 Mating and monitoring of $Nodal^{\Delta/+}$ females

In order to assess the pregnancy rate of $Nodal^{\Delta/+}$ mice, adult $Nodal^{\Delta/+}$ mice and control mice were mated with CD1 males overnight and the day of vaginal plug visualization was considered day 0.5 of pregnancy. Mice were monitored from day 13.5 of pregnancy until delivery to assess the pregnancy rate. In order to assess preterm birth, mice were monitored every 3 hours from day 15.5 of pregnancy until delivery. Births were considered preterm if mice delivered two days prior to term on day 18.5 of pregnancy.

In order to study the effect of LPS on $Nodal^{\Delta/+}$ mice, mice were divided into four groups: 1) control mice; 2) control+LPS mice; 3) $Nodal^{\Delta/+}$ mice; and 4) $Nodal^{\Delta/+}$ +LPS mice. On day 15.5 of pregnancy, females were i.p. injected with either 0.57 or 1.42 mg/kg of LPS (Sigma-Aldrich

Cat No. L2630). Twelve hours post-injection, placentas were dissected into PBS. Samples were used for RNA or protein extraction or fixed for immunofluorescence.

2.6.3 RNA isolation and RT^2 Profiler PCR Array for maternal decidual tissue

Placentas and maternal decidua tissue were carefully separated and collected 12 hours after LPS injection on day 16.5 of pregnancy. RNA was collected using Trizol (Thermofisher Cat No. 15596026), and a RNeasy Mini Kit (Qiagen Cat No. 74104), cDNA was synthesized using the Qiagen RT^2 First Strand Kit (Qiagen Cat No. 330404), and RT-PCR was performed using the Mouse Innate & Adaptive Immune Responses RT^2 Profiler PCR Array (Qiagen Cat No. PAMM-052Z) following the manufacturer's protocols.

2.6.4 Protein extraction and multiplex ELISA for maternal decidual tissue

Placentas were collected on day 16.5 of pregnancy, weighed, and rinsed in PBS. Protein was extracted by homogenizing 1.0 g of maternal tissue in 500 µl Lysis Buffer #2 (R&D Systems Cat No. 33076,) and 500 µl of PBS. Homogenates were incubated overnight at room temperature. Samples were then centrifuged (1,000 x g for 15 min). The supernatant was stored at -80°C until assayed. Protein concentrations of IL-1β, IL-6, IL-12p70, TNF-α, IFN-γ, and IL-10 were measured by multiplex ELISA using the Mouse Magnetic Luminex Screening Assay (R&D Systems Cat No. LXSAMSM-6) according to the manufacturer's instructions and the Bio-Rad CFX384 Real-Time System.

2.6.5 Immunofluorescence

Placentas decidual tissue were dissected into PBS on day 16.5 and fixed overnight at 4°C in 4% paraformaldehyde (PFA) in PBS. Samples were dehydrated with increasing concentrations of ethanol (two times 25, 50, 70, and 100% for 20 min each). Samples were then submerged in xylenes (two times for 15 min each) and placed in melted paraffin wax and xylenes (1:1) for 1 hour at 60°C followed by pure paraffin overnight under a vacuum. Samples were embedded at room temperature and blocks were solidified at -20°C. Seven-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 PBT (0.2% BSA and 2.5% TritonX-100 in PBS) (two times for 5 min each). Slides were then washed several times in PBS containing 0.1% Tween-20 (two times for 5 min each) and blocking with 5% heat-inactivated goat serum in PBT for 1 hour within a humidified chamber at room temperature and incubated with primary antibody at 4°C overnight. For detection of uNK cells, FITC-conjugated Dolichos biflorus agglutinin (DBA) lactin (1:300, BioWorld Cat No. 21761015-1) was used. For identification of macrophages, rat anti-mouse F4/80 (1:100, Bio-Rad Cat No. MCA497RT) was used. Then, slides were washed with PBS containing 0.1% Tween-20 three times, F4/80 was used to detect macrophages, slides were incubated with Alexa Fluor 488-labelled goat anti-rat secondary antibody (1:100, Life Technologies Cat No. 1301839) for 1 hour at room temperature. Slides were washed and mounted with Mowiol 4-88.

2.6.6 Generation of adult murine BMDMs

Bone marrow was harvested from both femurs and tibiae in RPMI supplemented with 2mML-glutamine (Wisent Cat No. 350-000-CL), 10% FBS (Wisent Cat No. 80150), 2%HEPES (Wisent Cat No. 330—050—EL), 1% non-essential amino acids (Wisent Cat No. 321-010-EL), 1% essential amino acids (Wisent Cat No. 321-011-EL), 0.14% 5N NaOH, 1mM sodium pyruvate (Wisent Cat No. 600-110-EL), 100U/ml penicillin, 100µg/ml streptomycin (Wisent Cat No. 450-115-EL), and 30% of L929-cell conditioned media (LCM) in petri dishes. After 3 days of incubation at 37°C in a 5% CO_2, fresh medium containing LCM was added. Cells were allowed to differentiate into macrophages for a total of 6 days and then were harvested by removing the supernatant and addition of 4ml cell stripper for 20 minutes at 37°C. (Fisher Scientific Cat No. MT25056CT).

2.6.7 Macrophage treatment, RNA isolation and Q-PCR

RAW 264.7 cells were grown to confluence in complete medium containing 10% FBS. (i)Cells were cultured for 1h in medium alone, (ii) treated for 1h with 300ng/ml of rNodal only (R&D Systems Cat No. 13-15-ND), (iii) stimulated with 100ng/ml LPS only for 6h, or stimulated for 1h with 300ng/ml of rNodal then cells stimulated with 100ng/ml LPS for 6h. Cells were then lysed in 1 ml of Trizol (Thermofisher Cat No. 15596026), and RNA isolated using an RNeasy Mini Kit (Qiagen Cat No. 74104). cDNA was synthesized using the Qiagen RT^2 First Strand Kit (Qiagen Cat No. 330404). Real-Time PCR was performed using the Roter-Gene SYBR Green PCR Kit (Qiagen Cat No. 204074) as described by the manufacturer's protocol.

The following primers were used: Il1b (251bp) 5'-TGCCACCTTTTGACAGTGATG-3' and 5'-AAGGTCCACGGGAAAGACAC-3'; Il6 (251bp) 5'- CAACGATGATGCACTTGCAGA-3' and 5'-TGTGACTCCAGCTTATCTCTTGG-3'; Tnfa (250bp) 5'-ACAGAAAGCATGATCCGCGA-3' and 5'-CCACTTGGTGGTTTGTGAGTG-3'; Il10 (288bp) 5'-ACTACCCAAAGCCACAAGGCA-3' and 5'-TGGCAACCCAAGTAACCCTTAAA-3'; Gapdh (209bp) 5'- CGAGGACTTTAAGGGTTACTTGG-3' and 5'- GCTCCACTGCCTTGCTCTTA-3'. Tnfa 5'-ACAGAAAGCATGATCCGCGA-3' and 5'-CCACTTGGTGGTTTGTGAGTG-3'; Il6 5'-CAACGATGATGCACTTGCAGA-3' and 5'-TGTGAXTCCAGCTTATCTCTGG-3'; Il10 5'-CCAAGCCTTATCGGAAAATGA-3' and 5'-CCAAGCCTTATCGGAAATGA-3';

2.6.8 Statistics

Statistical analyses of differences between experimental groups were performed using analysis of variance (ANOVA), two-sided Student's *t*-test, or Chi-square (χ^2) tests using GraphPad Prism 6 software. Bonferroni corrections were applied where appropriate. Data represent mean ± SEM. Differences were considered significant if *P*-value ≤ 0.05.

Study Approval

All experimental protocols were approved by the Animal Care Committee of the McGill University Health Centre. Mice were cared for in accordance with the regulations established by the Canadian Council on Animal Care.

Author Contributions

All contributing authors agreed to the submission of this manuscript for publication. TAA, LMS, and DD conceived and designed the study. TAA and LMS performed the experiments. TAA, LMS, and DD wrote the manuscript.

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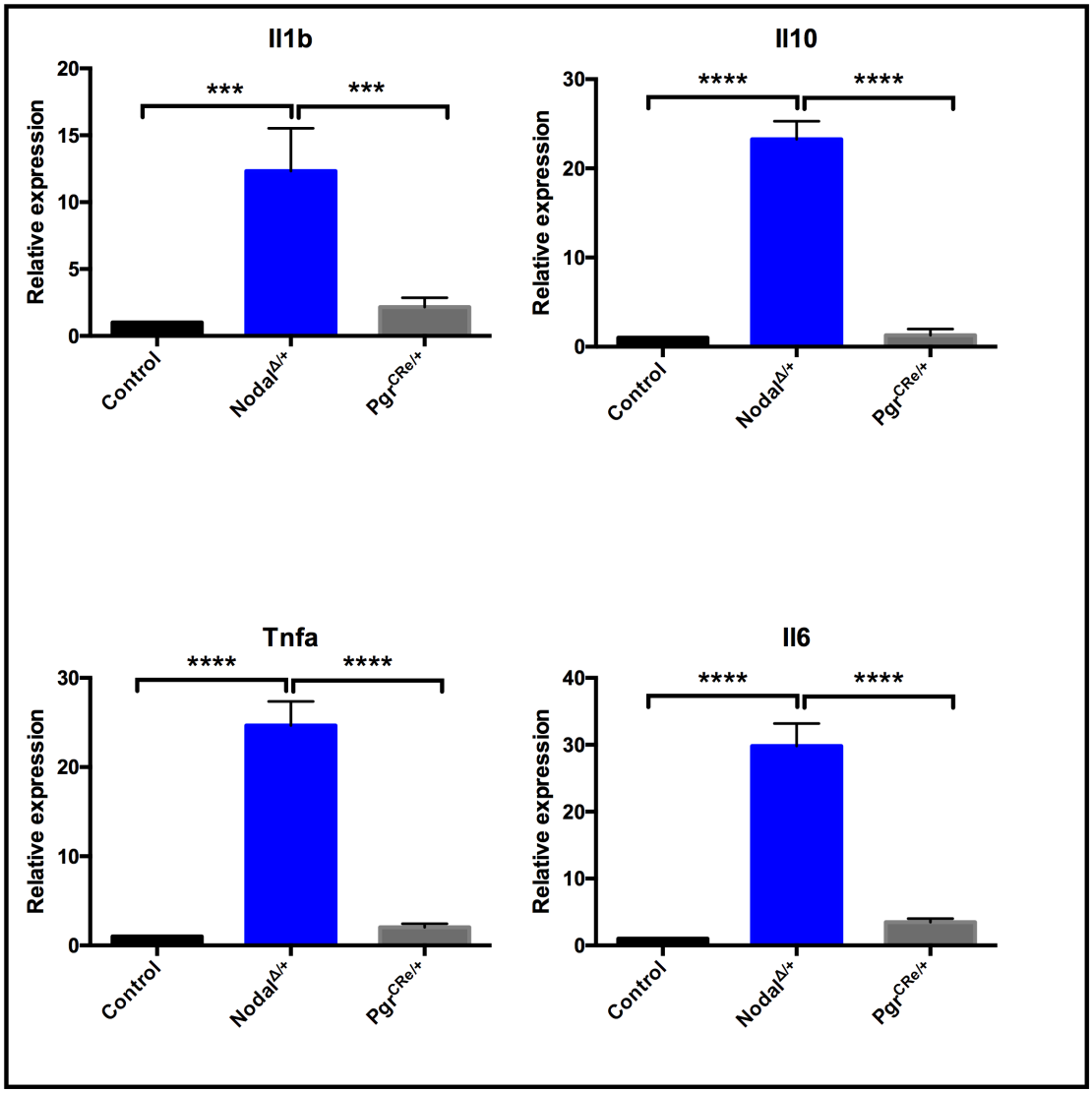
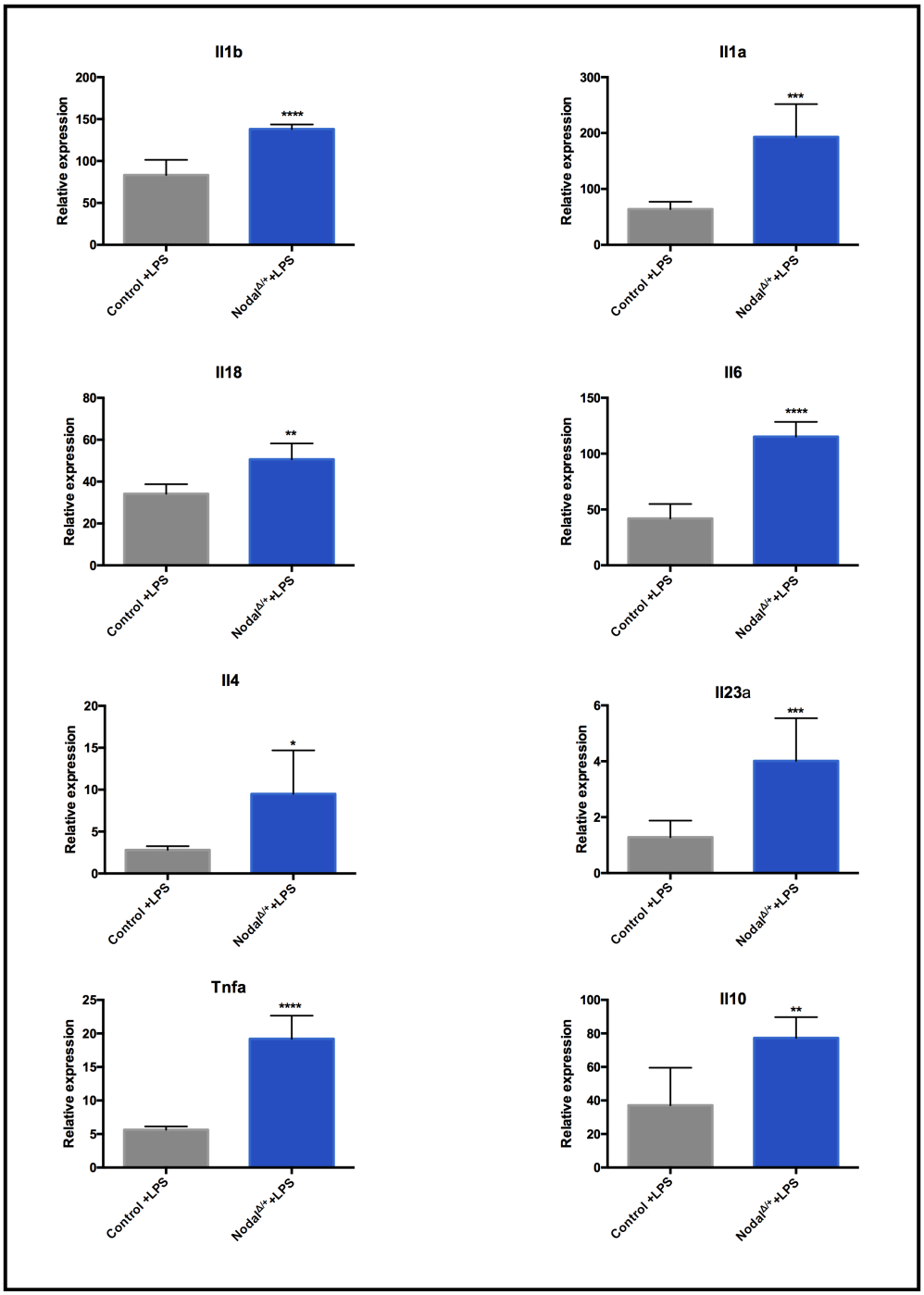
2.7 Supplemental Material



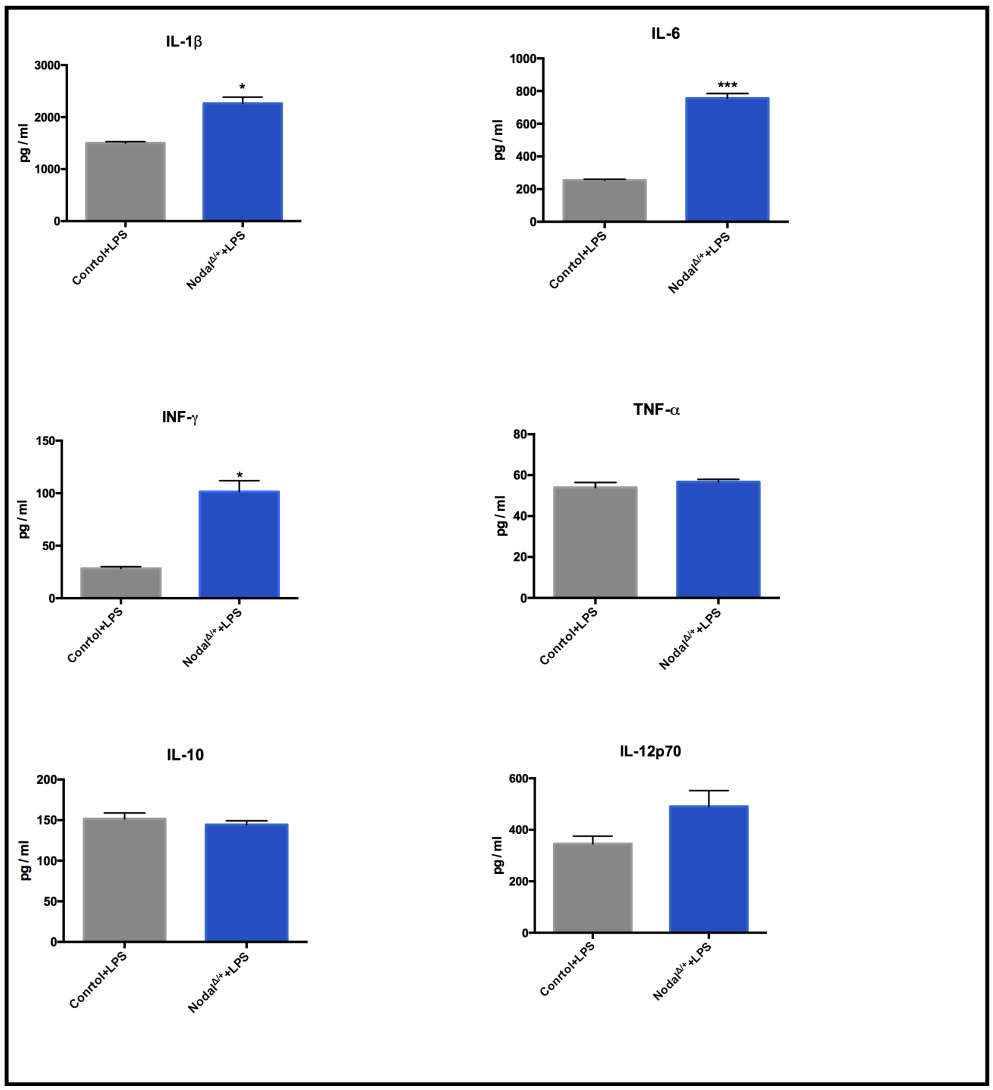
Supplementary Figure 2.1Basal levels of pro-inflammatory cytokines are not elevated in Pgr$^{cre/+}$ mice-uterine decidua

Basal levels of Il1b, Il6, Tnfa, and Il10 were significantly higher in Nodal$^{\Delta/+}$ (missing one allele of Nodal and one allele of Pgr) mice (N=3) compared to control (N=3) and Pgr$^{cre/+}$ (missing one allele of the Pgr) mice(N=3). Data were analyzed and compared using an unpaired Student's t-test. *$P < 0.05$. Three independent experiments.



Supplementary Figure 2.2 Inflammatory-related genes expression in maternal decidual tissue of LPS injected control and Nodal $^{\Delta/+}$ females

Stimulated levels of Ilb1, Il1a, Il18, Il6, Il4, Tnfa, Il23a and Il10 were significantly higher in Nodal$^{\Delta/+}$ +LPS compared to control+LPS mice**.** N=4 animals each genotype (4 different tissues from each animals). Data were analyzed using an unpaired Student's t-test. *$P < 0.05$.



Supplementary Figure 2.3 Pro-inflammatory cytokines expression in maternal decidual tissue of LPS injected control and Nodal $^{\Delta/+}$ females measured by multiplex ELISA

Levels of IL-1β, IL-6 and IFN-γ were significantly higher in Nodal$^{\Delta/+}$+LPS compared to control+LPS mice**.** No statistical differences were seen in the levels of TNF-α, the anti-inflammatory IL-10 and IL-12p70 between Nodal$^{\Delta/+}$ +LPS mice compared to Nodal control+LPS mice. N=4 animals each genotype (4 different tissues from each animals). Data were analyzed using an unpaired Student's t-test. *$P < 0.05$.

Gene ID	Control	Control+ LPS	Nodal$^{\Delta/+}$	Nodal$^{\Delta/+}$+LPS	P value
Il1r1	1	1.33	0.53	1.43	0.0676
Il18	1	31	20.99	43.47	0.0001
Il1a	1	63.7	53.47	139.1	0.0001
Il1b	1	84.31	51.27	138.4	0.0001
Ifngr1	1	1.18	3.86	2.69	0.043
Ifnar1	1	2.3	2.25	3.5	0.0001
Il10	1	37.12	55.25	77.26	0.0001
Il23a	1	1.36	0.36	4.01	0.0001
Il4	1	6.68	2.822	9.4	0.0001
Il6	1	41.94	29.83	115.26	0.0001
Tlr4	1	2.7	6.09	23.76	0.0001
Tlr2	1	2.88	4.5	6.5	0.0001
Tlr9	1	0.24	0.10	0.020	0.0001
Tlr1	1	0.57	0.082	0	0.0001
Tlr3	1	0.73	0.060	0	0.0001
Tlr5	1	0.72	0.067	0.15	0.0001
Tlr6	1	0.22	0.39	0.02	0.0001
Nfkbia	1	2.2	1.4	0.769	0.0001
Myd88	1	1.25	3.5	6.73	0.0001
Nfkb1	1	2.2	2.4	4.5	0.0001
Icam1	1	1.6	3.6	4.2	0.0001
Traf6	1	1.6	1.5	0	0.0001
Tnfa	1	5.6	4.5	19.19	0.0001
Irak1	1	1.1	1.5	2.02	
H2-T23	1	1.5	0.97	0.65	0.0001
Ddx58	1	2.4	2.7	1.6	0.002
C3	1	1.2	2.2	1.4	0.0001
Cxcl10	1	32.1	35.37	63.63	0.0001
Mapk8	1	1.06	1.3	2.4	0.0001
Mapk1	1	1.9	2.1	0.38	0.0001
Jak2	1	3.5	3.5	6.6	0.0001
Stat1	1	2.5	1.8	0.9	0.0001
Stat3	1	1.3	1.3	1.05	0.0001
Ly96	1	1.6	4.2	5.2	0.0001
Ccl5	1	4.65	2.1	17.8	0.0001
Ccl12	1	10.82	4.1	26.48	0.0001
Casp1	1	2.3	1.4	29.8	0.0001

Gata3	1	1.2	1.5	4.8	0.0024
Irf3	1	1.3	1.4	0.55	0.0001
Irf7	1	0.59	1.6	1.4	0.0001
Ccr5	1	2.48	8.3	10.44	0.0001
Cd14	1	2.43	5.11	7.17	0.0001
Cd40	1	2.3	3.5	6.9	0.0001
Mop	1	0	3.95	3.29	0.0001
Ticam1	1	1.9	1.1	0	
C5r1	1	2.1	0.11	2.4	0.0001
Ifna2	0	0	0	0	
Ifnb1	0	0	0	0	
Ifng	0	0	0	0	
Apcs	0	0	0	0	
Ccr4	0	0	0	0	
Ccr6	0	0	0	0	
Ccr8	0	0	0	0	
Cd4	0	0	0	0	
Cd40lg	0	0	0	0	
Cd80	0	0	0	0	
Cd86	0	0	0	0	
Cd8a	0	0	0	0	
Crp	0	0	0	0	
Csf2	0	0	0	0	
Cxcr3	0	0	0	0	
Foxp3	0	0	0	0	
Fasl	0	0	0	0	
Il17a	0	0	0	0	
Il13	0	0	0	0	
Il2	0	0	0	0	
Il5	0	0	0	0	
Mbl2	0	0	0	0	
Mx1	0	0	0	0	
Nlrp3	0	0	0	0	
Nod1	0	0	0	0	
Reg1	0	0	0	0	
Slc11a1	0	0	0	0	
Stat4	0	0	0	0	
Stat6	0	0	0	0	
Tbx21	0	0	0	0	
H2-Q10	0	0	0	0	
Itgam	0	0	0	0	

Nod2	0	0	0	0	
Rorc	0	0	0	0	
Tyk2	0	0	0	0	
Tlr 7	0	0	0	0	
Tlr 8	0	0	0	0	
Lyz2	1	0	0.67	0	

Supplementary Table 2.1 Expression of 84 genes in the RT2 PCR Profile array in maternal decidual tissue of all four groups. (control, control+LPS, Nodal$^{\Delta/+}$, and Nodal$^{\Delta/+}$+LPS) at, *P*-value≤0.05 by 2-way ANOVA with Bonferroni's multiple comparisons test.

Gene ID	Control+LPS	Nodal$^{\Delta/+}$+LPS	P value
Il1b	84.31	138.47	0.0005
Il1a	63.7	193.1	0.002
Il6	41.94	115.26	0.0001
Il18	31	43.47	0.01
Il10	37.12	77.26	0.01
Il23a	1.36	4.01	0.007
Il4	6.68	9.4	0.02
Tnfa	5.6	19.1	0.0001
Chemokines			
Cxcl10	32.1	63.6	0.0001
Ccl5	4.65	17.8	0.0001
Ccl12	10.82	26.48	0.0001
Tlr2	2.88	6.5	0.0001
Tlr4	2.7	23.76	0.007
Receptor, Co-receptors and Transcription Factors			
Ifngr1	1.18	2.69	0.0006
Ifnar1	2.37	3.54	0.0001
Ccr5	2.48	10.44	0.0003
C5r1	2.01	2.4	Na
Cd14	2.43	7.17	0.0001
Cd40	2.3	6.9	0.008
Myd88	1.25	6.73	0.0001
Other			
Ly96	1.6	5.2	0.0001
Icam1	1.6	4.2	0.0001
Mapk8	1.06	2.4	0.01
Irf7	0.59	1.4	0.002
Jak2	3.5	6.6	0.001
Nfkb1	2.2	4.5	0.03

Table 2.2 Expression of genes in the RT2 PCR Profile array that were significantly up-regulated in maternal decidual tissue of Nodal$^{\Delta/+}$+LPS mice and with 1.42 mg/kg LPS relative to control+ LPS mice

Data were analyzed and compared using an unpaired Student's t-test. *$P < 0.05$

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Chapter 3: NODAL Regulates the LPS-Induced Inflammatory Response in Macrophages through NFκB and MAPK Pathways

NODAL Regulates the LPS-Induced Inflammatory Response in Macrophages through NFκB and MAPK Pathways

Taghreed A. Ayash[1] and Daniel Dufort[1,2]

[1]Division of Experimental Medicine, McGill University, Montreal, Quebec, Cananda.

[2]Department of Obstetrics and Gyneocology, McGill University, Montreal, Quebec, Canada.

Corresponding author: Daniel Dufort, Research Institute of the McGill University Health Centre, Glen Site, EM0.3211, Montreal, Quebec, Canada. Tel: 1-514-934-1934 x34743. E-mail: Daniel.dufort@mcgill.ca

Taghreed.heba@mail.mcgill.ca

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Key words: *NODAL*, inflammation, Smad independent pathway, TLR receptor signaling

3.1 Preface

The TGF-ß superfamily is comprised of more than 33 growth and differentiation factors. Nodal, a TGF-ß superfamily morphogen, is well known in many embryonic development processes and has been implicated in different mammalian reproduction events including implantation, placentation and timing of parturition. Also, NODAL and components of its canonical signaling pathway have been implicated in modulating the immune system. In addition to Nodal and other TGF-ß family member also can activate different pathways through the non-canonical signaling of ERK, JNK, MAPKp38 and AKT. Despite previous findings that CRIPTO can modulate the LPS-induced production of cytokines through NFkB,(Zhang et al 2016), little is known about the mechanisms by which Nodal affect the immune system.

Our data reveal an inhibitory effect of Nodal through the non-canonical pathways. First, we found that Nodal regulates LPS-induced cytokine production in macrophages. Indeed, we demonstrated that Nodal significantly decreases the phosphorylation of proteins in RAW 264.7 macrophages after treating the cells with rNodal for two minutes. We determined that these differentially phosphorylated proteins were linked to many signaling pathways. Most importantly, the MAPK and NFκB pathways were downregulated in response to rNodal treatment, which suggesting that both signaling pathways are key players in these inflammatory processes. Furthermore, we showed that Nodal significantly inhibits the phosphorylation of NFκB p65 as well as MAPK p38. Together, these results highlight a mechanism by which Nodal can influence inflammation. This finding may prove useful in the development of new therapies for various inflammatory diseases.

3.2 Abstract

Nodal is a member of the TGF-ß superfamily which plays a crucial role in embryonic development as well as in reproduction. In mice, Nodal is expressed throughout the uterus during early pregnancy and is associated with numerous events such as implantation, placentation and timing of parturition. Interestingly, Nodal appears to be involved in maintaining the uterine environment in an anti-inflammatory state as the loss of one allele of *Nodal* results in high basal levels of pro-inflammatory cytokines and an increased susceptibility to LPS induced preterm birth. To elucidate the mechanism by which Nodal modulates pro-inflammatory cytokine levels, we have used the macrophage cell line RAW264.7 and demonstrated that pre-treating these cells with recombinant Nodal (rNodal) reduced pro-inflammatory gene expression in response to LPS treatment. Moreover, proteomic analysis showed that treating macrophages with rNodal for only two minutes resulted in a significant decrease in global protein phosphorylation compared to the untreated group. Specifically, 295 phosphorylated proteins were uniquely present upregulated in the control group whereas only 40 were uniquely phosphorylated in the Nodal treated group. Furthermore, we demonstrate that Nodal prevents the phosphorylation of the nuclear NFκB p65 as well as p38MAPK in response to LPS. Our results suggest that one mechanism by which NODAL prevents the LPS-induced inflammation is through the downregulation of the NFκB and MAPK pathways.

3.3 Introduction

The immunological status of the female reproductive tract changes dynamically during pregnancy and is either in a pro-inflammatory or anti-inflammatory state depending on the stage of pregnancy (Mor & Cardenas 2010). At the early stages, including, peri-implantation, implantation, and placentation, a robust pro-inflammatory state is required for these processes to occur successfully (Zhang et al 2017). During this phase, macrophages and other immune cells secrete pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-1 ß (IL-1 ß) and tumor necrosis factor-α (TNF-α) (Mor & Cardenas 2010). During the fetal growth and development phase, the immunological state shifts to an anti-inflammatory state following release of anti-inflammatory mediators such as interleukin-10 (IL-10) and transforming growth factor-ß (TGF-ß) (Mor & Cardenas 2010). Finally, in order to initiate the parturition cascade, during the final stage of pregnancy, a critical shift occurs in the immunological state: the release of pro-inflammatory mediators (IL-6, IL-1 ß, and TNF-α) are involved in activation of the labor cascade (Mor & Cardenas 2010, Zhang et al 2017), resulting in the marked shift from an anti-inflammatory state to a pro-inflammatory one.

Growing evidence suggests that macrophages play a crucial role in activating the parturition cascade at term and as well as in preterm delivery via secretion of many pro-inflammatory mediators that are involved in initiating the parturition cascade, such as TNF-α, IL-1 ß, IL-6, matrix metalloproteinases (MMP), prostaglandins (PGE2) and nitric oxide (NO)(Gomez-Lopez et al 2014, Huang et al 2012, Vos et al 2000).

NODAL is a morphogen belonging to the TGF-β superfamily. It plays a crucial role during embryonic development (Brennan et al 2002, Takaoka et al 2006) and has been involved in many

mammalian reproduction events including implantation, placentation and timing of parturition in mice (Park et al 2012, Park & Dufort 2011, Park & Dufort 2013). Like other members of the TGF-ß superfamily, Nodal signals through both the canonical (SMAD-dependent) pathway and/or noncanonical (SMAD-independent) pathway which encompass the PI3K/AKT, MAPK (JNK, ERK, and p38MAPK) and NFκB pathways (Neuzillet et al 2015). MAPK and NFκB pathways are well known as key players in the innate and adaptive immune systems (Ruland 2011, Tammi et al 2011, Zhang & Dong 2005) and are involved in the activation of the inflammation cascade by producing numerous pro-inflammatory agents, including cytokines and chemokines (Liu et al 2017, Zhang & Dong 2005). In the canonical pathway, NODAL and its signaling components have been implicated in immune system regulation, as NODAL and its co-receptor CRIPTO polarize cultured macrophages towards an anti-inflammatory state (Wang et al 2014, Zhang et al 2016).

Previous work in our laboratory has revealed that conditional uterine Nodal heterozygous pregnant mice experience a higher basal level of cytokines and chemokines which make them more susceptible to lipopolysaccharide (LPS)-induced preterm births in contrast to Nodal control mice which deliver at term after injection with the same dose of LPS (Ayash, et al. manuscript submitted). Moreover, Nodal heterozygous mice showed a dramatic increase in the number of macrophages in maternal-decidual tissues post LPS injection compared to controls. We further demonstrated that pre-treatment of bone marrow-derived macrophages (BMDM) with rNodal downregulated the expression of pro-inflammatory cytokines produced in response to LPS.

In this manuscript, we have addressed the mechanism by which Nodal regulates pro-inflammatory cytokine expression. Using the murine macrophage RAW264.7 cell line, we demonstrate that pretreatment with rNodal for two minutes significantly reduces the number of phosphoproteins in RAW264.7 cells. Interestingly, two of the pathways that have decreased

phosphorylation are the NFκB and MAPK, which are known to be involved in pro-inflammatory cytokine expression. We demonstrate that Nodal prevents the phosphorylation of nuclear NFκB p65 as well as p38MAPK in response to LPS.

3.4 Results

3.4.1 Recombinant Nodal downregulates the expression of pro-inflammatory cytokines

Our previous work has shown that recombinant Nodal downregulates the expression level of several cytokines, including IL-1 β, IL-6, IL-10 and TNF-α in BMDM cells treated with lps (Ayash, et al. manuscript submitted). To further confirm the inhibitory effect of Nodal on cytokine expression, we have used the mouse macrophage cell line RAW264.7. As we have previously observed in bone marrow derived macrophages, RAW264.7 cells do not express Il1b, Il6, Il10 and Tnfa and the addition of rNodal does not increase expression levels of these genes. However, addition of LPS significantly increased pro-inflammatory cytokine expression whereas pre-treatment with rNodal prior to LPS significantly reduced expression (Figure 3.1). This result confirms that Nodal plays an inhibitory function and attenuates the expression of these cytokines in macrophage cells.

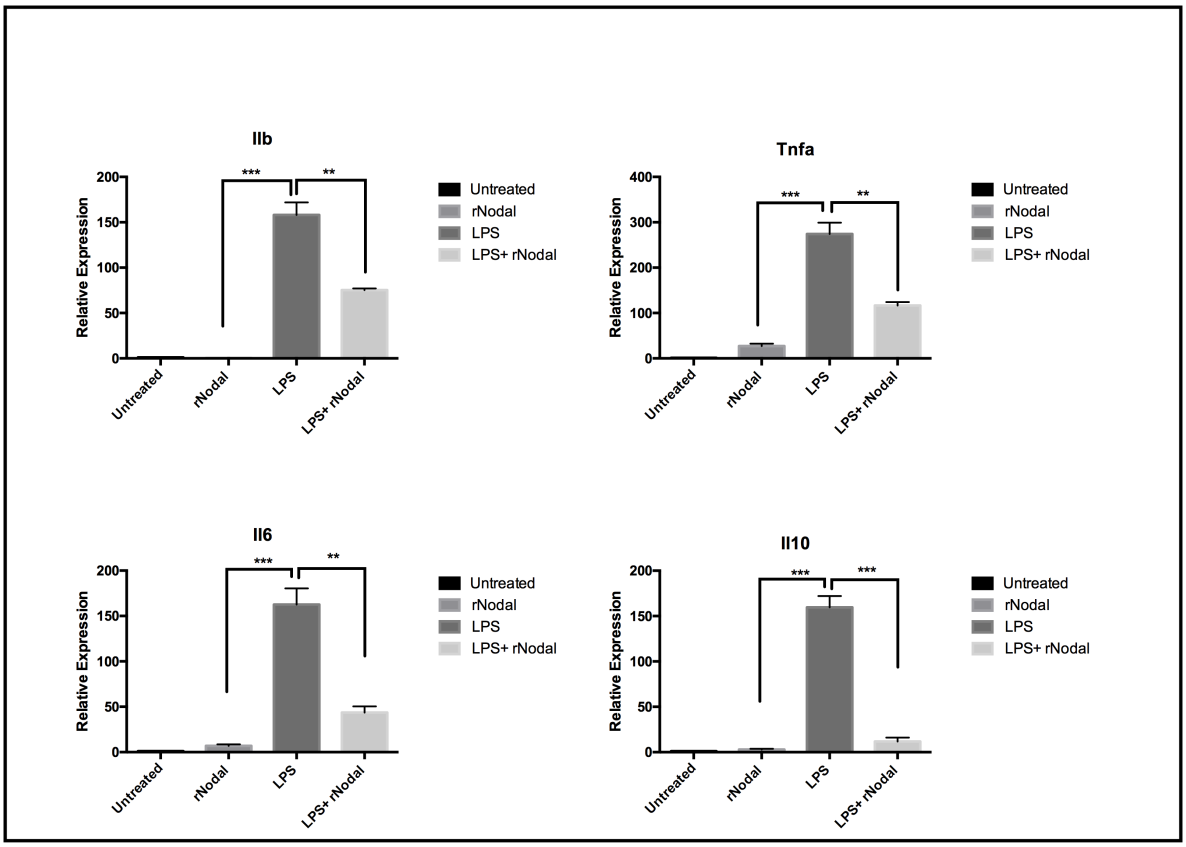


Figure 3.1 Recombinant Nodal downregulates the expression of cytokines in the macrophages cell line RAW264.7

mRNA levels of cytokines (Ilb1, Tnfa, Il6 and Il10) were low in both untreated cells and those treated with 300ng/ml of rNodal for 1h. mRNA levels of cytokines were significantly higher when cells were stimulated with 100ng/ml LPS for 6h. Cytokine levels were significantly lower when cells were stimulated for 1h with 300ng/ml of rNodal and then stimulated with 100ng/ml LPS for 6h. rNodal was able to suppress the expression of these cytokines. Three independent experiments. Data were analyzed and compared using an unpaired Student's t-test. $*P < 0.05$

3.4.2 rNodal inhibits expression of COX-2 in RAW 264.7 macrophage and BMDM cells

COX-2 expression is strongly induced in macrophages following appropriate stimulation including LPS, growth factors and several cytokines (Giroux & Descoteaux 2000). In order to investigate the role of rNodal on COX-2 in post-LPS stimulation, immunofluorescence staining using a COX-2 specific antibody was performed. COX-2 expression in RAW 264.7 cells was not detected in the untreated group nor in the rNodal stimulated group (Figure 3.2 A, B) nor was it detected in BMDM cells (Figure S3.1 A, B). Interestingly, LPS stimulation showed a significant increase in the cytosolic expression of COX-2 in both RAW 264.7 (Figure 3.2C) and BMDM cells (Figure S3.1 C). Remarkably, treating the cells for 1h with rNodal prior to the 6h incubation with LPS showed a dramatic decrease in COX-2 expression in both RAW 264.7 (Figure 3.2 D) and BMDM (Figure S3.1 J-L). Similarly, mRNA levels of COX-2 were significantly induced in response to LPS while treatment with rNodal showed a significant decrease in mRNA levels of COX-2 in both RAW 264.7 (Figure 3.1E) and BMDM cells (Figure S3.1E). Together, these data strongly underscore the inhibitory effect of Nodal on COX-2, a known marker of inflammation.

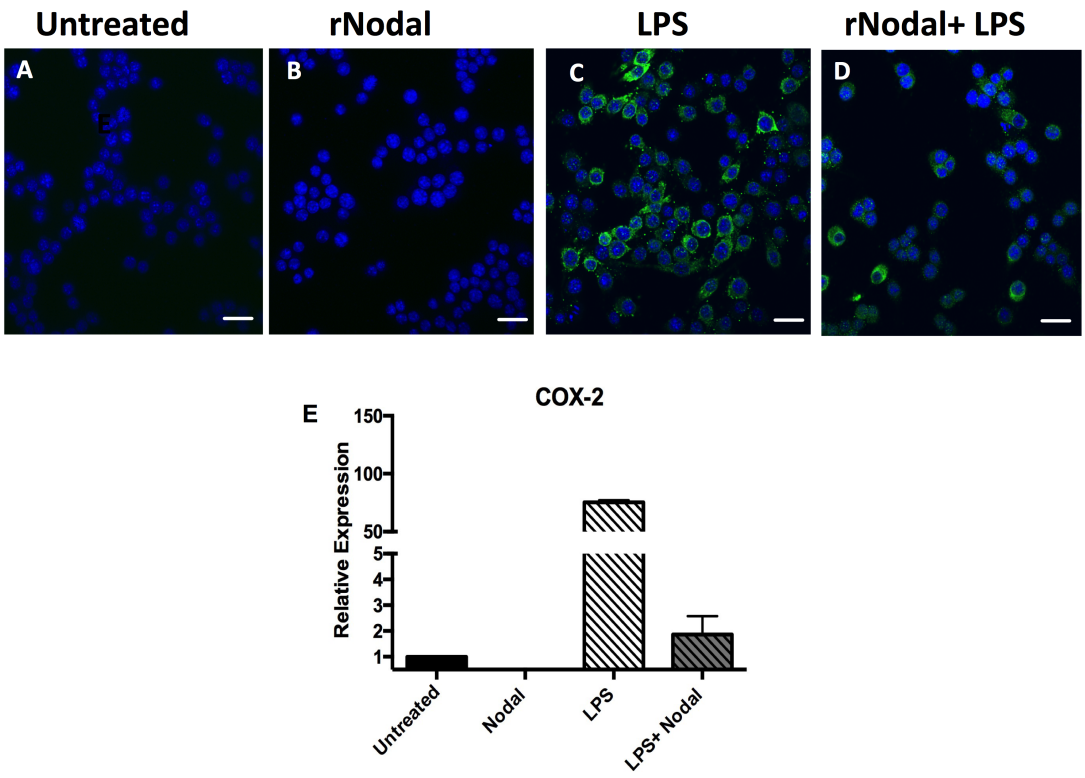


Figure 3.2 rNodal inhibited expression of COX-2 in RAW 264.7 macrophages cells
Representative images of immunofluorescence of RAW 264.7 (A&B): COX-2 expression in RAW 264.7 cells was not detected in the untreated group or in the in the 300ng/ml of rNodal incubated groups. (C): COX-2 expression significantly increased post 100ng/ml LPS stimulation. (D): COX-2 expression significantly decreased with prior treatment with 1h with 300ng/ml of rNodal prior to the 6h incubation with 100ng/ml LPS in RAW 264.7 cells. Merged images: nuclear staining for DNA (DAPI) is shown in blue and staining for COX-2 is shown in green. Scale bar = 200µm. (E): q-PCR analysis showed mRNA levels of COX-2 in RAW 264.7 were significantly induced in response to LPS while treatment with rNodal showed a significant decrease in mRNA levels. Three independent experiments. Data were analyzed and compared using an unpaired Student's t-test. $*P < 0.05$

3.4.3 Nodal significantly decreased global protein phosphorylation in RAW264.7 cells

Our findings indicate that Nodal downregulates the expression of different cytokines that are induced by LPS. In order to determine how Nodal regulates the expression of cytokines secreted by macrophages and what pathways were be associated with Nodal function we performed a proteomic analysis to identify proteins in phosphorylated response to Nodal. RAW264.7 cells were treated with rNodal for 2 minutes and we compared the level of global protein phosphorylation between the treated and non-treated control groups. A combined total of 1366 phosphorylated proteins were identified. Interestingly, 295 and 40 differentially phosphorylated proteins were present in the control and the Nodal treated groups, respectively (Figure 3.3B). The volcano plot illustrates the differential protein phosphorylation (corrected p value < 0.05) in each group. The left side of the volcano plot represents differentially phosphorylated proteins in the control group, whereas the right side represents differentially phosphorylated proteins in the Nodal treated group (Figure 3.3 A).

Additionally, we sought to determine the proteins and associated pathways that were differentially phosphorylated. Interestingly, our findings suggest that multiple pathways are affected reactivity by rNodal treatment (Figure 3.2S), whereas fewer pathways were affected positively by rNodal treatment. (Figure 3.3S).

Although spectral counting is suitable for major peptide signals, it is less reliable for peptides with lower counts. Two proteins of interest (MAP3K2 and NFκB1) were quantified using MS1 XIC (extracted ion chromatogram) methods and the Pinnacle program (optys Tech, Boston).

Figure 3.3 C,D show an example of phosphorylated peptides for both MAP3K2 and NFκB1, clearly demonstrating a decreased amount upon Nodal treatment. The top three biological repeats represent the signals from the controls while the bottom three represent the rNodal treated group (Figure 3.3C,D)

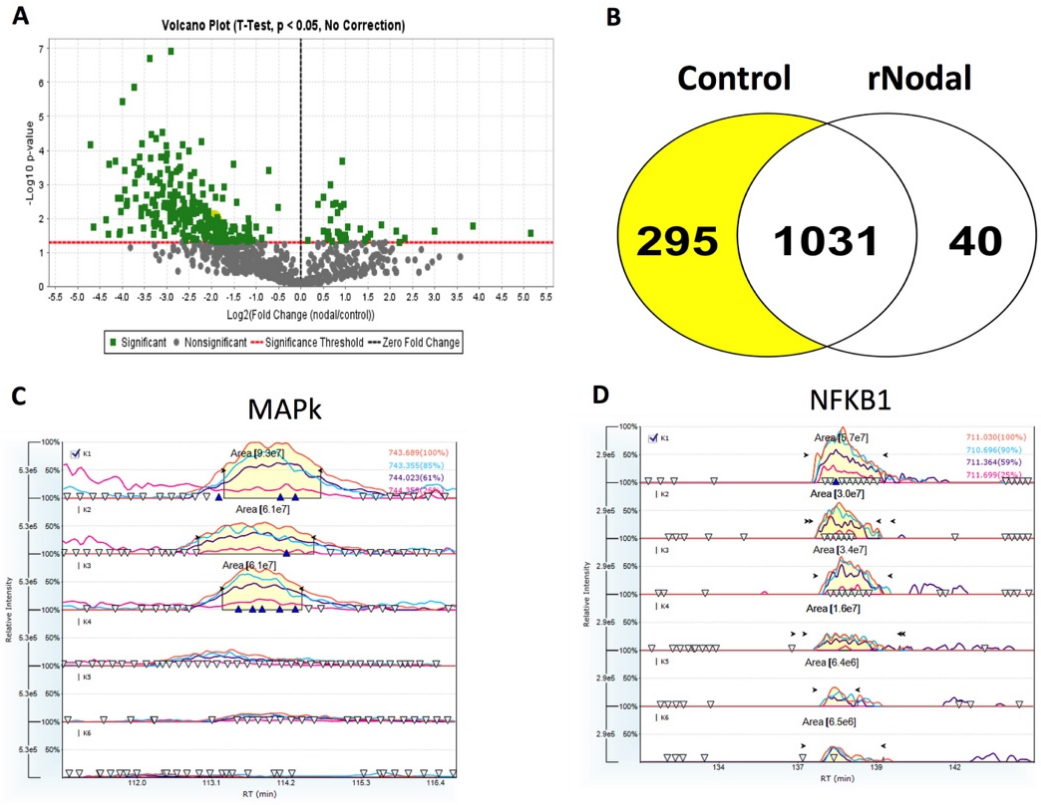


Figure 3.3Nodal significantly decreased global protein phosphorylation in RAW264.7 cells

(A): Volcano plot illustrating the differential protein phosphorylation (corrected p value < 0.05) in each group. The left side of the volcano plot represents differentially phosphorylated proteins in the control group, whereas the right side represents differentially phosphorylated proteins in the Nodal treated group (B): A Venn diagram showing combined total of 1366 phosphorylated proteins were identified. 295 unique phosphorylated proteins were upregulated in the control versus only 40 unique phosphorylated proteins were upregulated in the Nodal treated groups. (C): Phosphorylated peptide for MAP3K2 was decreased upon Nodal treatment. (D): Phosphorylated peptide for NFκB1 was decreased upon Nodal treatment. In Both MAP3K2 and NFκB1 the top three biological repeats represent the signals from the controls while the bottom three represent the rNodal treated groups. Three independent experiments.

NFkß plays an essential role in the inflammation cascade and is therefore a key player in the innate and adaptive immune systems (Ruland 2011, Tammi et al 2011). It is well known that it can induce the expression of many pro-inflammatory agents including cytokines and chemokines (Liu et al 2017). We examined the expression of the downstream NF-kB signaling pathway, including the nuclear transduction of phosphorylated NFkßp65. To address the role of Nodal in the regulation of the NF-kB signaling pathway, we examined the phosphorylation state of NFkß-p65 by immunofluorescence. Using an antibody that specifically recognized the phosphorylated state of NFkß-p65, we detected low levels of phosphorylated NFkß-p65 in both the untreated control and rNodal treated groups. (Figure 3.4C, F). In contrast, addition of LPS resulted in high levels of phosphorylated NFkß-p65. (Figure 4I). Interestingly, incubation of the cells with rNodal for 15 mins prior to the addition of LPS significantly decreased the levels of phosphorylated NFkß-p65 (Figure 3.4L). The immunofluorescence was quantitated using ImageJ software (Figure 3.4M). As a control, we used an antibody the recognizes total NFkß-p65 and found that the expression of the total NFkß-p65 was equal in all the groups mentioned above (Figure 3.5C, F, I, and L) as quantitated using ImageJ software (Figure 3.5M). Altogether, these findings highlight a clear, inhibitory role for Nodal in the NFkß pathway by decreasing the phosphorylation of NFkß-p65 in response to LPS.

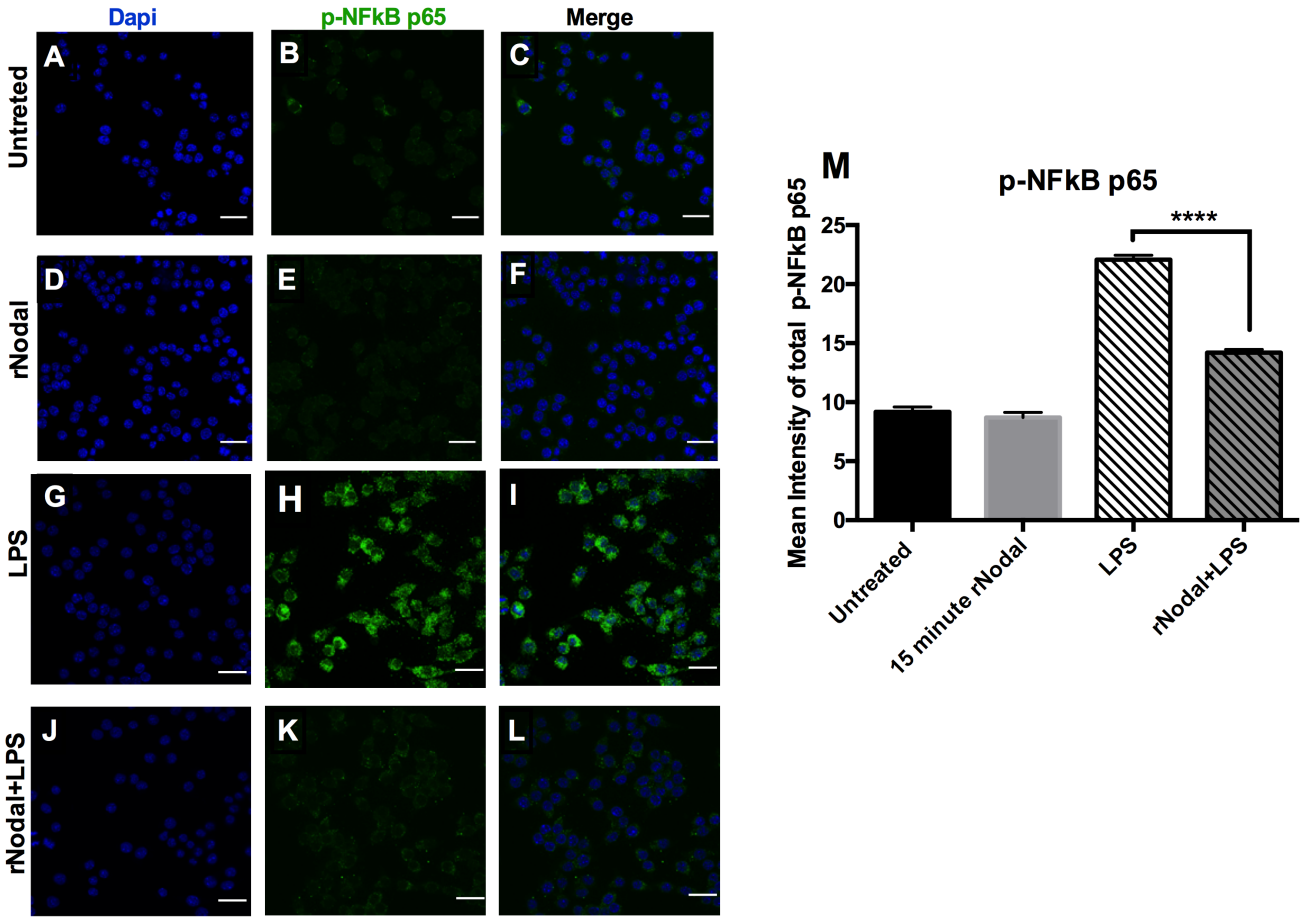


Figure 3.4rNodal significantly decreased phosphorylated nuclear NFkßp65 in RAW264.7 cells

Representative immunofluorescence images of RAW 264.7 cells **(A-C):** Untreated control group in RAW 264.7cells showed low levels of phosphorylated NFkß-p65 **(D-F):** 300ng/ml of rNodal was used to treat group in RAW 264.7cells showed low levels of phosphorylated NFkß-p65 **(G-I):** High levels of phosphorylated NFkß-p65 post LPS stimulation **(J-L):** Significant decrease in the levels of phosphorylated NFkß-p65 when cells incubated with rNodal for 15 mins prior to the addition of LPS 30 min. nuclear staining for DNA (DAPI) is shown in blue and staining for phosphorylated NFkß-p65 is shown in green. Scale bar = 200µm. **(M)**: immunofluorescence quantification using ImageJ software showed phosphorylated NFkß-p65 significantly induced in response to LPS while treatment with rNodal showed a significant decrease NFkß-p65 level in

RAW 264.7 cells. Three independent experiments. Data were analyzed and compared using an unpaired Student's t-test. $*P < 0.05$

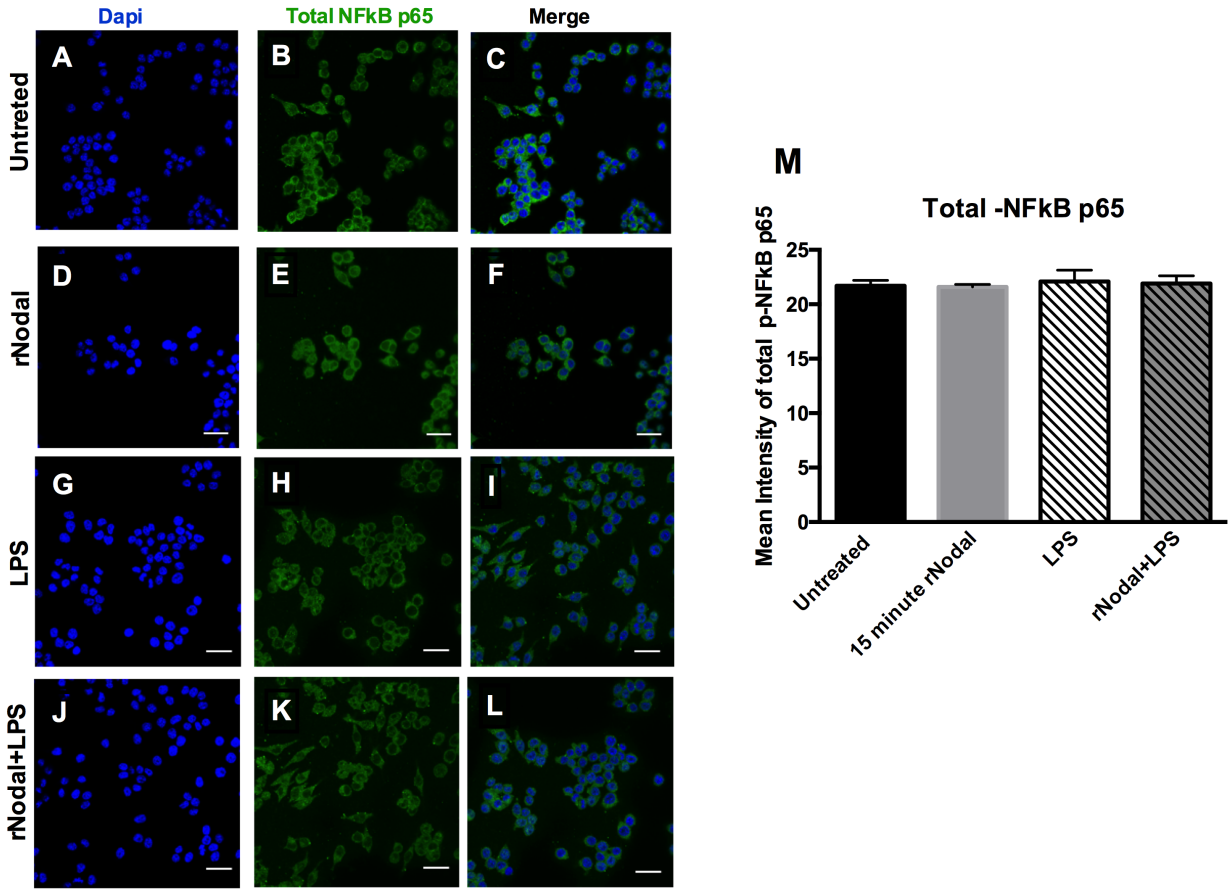


Figure 3.5rNodal effect on levels of total nuclear NFkß p65 RAW264.7 cells
Representative immunofluorescence images of of RAW 264.7 **(C,F,I, and L):** Merged images for the expression of the total NFkß-p65 was effect on Untreated, with 300ng/ml rNodal treated, 100ng/ml of LPS treated and incubated with 300ng/ml rNodal for 15 mins prior to the addition of 100ng/ml of LPS for 30 min **(A,D,G, and J):** nuclear staining for DNA (DAPI) is shown in blue for all the groups. **(B,E,H, and K):** Staining for total NFkß-p65 is shown in green in RAW 264.7cells. Scale bar = 200µm. **(M)**: immunofluorescence quantification using ImageJ software showed total NFkß-p65 was effect on all the groups mentioned above. Three

independent experiments. Data were analyzed and compared using an unpaired Student's t-test. *$P < 0.05$

3.4.4 rNodal significantly decreased phosphorylated p38 MAPK

MAPK signaling has also been implicated in the modulation of the inflammatory system including cytokine production (Zhang & Dong 2005), To further investigate the role of rNodal in modulating the MAPK pathway, we examined the phosphorylation of p38 as previously done for phosphorylated NFkß-p65. Using an antibody that specifically recognized phosphorylated p38 we found low levels in both the untreated control and rNodal treated groups (Figure 3.6C, F) and addition of LPS for 30 min resulted in high levels of phosphorylated p38 (Figure 3.6I). Similar to what was observed for phosphorylated levels of NFkß-p65, incubation of the cells with rNodal for 15 mins prior to the addition of LPS significantly decreased the levels of phosphorylated p38 (Figure 3.6L). The immunofluorescence was quantified using ImageJ software Figure 3.6M). On the other hand, we used the total p38 antibody to stain the macrophages cell line. Using an antibody that recognizes total p38, we found no change in the total levels of p38 (Figure 3.7C, F, I, and L and M). Taken all together, our findings indicate that Nodal attenuates the LPS-induced inflammatory response through both the MAPK and NFkß pathways. Thus, it appears that Nodal plays an important role in preventing the activation of these pathways and, therefore, in blocking the onset of the inflammatory response.

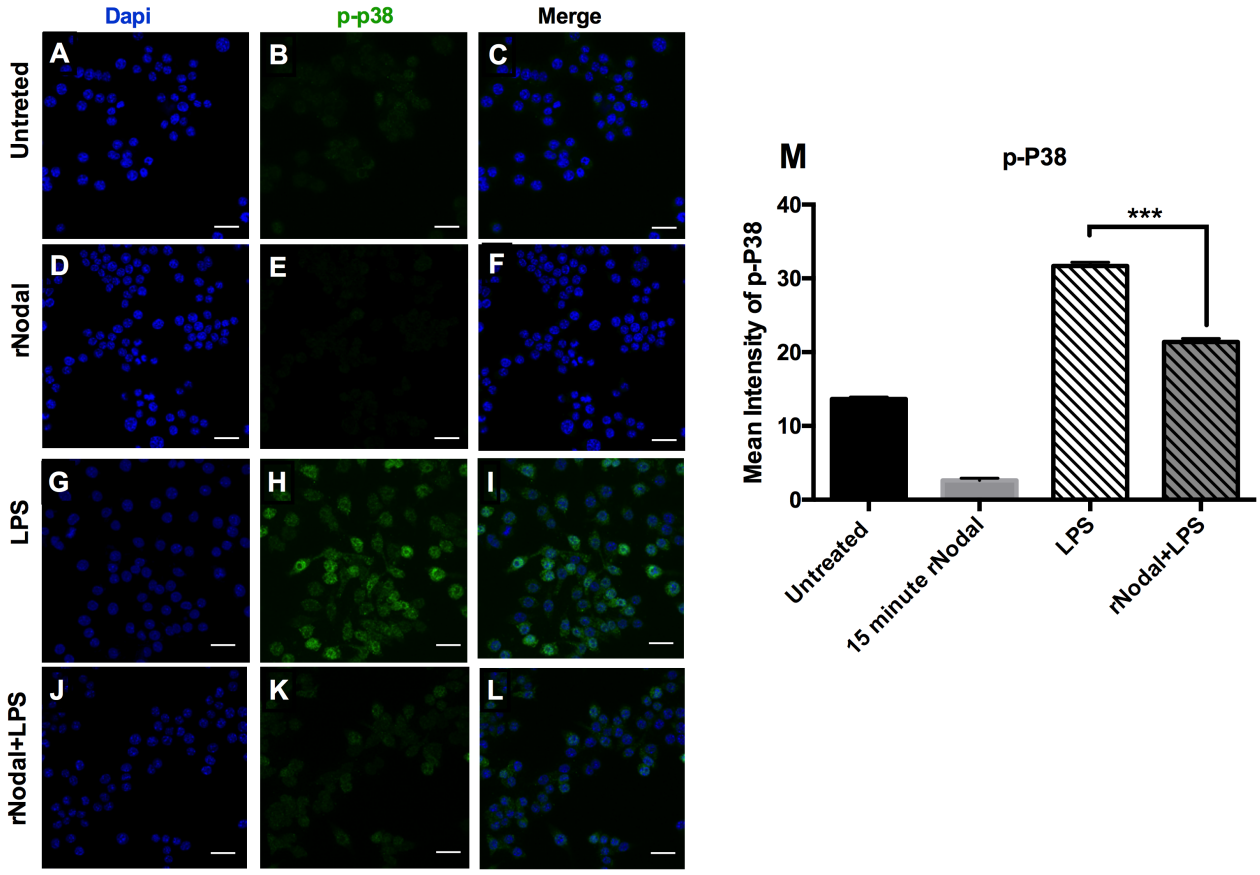


Figure 3.6 rNodal significantly decreased phosphorylated p38MAPk in RAW 264.7 cell
Representative immunofluorescence images of of RAW 264.7 cells (A-C): Untreated control group showing low levels of phosphorylated p38MAPK. (D-F): 300ng/ml rNodal was used to treat group showing very low levels of phosphorylated p38MAPK (G-I):) High levels of phosphorylated p38MAPK post 100ng/ml LPS stimulation (J-L): Significant decrease the levels of phosphorylated p38MAPK when cells were incubated with 300ng/ml rNodal for 15 mins prior to the addition of 100ng/ml LPS 30 min. (M): immunofluorescence quantification using ImageJ software. Nuclear staining for DNA (DAPI) is shown in blue and staining for phosphorylated p38MAPK is shown in green. Scale bar = 200µm. Three independent experiments. Data were analyzed and compared using an unpaired Student's t-test. $*P < 0.05$

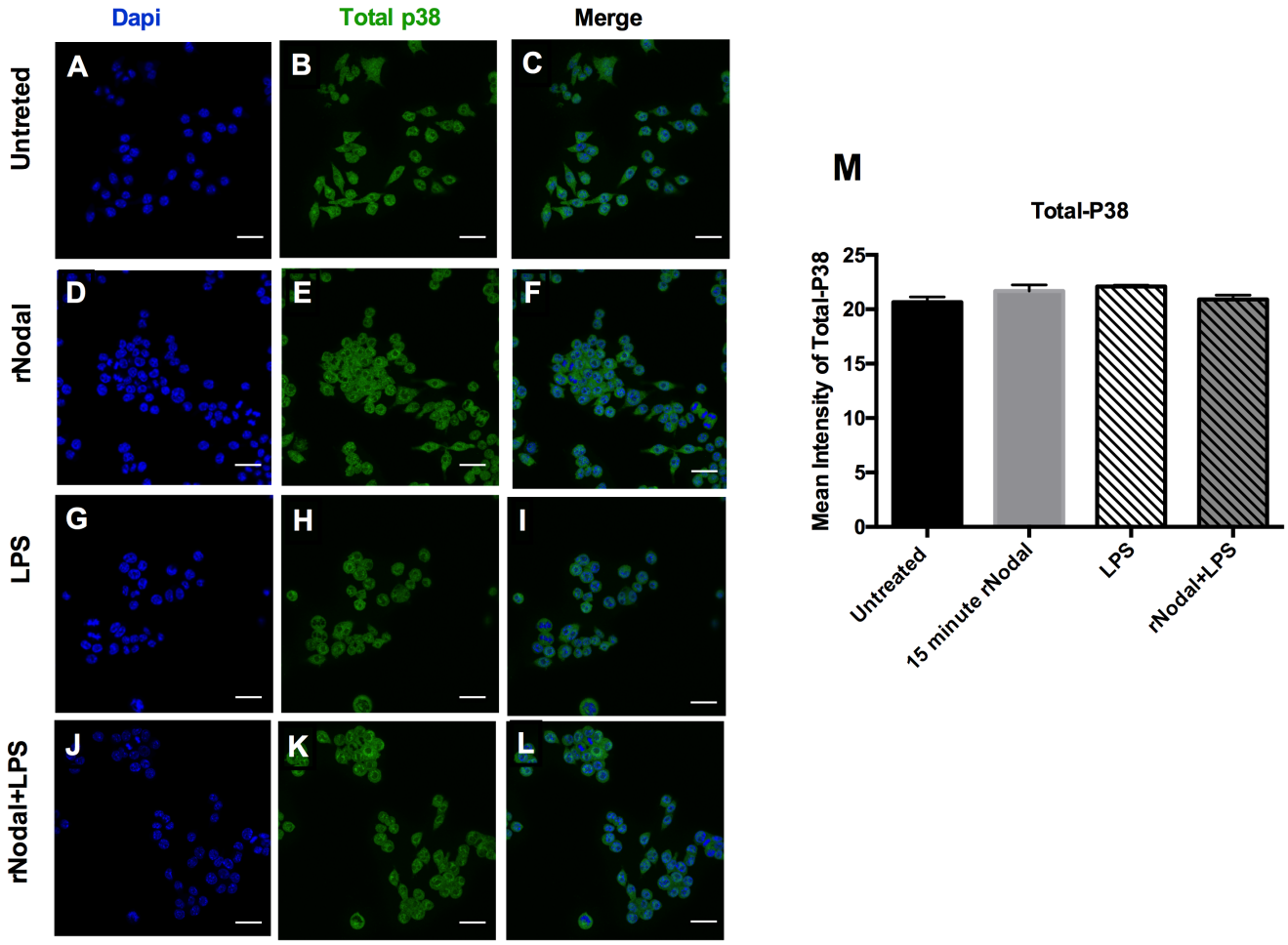


Figure 3.7 rNodal effect on levels of total p38MAPK in RAW264.7 cells
Representative immunofluorescence images of of RAW 264.7 **(C,F,I, and L):** Showed Merged images for the expression of the total p38 was effect on in untreated, with 300ng/ml rNodal treated, with 100ng/ml LPS treated cells incubated with 300ng/ml rNodal for 15 mins prior to the addition of 100ng/ml LPS 30 min groups respectively. **(A,D,G, and J):** nuclear staining for DNA (DAPI) is shown in blue for all the groups. **(B,E,H, and K):** showed staining for total p38 is shown in green in RAW 264.7cells. Scale bar = 200µm. **(M)**: immunofluorescence quantification using ImageJ software showed total p38 was effect on all the groups

mentioned above. Three independent experiments. Data were analyzed and compared using an unpaired Student's t-test. $*P < 0.05$

3.5 Discussion

In our current study using RAW264.7 macrophage cells, we demonstrate that Nodal plays an essential anti-inflammatory role by downregulating the phosphorylated state of key member of the NFκB and MAPK pathways. We have shown that treating macrophages with rNodal prior to LPS inflammatory challenge results in a significant decrease in the production of different pro-inflammatory cytokines, including IL-1ß, IL-6, IL-10, and TNF-α and COX-2 at the mRNA level. Using proteomics analysis, we found that Nodal significantly decreased the phosphorylation of multi proteins after treating the macrophages with rNodal for two minutes compared to untreated macrophages. Most importantly, we showed that treating with rNodal prior to LPS results in a significant decrease in the phosphorylation of NFκB p65 and MAPK p38 in RAW264.7 macrophages.

Previous studies have shown that the TGF-ß superfamily exerts a variety of vital functions in the immune system as these factors alter the immune system under different circumstances (Sanjabi et al 2017). A different of aspect immunology regulation, for instance, TGF-ß has been found to suppress IL-12 secretions in T cells (Brabletz et al 1993) and it has been implicated in regulating the activation, survival and proliferation of B cells and is involved in the growth and function of other innate cells (Li et al 2006). TGF-ß has also been shown to inhibit the function and development of NK cells (Marcoe et al 2012) and prevents the pro-inflammatory effect of macrophages triggered by cytokines or other inflammatory mediators (Li et al 2006).

There is currently very little that is known on the role of Nodal in immune modulation. One study has shown the Nodal is involved in macrophage polarization and regulation and has been shown to downregulate the production of IL-12 in macrophages (Wang et al 2014). Similarly, Cripto, the co-receptor of NODAL, has also been implicated in modulating macrophage cytokine production, including IL-1ß, IL-6, IL-10, and TNF-α (Zhang et al 2016). Notably, in agreement with previous studies, our lab shown that Nodal is involved in the modulation of the immunological state during pregnancy as Nodal heterozygous mice have significantly higher basal levels of pro-inflammatory cytokines and chemokines compared to the control mice (Ayash, et al. manuscript submitted). Moreover, pre-treatment of bone marrow-derived macrophages (BMDM) with rNodal before exposing them to LPS showed a dramatic decrease in the levels of various cytokine including IL-1ß, IL-6, IL-10, and TNF-α. Ultimately, these results support our current findings of the anti-inflammatory role of Nodal in preventing cytokine production in macrophages. This could open a new avenue in using Nodal as an anti-inflammatory agent in many therapeutic areas.

Studies have shown that Nodal, like other members of the TGF-ß superfamily, signal through the heteromeric receptor and Smad complexes (known as the SMAD depending signaling pathway). However, Nodal can also activate many Smad-independent signaling pathways including ERK, JNK, p38, PI3K and AKT (Derynck & Zhang 2003, Massague 2012). Our proteomic data showed that Nodal significantly decrease the phosphorylated pathways state of key member of NFκB and MAPK. Studies have demonstrated that both NFκB (Yoon et al 2010) and MAPK pathways are critical players in inflammatory events (Hommes et al 2003, Yoon et al 2010). Specifically, our current findings highlight significant decreases in phosphorylation of both NFκB p65 and MAPKp38 when macrophages are treated with rNodal, prior to LPS exposure.

Our study has shown novel role for Nodal in decreasing the phosphorylated state of multiple proteins. Also, our results suggest a role for Nodal as an anti-inflammatory by signaling through two non-Smad signaling pathways (NFκB and MAPK). Further studies are required to determine how Nodal prevents phosphorylation. we showed also check the upstream and downstream components for both pathways to confirm after precisely Nodal in of Nodal through pathways.

3.6 Material and Methods

3.6.1 Macrophage cell line culture

RAW 264.7 macrophage cell line (a generous gift from Dr. Marianna Orlova, McGill University Health Center). Cells were maintained in complete medium composed of DMEM (Multicell Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Wisent Cat No. 80150) and 1% penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere.

3.6.2 Bone Marrow Derived Macrophage (BMDM) Isolation

BMDM were isolated from wild-type CD1 mice aged 6-8 weeks. Briefly, after mice were sacrificed and disinfected with 70% ethanol, bone marrow was flushed from the femurs. The cells were cultured in RPMI medium without antibiotics, 10% fetal bovine serum (FBS) and L929 cell condition medium (macrophage colony-stimulating factor). At day 4 in culture, RPMI medium with antibiotics, 10% fetal bovine serum (FBS) and L929 was added to the cells. Cells were at 37°C incubator with a 5% CO2 atmosphere. At day 6 in culture, cells were washed; the adherent BMDM cells were plated in a plate at density of 1X106 cells/well, after day 7 in cells differentiated to a mature macrophage phenotype.

3.6.3 Macrophage cell line and RAW 264.7 Treatment, RNA Isolation and Q-PCR

RAW 264.7 cells were grown to confluence in an appropriate complete medium containing 10%FBS. (1) Cells were cultured for 1h in medium alone, (2) treated 1h with 300ng/ml of rNodal only (R&D Systems Cat No. 13-15-ND), (3) stimulated with 100ng/ml LPS only for 6h, (4) or 1h with 300ng/ml of rNodal then stimulated with 100ng/ml LPS for 6h. Cells were then lysed in 1 ml of Trizol (Thermofisher Cat No. 15596026), and the RNA extracted using an RNeasy Mini Kit (Qiagen Cat No. 74104), cDNA was synthesized using the Qiagen RT2 First Strand Kit (Qiagen Cat No. 330404). Real-Time PCR was performed using the Roter-Gene SYBR Green PCR Kit (Qiagen Cat No. 204074) as described by the manufacturer's protocol.

3.6.4 Cell Preparations for Proteiomics analysis

RAW 264.7 cells were grown to confluence in complete medium containing 10%FBS. On ice, cells were washed gently with ice cold PBS, then carefully scraped with a cell scraper in 1 ml ice cold PBS. Dispersed cells in PBS were transferred into an Eppendorf tube and spun for 3 minutes at 300 rpm at 4°C. Supernatant was aspirated and discarded; cell pellets were sent for proteomic analysis. Cells were analysed via liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS) at the Proteomic Platform of the Research Institute of McGill University Health Center.

3.6.5 uHPLC-MS/MS Parameters:

After running on an SDS PAGE stacking gel, the peptides were reduced, alkylated and digested. Phosphopeptides were enriched with TiO2 beads (GL Sciences) using the method of

Thingholm et al 2006, and loaded onto a Thermo Acclaim Pepmap (Thermo, 75uM ID X 2cm C18 3uM beads) precolumn. This was followed by an Acclaim Pepmap Easyspray analytical column (Thermo, 75uM X 15cm with 2uM C18 beads) separation using a Dionex Ultimate 3000 uHPLC at 220 nl/min with a gradient of 2-35% organic (0.1% formic acid in acetonitrile) over 3 hours . Peptides were analyzed using a Thermo Orbitrap Fusion mass spectrometer operating at 120,000 resolution.

The raw data were converted into *.mgf format (Mascot generic format) searched using Mascot 2.3 against Uniprot mouse sequences. The database search results were loaded onto Scaffold Q+ Scaffold_4.4.8 (Proteome Sciences) for spectral counting, statistical treatment and data visualization. Some peptides were quantified using Pinnacle (Optys tech) method " Targeted Quantification - Label Free DDA" by combining the Mascot*dat files with the Thermo *.raw files inside Pinnacle.

3.6.6 Getting proteomics data into the Reactome

Peptide spectral counting data (from Scaffold) or peptide MS1 XIC data (from Pinnacle) were exported into Excel as a simple list of protein + amount (spectral counts or MS1 integrated counts). To help provide better visualization of changes between samples, all data were converted to a log scale. Data were cut and pasted onto the Reactome online Pathway Analysis Tool (http://reactome.org/PathwayBrowser/#TOOL=AT).

3.6.7 Immunofluorescence

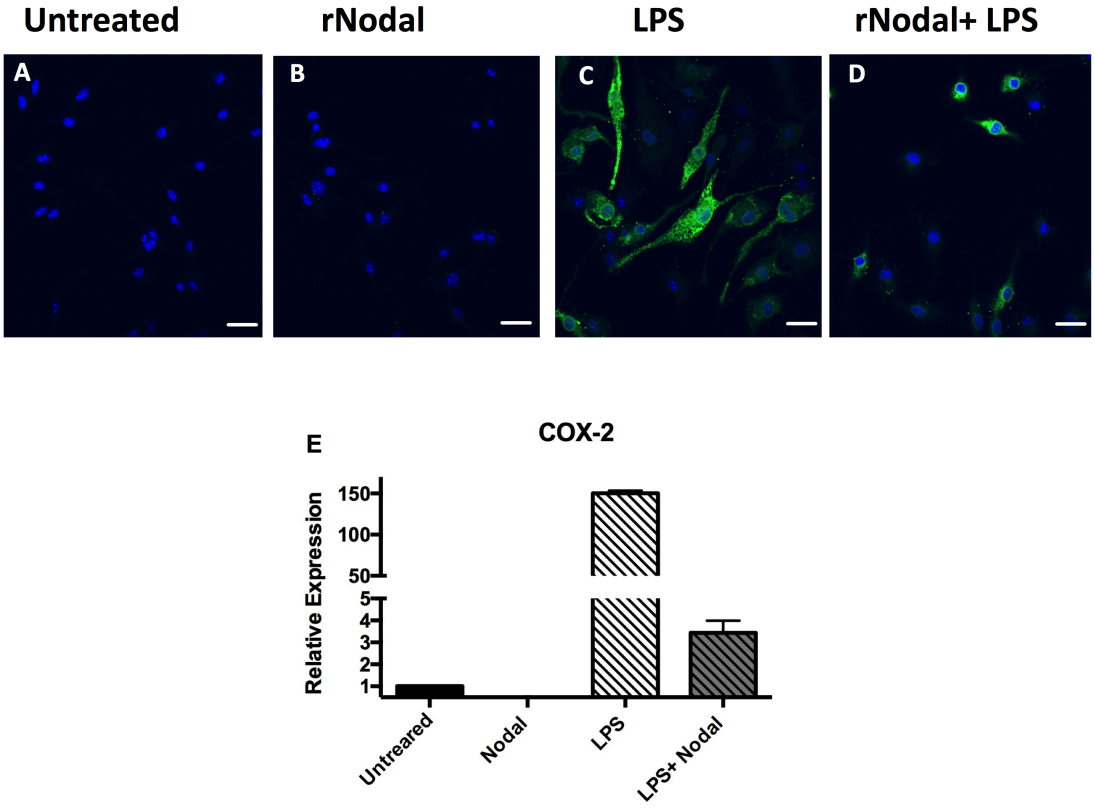
RAW 264.7 cells were grown on coverslips for 24h. Cells were washed with 1X PBS, then fixed for 15 min with 4% PFA at room temperature, followed by 5 min permeabilization with 0.1% Triton-X100 in 1X PBS. Cells were then washed with PBS containing 0.1% Triton-X100 three

times then blocked with 5% heat-inactivated goat serum in PBS for 1 hour at room temperature. Macrophage cells were subsequently immunostained overnight with the following primary antibodies at 4°C :Cox-2 (Cayman; 1:200, Cat No. aa584-598), p-NFkB-p65 (Cell signaling 1:200, Cat No. 3033), total NFkB-p65 (1:200, Santa Cruz Cat No. SC-800), p-p38 (1:200, Cell Signaling Cat No. 9211), or total p38 (Cell Signaling; 1:250 Cat No.8690). Cells were then washed several times in PBS containing 0.1% Tween-20 and incubated with Alexa Fluor 488 secondary antibody (1:100, Invitrogen Cat No. A-11008), and Dapi for 1h at room temperature. Coverslips were washed and mounted on slides with Mowiol 4-88 and stored at 4°C. Confocal microscopy was performed using Zeiss LSM 780 confocal microscopy.

3.6.8 Statistics

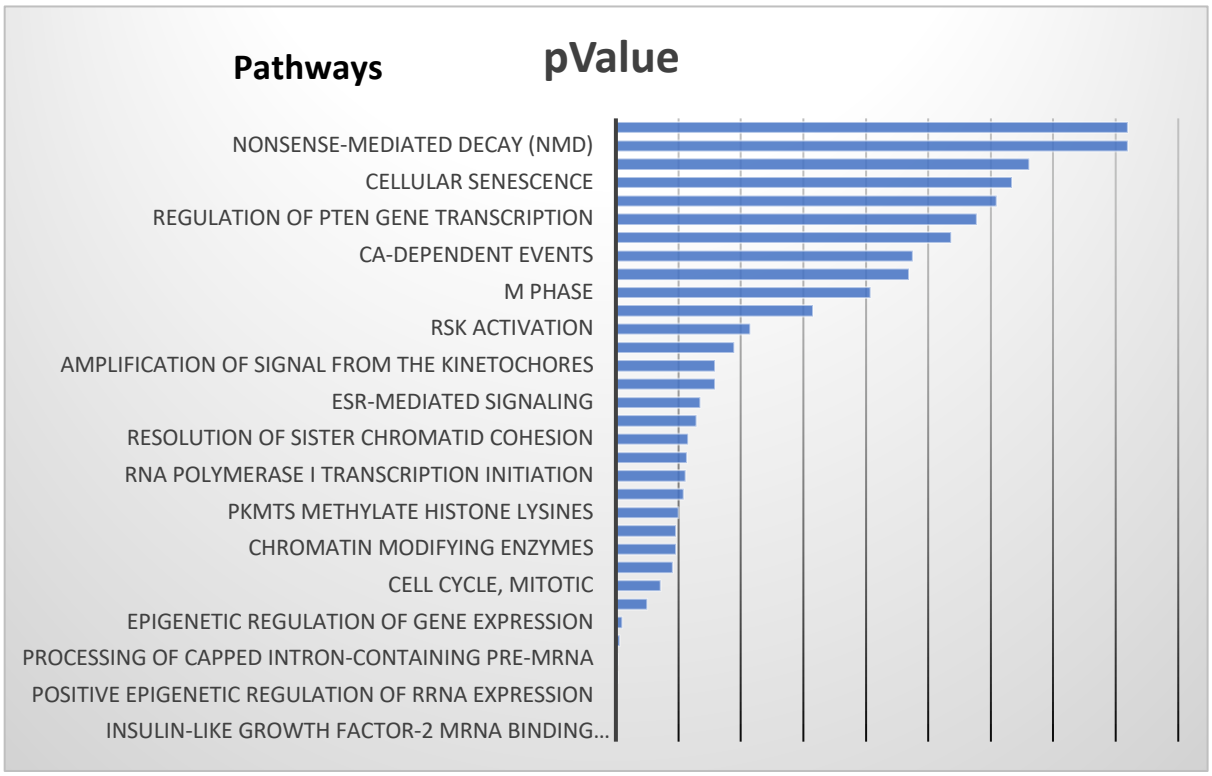
Statistical analyses of differences between experimental groups were performed using analysis of two-sided Student's *t*-test, or Chi-square (χ^2) tests using GraphPad Prism 6 software. Data represent mean ± SEM. Differences were considered significant if *P*-value ≤ 0.05.

3.6.9 Supplemental Material

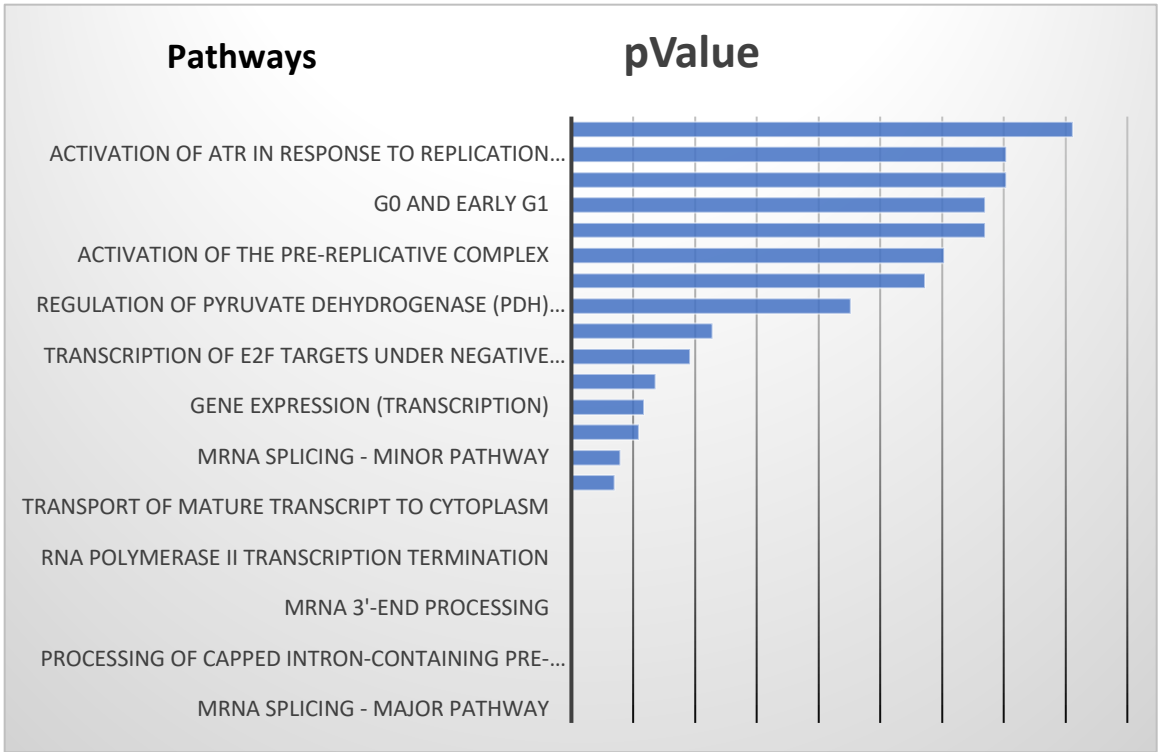


Supplementary Figure 3.1 rNodal inhibited expression of COX-2 in BMDM cells

(A & B): COX-2 expression in BMDM cells was not detected in the untreated group nor in the rNodal stimulated group. (C): COX-2 expression significantly increased post LPS stimulation. (D): COX-2 expression significantly decreased with 1h of rNodal incubation prior to the 6h incubation with LPS in BMDM cells. (E): q-PCR analysis showed mRNA level of COX-2 in BMDM cells. Merged images: nuclear staining for DNA (DAPI) is shown in blue and staining for COX-2 is shown in green. Scale bar = 200µm. Three independent experiments. Data were analyzed and compared using an unpaired Student's t-test. $*P < 0.05$



Suplementary Figure 3.2Bar chart showing the multiple pathways upregulated in control cells (RAW264.7 cells) Data were analyzed and compared by p values *P < 0.05



Supplementary Figure 3.3 Bar chart showing few pathways were upregulated when RAW 264.7 cells treated by rNodal. Data were analyzed and compared by p values *P < 0.*05

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Chapter 4: Evidence of a gene-environment interaction of *NODAL* variants and inflammation in preterm birth

Evidence of a gene-environment interaction of *NODAL* variants and inflammation in preterm birth

Lisa M. Starr, PhD, Department of Obstetrics and Gynecology, McGill University, Montreal, Quebec, Canada

Taghreed Ayash, MSc, Division of Experimental Medicine, McGill University, Montreal, Quebec, Canada

Daniel Dufort, PhD, Departments of Obstetrics and Gynecology and Division of Experimental Medicine, McGill University, Montreal, Quebec, Canada

Corresponding author: Daniel Dufort, Research Institute of the McGill University Health Centre, Glen Site, EM0.3211, Montreal, Quebec, Canada. Work phone: 1-514-934-1934 x34743. E-mail: daniel.dufort@mcgill.ca

Lisa.starr@mail.mcgill.ca , Taghreed.ayash@mail.mcgill.ca

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4.1 Preface

In our previous study, we identified the role of Nodal in preterm birth in mice. Significantly, we found that the loss of one Nodal allele results in increased susceptibility to inflammation. Which, in turn induces preterm birth. Specifically, Nodal heterozygous mice were found to have higher basal levels of cytokine and chemokines in maternal decidua tissue compared to controls. Additionally, they showed more macrophages compared to control mice. In this chapter, we investigate the link between NODAL mutations and preterm birth in the human population.

Employing a secondary analysis of a nested case-control study from a prospective birth cohort study, we determined the association between preterm birth and the genetic variants of the NODAL gene. We showed that there is no association between NODAL SNPs and preterm birth; however, when we included bacterial vaginosis (BV) or placental inflammation (PI) in our models, interestingly we uncovered that three SNPs had a differential effect on preterm birth risk coupled conditionally with the presence of infection or inflammation. In particular, rs2231947 increased the risk of preterm birth for women diagnosed with BV. In contrast, rs1904589 increased the risk of preterm birth in women who did not have placental inflammation, while rs10999338 decreased the risk of preterm birth in women who did have placental inflammation.

4.2 Abstract

Objective: NODAL has been implicated in timing of parturition and immune regulation. We investigated the relationship between *Nodal* polymorphisms, infection/inflammation, and preterm birth.

Study Design: In this secondary analysis, 613 women (189 preterm and 424 term) from the Montreal Prematurity Study were genotyped for *NODAL* polymorphisms and assessed for bacterial vaginosis and placental inflammation.

Result: *NODAL* polymorphisms were not associated with preterm birth. However, the rs2231947 variant allele was associated with increased risk for preterm birth among women with bacterial vaginosis (odds ratio 2.76, 95% confidence interval 1.12−6.85). Among women without placental inflammation, the rs1904589 variant allele was associated with increased risk of preterm birth (odds ratio 1.31, 95% confidence interval 1.02−1.70). Among women with placental inflammation, the rs10999338 variant allele was associated with reduced risk of preterm birth (odds ratio 0.50, 95% confidence interval 0.29−0.87).

Conclusion: The effect of *NODAL* polymorphisms on preterm birth depends on maternal infection/inflammation status.

Keywords: Bacterial vaginosis, gene-environment interaction; genetic association study; bacterial vaginosis; Nodal; placental inflammation; preterm birth; single nucleotide polymorphisms

4.3 Introduction

Preterm birth, defined as delivery before 37 weeks of gestation, is a major public health issue that affects up to 12% of pregnancies worldwide and accounts for approximately 75% of all neonatal deaths.[1,2] Premature babies that survive are at an increased risk of developing neurodegenerative disorders, including cerebral palsy, learning disabilities, impaired vision, and behavioural and emotional problems.[3] However, the mechanisms that underlie preterm birth remain largely unknown.

Growing evidence suggests that infection and/or inflammation may be a key mechanism leading to preterm birth.[4,5] Similar to the process of normal parturition, intrauterine infections stimulate the localized release of cytokines, including IL-1β, TNF-α, IL-6, and IL-8 by the decidua and fetal membranes. This results in the production of prostaglandins, chemokines, and metalloproteases and stimulates uterine contractions and membrane rupture.[5] However, the predictive value of infection for preterm birth is highly variable and differs among populations. For example, only a subset of the 15% to 20% of pregnant women who are diagnosed with bacterial vaginosis (BV) is at risk for preterm birth.[6]

Variability in susceptibility to infection-induced preterm birth may be related to genetic predispositions. Polymorphisms that amplify inflammatory responses have been associated with preterm birth, including in Selenoprotein S (*SEPS1*)[7] and *TNFA*[8] genes. Genetic factors may also influence the response to the environment. For example, synergistic effects of *TNF* variants and BV on preterm birth have been reported by Macones et al.[9] Thus, analyzing the interaction of genetic and environmental factors such as maternal infection could improve the ability to predict preterm birth.

NODAL, a morphogen in the TGF-β superfamily, plays a critical role during embryonic development and has been implicated in the timing of parturition in mice.[10,11] In the mouse, *Nodal* is expressed throughout the uterus during early pregnancy[12] and this expression pattern is conserved in the human endometrium.[13] NODAL has been implicated in many events associated with mammalian reproduction, including implantation, placentation, and parturition.[14] In conditional uterine *Nodal* knockout mice approximately 80% of mice who become pregnant give birth prematurely (day 17.5 rather than day 19.5).[15] Components of the NODAL signalling pathway have also been linked to regulation of the immune system. Both NODAL and its co-receptor CRIPTO polarize cultured macrophages.[16,17] In our laboratory, we have demonstrated that pregnant conditional uterine *Nodal* heterozygous knockout mice have higher basal cytokine levels and are susceptible to preterm birth in response to low doses of lipopolysaccharide that do not cause preterm birth in control mice (unpublished data). However, the role of NODAL during human pregnancy has not been determined.

Since NODAL has been associated with the timing of parturition in mice, we tested whether variation in the human *NODAL* gene is associated with preterm birth. Given the suggested role of NODAL in regulating immune cell function, we hypothesized that *NODAL* single nucleotide polymorphisms (SNPs) increase susceptibility to inflammation-induced preterm birth. We examined this hypothesis using validated data on placental inflammation and BV in pregnant women from the Montreal Prematurity Study (MPS).[18]

4.4 Materials and Methods

This was a secondary analysis of data collected by MPS,[18] which combined a prospective birth cohort study with a nested case-control design study and focused on characteristics and risk factors of preterm birth. The study recruited women ≥ 18 years who were pregnant with a singleton fetus and had no chronic illness at 8−24 weeks of gestation at four hospitals in the Montreal Census Metropolitan Area between October 1999 and April 2004. Subjects with preeclampsia or indicated preterm birth were excluded. Data and samples collected at the initial visit, at 24−26 weeks of gestation and after delivery were used in the present analysis. During data collection, participants self-reported their ethnicity as European, African/Caribbean, Asian, Middle Eastern, or Latin American. Preterm birth was classified as spontaneous delivery before 37 completed weeks (based on the last menstrual period if confirmed ± 7 days by early ultrasound, otherwise by ultrasound estimate).[19] From the larger cohort, 613 participants, 189 of whom had preterm birth, were included in our analysis. The current analysis was approved by the Research Ethics Board of the McGill University Health Centre, Canada (approval #14-062-PED). Informed consent was obtained from all women and patient records/information were anonymized and de-identified prior to analysis.

Women were assessed for BV for a previous study.[20] Briefly, vaginal swabs were obtained by a research nurse at 24−26 weeks of gestation from the posterior fornix. Smears were prepared by rolling the swab on a slide, air-drying, fixing in methanol, and freezing at -70°C. Swabs were processed using a standard Gram stain and assessed at a clinical microbiology laboratory for the presence of BV using the Nugent criteria.[21] Inter-observer agreement for the assessment of these samples was found to be high using these criteria.[20]

Data on the presence of placental inflammation in the MPS population were obtained for a previous study.[22] Briefly, three transmural sections of 3 mm thickness (near the insertion of the umbilical cord, near a placental margin, and mid-way in-between) were cut from fresh, delivered placentas and assessed by a placental pathologist for inflammation (membrane inflammation, and/or funisitis, and/or umbilical cord vasculitis). As previously reported, these assessments had moderate to high intra- and inter-observer agreement.[22] Previous analysis of MPS data showed that neither BV nor placental infection/inflammation were associated with preterm birth (< 37 weeks), although placental infection/inflammation was associated with both early (< 34 weeks) and very early (< 32 weeks) birth.[23]

Maternal genomic DNA was isolated from peripheral blood samples and genotyped by Sanger sequencing at the McGill University and Genome Quebec Innovation Centre. Primer sequences are listed in Table S1. Variant alleles were assigned based on the reference genome (NCBI NM_018055.4). Variant allele frequency (VAF) was calculated as the relative frequency of the variant allele within the population. SNPs with VAF > 1% were selected for further analysis.

Population structure of genetic variation in *NODAL* was assessed using publicly available data from the 1000 Genomes Project, a catalogue of genetic variants in the genomes of healthy individuals,[24] from ethnic groups represented by our study cohort: African, Native American, European, East Asian, and South Asian. Hardy-Weinberg equilibrium (HWE) was tested for each SNP in MPS and across combined and individual populations in the 1000 Genomes Project using Chi-square (χ^2) tests. Weir and Cockerham's fixation index (F_{ST}) were calculated as a weighted average across all populations from the 1000 Genomes Project to determine genetic difference between populations.

Demographic characteristics were compared in term and preterm groups using Student's T-test or χ^2 tests. For genetic analysis, χ^2 or Fisher's exact tests were used to determine the association between the four common *NODAL* SNPs (rs2231947, rs1904589, rs10999338, and rs74139636) and preterm birth under different inheritance models: genotypic (genotype as a 3-level categorical variable), dominant (genotype as a 2-level categorical variable; heterozygous grouped with homozygous), or recessive (heterozygous grouped with wildtype). Cochran-Armitage tests were used to test for trends toward preterm birth with increasing variant alleles. Logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI) for preterm birth for each SNP under a multiplicative genetic model (0, 1, or 2, indicating the number of variant alleles). A SNP by infection/inflammation interaction analysis was performed by logistic regression using a multiplicative genetic model and including an interaction term for SNP by infection/inflammation. Stratified analysis was conducted to determine the OR and CI for preterm birth for infected and uninfected women separately. According to sample size calculations based on Peduzzi et al,[25] this study was slightly underpowered for stratification by BV but adequate powered for stratification by placental inflammation status.

VCFtools v0.1.13[26] was used to calculate F_{ST}. All other statistical analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC, US). *P* values < 0.05 were considered significant.

4.5 Results

Summary statistics of study participants (n = 613) are shown in Table 4.1. Neither maternal age nor maternal region of origin had an effect on preterm birth rates, but the proportion of male infants was higher in preterm compared to term births ($P = 0.0215$).

We identified 12 known SNPs in the *NODAL* gene of participants in the study population (Figure 4.1). Of these, four had VAF > 1% and were included in further analyses (Table 4.2). For each of the four common SNPs, cases and controls were found to be in HWE both individually and overall within the study population (Table S4.2).

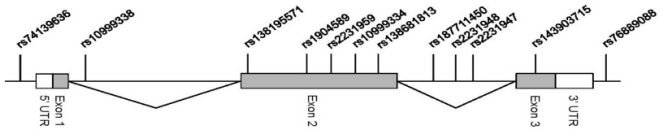

Figure 4.1 Schematic representation of NODAL genome structure and location of coding variants identification in study cohort

Table 4.1 Demographic characteristics of study population

Characteristic	Term[1] (*n* = 424)	Preterm (*n* = 189)	*P* value
Maternal age, y[2]	28.7 ± 0.3 (18−43)	28.7 ± 0.4 (18−43)	0.9804
Infant sex[3,4]			
Male	214 (65)	114 (35)	
Female	209 (74)	74 (26)	0.0215
Maternal Origin[3,5]			
European	345 (69)	157 (31)	
African/Caribbean	30 (71)	12 (29)	
Asian	16 (70)	7 (30)	
Middle Eastern	11 (65)	6 (35)	
Latin American	19 (76)	6 (24)	0.9311

[1] Term, births ≥ 37 weeks; Preterm, births < 37 weeks.
[2] Mean ± SEM (range). T-test *P* value.
[3] Count (%). χ^2 test *P* value.
[4] Missing values: 1 term, 1 preterm.
[5]Missing values: 3 term, 1 preterm.

Table 4.2 list of common SNPs in the NODAL gene identification in the MPS study

						VAF	
SNP	**Nucleotide Variation[1]**	**Type**	**Amino Acid Substitution**	**Effect**	**Position[2]**	**MPS**	**1000GP**
rs2231947	c.892-1010C>T	Intronic	-	Alternative splicing[3]	10:70434098	0.13	0.14
rs1904589	c.494A>G	Missense	H165R	50% activity[4]	10:70435683	0.59[5]	0.33
rs10999338	c.193+12C>T	Intronic	-	Unknown	10:70441463	0.41	0.40
rs74139636	c.-109T>C	5' Upstream	-	Unknown	10:70441776	0.08	0.15

Table 4.2 list of common SNPs in the NODAL gene identification in the MPS study
1000GP, 1000 Genomes Project; MPS, Montreal Prematurity Study; SNP, single nucleotide polymorphism; VAF, variant allele frequency.

[1] NCBI reference sequence NM_018055.4.

[2] Genomic position according to NCBI Build 38 GRCh38.p2 assembly.

[3] According to reference 33.

[4] According to reference 29.

[5] The minor allele of rs1904589 in the MPS population has been confirmed to be the normal version of the gene by functional studies.[30]

In combined 1000 Genomes Project populations, HWE was rejected for each of the four SNPs assessed, suggesting population stratification (Table S4.2). After subdividing populations, HWE was satisfied for all subgroups except the African population for SNP rs1904589. High F_{ST} values for SNPs rs1904589 and rs10999338 suggested a high level of differentiation between populations (Table 4.4).

Table 4.4 Population differentiation in NODAL due to genetic structure across all included 1000 Genome Project populations

SNP	F_{ST}
rs2231947	0.0513
rs1904589	0.1489
rs10999338	0.2041
rs74139636	0.0484

F_{ST}, Weir and Cockerham's global fixation index; SNP, single nucleotide polymorphism.

No associations were found for any SNP under genotypic, dominant, or recessive inheritance models (Table S3), using Cochran-Armitage tests for trends (Table S4.3), or under multiplicative inheritance models (Table 4.3). Logistic regression analyses including an interaction term indicated that rs2231947 had a significant SNP by BV interaction effect (P = 0.0231). Additionally, rs1904589 and rs10999338 both had significant SNP by placental inflammation interaction effects (P = 0.0224 and 0.0230, respectively). To further explore the nature of these interactions, we conducted stratified analyses to test the association between SNPs and preterm birth among infected versus uninfected women (Table 4.3). The rs2231947 variant allele was associated with increased risk for preterm birth among women with BV (OR

2.76, 95% CI 1.12−6.85). Among women without placental inflammation, the rs1904589 variant allele was associated with greater risk of preterm birth (OR 1.31, 95% CI 1.02−1.70), whereas the rs10999338 variant allele was associated with a reduced risk of preterm birth among women with placental inflammation (OR 0.50, 95% CI 0.29−0.87).

Table 4.3 Logistic regression model of the association between common NODAL variants and preterm birth (<37weeks) for all women and stratified by infection/inflammation status under a multiplicative inheritance model

				BV			PI
SNP	Overall[1] (n = 613)	BV (–)[2] (n = 515)	BV (+)[2] (n = 94)	Interaction *P* value	PI (–)[2] (n = 449)	PI (+)[2] (n = 140)	Interaction *P* value
rs2231947	0.82 (0.56−1.19)	0.68 (0.45−1.03)	2.76 (1.12−6.85)	0.0231	0.78 (0.53−1.15)	1.71 (0.74−3.98)	NS
rs1904589	1.16 (0.90−1.48)	1.16 (0.90−1.50)	1.00 (0.53−1.91)	NS	1.31 (1.02−1.70)	0.67 (0.40−1.13)	0.0224
rs10999338	1.04 (0.82−1.33)	1.04 (0.80−1.35)	1.13 (0.62−2.07)	NS	1.13 (0.88−1.46)	0.50 (0.29−0.87)	0.0230
rs74139636	1.22 (0.78–1.92)	1.23 (0.75−2.01)	1.07 (0.33−3.42)	NS	1.19 (0.70–2.01)	1.35 (0.53–3.48)	NS

–, no infection/inflammation detected; +, infection/inflammation detected; BV, bacterial vaginosis; CI, confidence interval; NS, non-significant; OR, odds ratio; PI, placental inflammation; SNP, single nucleotide polymorphism.

[1] OR (95% CI) representing unadjusted increase of odds for preterm birth for each additional variant allele for entire MPS population.

[2] OR (95% CI) representing unadjusted increase of odds for preterm birth for each additional variant allele in MPS population stratified by placental inflammation or BV

4.6 Discussion

We report the results of a genetic association study of *NODAL* variants and preterm birth in a secondary analysis of a nested case-control study from a prospective birth cohort. Although initially there was no association between *NODAL* SNPs and preterm birth, when BV or placental inflammation were included in our models, we found that three SNPs had a differential effect on preterm birth risk depending on the presence of infection or inflammation. Specifically, rs2231947 increased the risk of preterm birth for women diagnosed with BV. In contrast, rs1904589 increased the risk of preterm birth in women who did not have placental inflammation, whereas rs10999338 decreased the risk of preterm birth in women who did have placental inflammation.

Previous studies have suggested that NODAL may play a role in regulation of the immune system. Both NODAL and its co-receptor CRIPTO have been shown to polarize cultured macrophages towards an anti-inflammatory phenotype.[16,17] Work in our lab has shown that a low dose of lipopolysaccharide that has no effect on control mice causes conditional uterine *Nodal* heterozygous knockout mice to give preterm birth (unpublished data). These results are in line with our current findings, which seem to support a role for human NODAL in the regulation of the immune response within the reproductive tract. However, while this regulation may be required during normal pregnancy, it may be advantageous to deregulate inflammation in the presence of infections or other inflammatory signals within the genital tract, as we suspect is the case in women with the rs10999338 SNP. Other studies have found similar effects of SNPs in genes that mediate pro-inflammatory responses, such as Toll-like receptor pathway adapter variant (TIRAP rs8177314)[27] and the IL-6-174 C/C variant.[28] These variants are

thought to protect against preterm birth by decreasing the inflammatory response to bacterial ligands that lead to premature initiation of the parturition cascade. Interestingly, it has been suggested that preterm birth may be beneficial to the mother in the presence of infection, as carrying an infected fetus may put her at risk.[29] In this scenario, a gene variant that prevents preterm birth in the presence of infection would be maladaptive. Nevertheless, prevention is required to ameliorate the suffering and economic costs associated with preterm birth. Further studies on the role of NODAL in the immune response will provide necessary insight into the potential risks and benefits of *NODAL* variants in pregnant women.

Initially, we were surprised to find high heterozygosity for rs1904589 and rs10999338, despite the fact that rs1904589 has been shown to reduce NODAL activity by ~50%[30] and rs10999338 is located in intron 1, which in the mouse contains an enhancer element.[31] These observations suggest that there may in fact be an advantage conferred by these SNPs. Indeed, our results showed that the rs10999338 SNP reduced the risk of preterm birth in women with placental inflammation. In order to test whether these SNPs may be under a selection pressure, we used 1000 Genomes Project data to assess population differences at these loci. For both rs1904589 and rs10999338, variant allele frequencies were highest in African and European populations. Interestingly, African ancestry is a risk factor for preterm birth and this has been linked to genetic variation in inflammation-related genes in these individuals.[32] Additionally, population stratification was suggested both by the rejection of HWE in pooled populations and by high F_{ST} values for these two SNPs, indicating a high level of population differentiation and a possible selective pressure on particular populations.

In contrast to rs1904589 and rs10999338, the rs2231947 SNP increased the risk of preterm birth in women with BV. This discrepancy was surprising, given that BV also induces an

inflammatory immune response.[33] rs2231947 is known to directly regulate alternative splicing of *NODAL* and may causing signalling through non-canonical pathways.[34] Furthermore, rs2231947 did not exhibit high heterozygosity or population differentiation. Thus, alternative splicing caused by the rs2231947 SNP likely results in different protein functioning compared to other *NODAL* variants, leading to different pregnancy consequences. Further studies are required to determine whether alternative splicing of *NODAL* has an impact on the immune system or if other functions that may affect preterm birth are involved.

Although ethnicities reported in the MPS study were based on self-reports of maternal region of origin and may not reflect genetic differences, ethnic diversity was very low within MPS. This in turn reduced population stratification in our study population, which improved our ability to detect differences in rates of preterm birth given the stratification we observed using 1000 Genomes Project data. However, similar analyses should be performed in more diverse populations to confirm the relationship between *NODAL* variants and infection/inflammation, especially given the relatively low rate of infection/inflammation in MPS. In addition, it would be interesting to determine whether this gene-environment interaction has differential effects on early (< 34 weeks) versus late preterm births.

In conclusion, we identified possible gene-environment interactions in which three *NODAL* SNPs modify the risk of preterm birth depending on BV and placental inflammation status. We also suggest that the *NODAL* gene may have been subject to selection pressure in certain populations, leading to high heterozygosity of certain *NODAL* SNPs. Additional studies are needed to identify the mechanisms by which interactions between NODAL and infection/inflammation influence preterm birth.

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Conflict of Interest

The authors declare no conflict of interest.

4.7 Supplementary Information

Supplementary Table 4.1 Primers used for genotyping

Primer	Forward (5'–3')	Reverse (5'–3')	SNPs covered
NODALE01	GCACCAGGCTCAGGTCTC	CCAACCCACAGCACTTCC	rs74139636, rs10999338,
NODALE02a	CCAGCAAGAGCTATGGTGGT	GCTTCCCACAGCAAGGTG	rs138195571, rs1904589,
			rs2231959, rs10999334
NODALE02b	AGCATGGTTTTGGAGGTGAC	GGAACATAGTCATTTAATAGCAAAGC	rs1904589, rs2231959,
			rs10999334, rs138681813,
			rs187711450
NODALE03	AAATACTATTCTGACCTGCCCATC	CAGACTCCACTGAGCCCTTC	rs76889088, rs143903715
rs2231947	GACTGTCTGGG4TCTTTGTGG	ATGGGACCAAAATGTTTTCA	rs2231947, rs2231948

SNP, single nucleotide polymorphism.

Supplementary Table 4.2Tests for Hardy-Weinberg equilibrium in global populations from the 1000 Genomes Project and MPS

	Allele		Genotype			Total	
Population[1]	**Frequency**		**Frequency**			**Samples**	**HWE[2]**
rs2231947	**C**	**T**	**CC**	**CT**	**TT**		
ALL	0.86	0.14	0.74	0.23	0.03	2504	0.0005
AFR	0.81	0.19	0.66	0.30	0.04	661	0.8088
AMR	0.89	0.11	0.80	0.19	0.01	347	0.8988
EAS	0.99	0.01	0.98	0.02	0.00	504	0.9746
EUR	0.80	0.20	0.64	0.32	0.04	503	1.0
SAS	0.82	0.18	0.67	0.30	0.03	489	0.9374
MPS	0.87	0.13	0.73	0.25	0.02	524	0.3480
rs1904589	**A**	**G**	**AA**	**AG**	**GG**		
ALL	0.33	0.67	0.15	0.38	0.48	2504	$4.33x10^{15}$
AFR	0.49	0.51	0.27	0.45	0.29	661	0.0182
AMR	0.32	0.68	0.10	0.43	0.46	347	0.9775
EAS	0.04	0.96	0.00	0.09	0.91	504	0.3636
EUR	0.47	0.53	0.21	0.51	0.28	503	0.8136
SAS	0.30	0.70	0.09	0.41	0.50	489	0.8975
MPS	0.41	0.59	0.17	0.48	0.34	613	0.9291
rs10999338	**C**	**T**	**CC**	**CT**	**TT**		
ALL	0.66	0.34	0.48	0.37	0.16	2504	$5.54x10^{-20}$
AFR	0.91	0.09	0.84	0.15	0.01	661	0.5554

AMR	0.59	0.41	0.33	0.51	0.15	347	0.4735
EAS	0.37	0.63	0.14	0.46	0.41	504	0.8629
EUR	0.58	0.42	0.35	0.45	0.19	503	0.3275
SAS	0.76	0.24	0.57	0.37	0.06	489	0.9352
MPS	0.59	0.41	0.35	0.48	0.17	608	0.9814
rs74139636	**T**	**C**	**AA**	**AG**	**GG**		
ALL	0.85	0.15	0.72	0.25	0.03	2504	0.0382
AFR	0.91	0.09	0.83	0.16	0.01	661	0.8370
AMR	0.79	0.21	0.63	0.32	0.05	347	0.8030
EAS	0.77	0.23	0.59	0.37	0.04	504	0.3776
EUR	0.93	0.07	0.87	0.13	0.002	503	0.6445
SAS	0.78	0.22	0.61	0.33	0.05	489	0.8710
MPS	0.92	0.08	0.84	0.16	0.002	608	0.1016

HWE, Hardy-Weinberg equilibrium; MPS, Montreal Prematurity Study.

[1] Allele and genotype frequencies obtained from 1000 Genomes sequence data. ALL, all included 1000 Genomes Project populations combined; AFR, African ancestry (ACB, ASW, ESN, LWK, MAG, MSL, YRI); AMR, Native American ancestry (CLM, MXL, PEL, PUR); EAS, East Asian ancestry (CDX, CHB, CHS, JPT, KHV); EUR, European ancestry (CEU, FIN, GBR, IBS, TSI); SAS, South Asian ancestry (BEB, GIH, ITU, PJL, STU).

[2] Probability that observed genotype frequencies are in Hardy-Weinberg equilibrium.

Supplementary Table 4.3 Test of association between common NNODAL variants and preterm birth (< 37weeks) under different inheritance models

SNP	Variant Allele	Genotype[1]	Dominant[2]	Recessive[3]	Trend[4]
rs2231947	T	0.4894	0.4353	0.3615	0.2934
rs1904589	G	0.4883	0.4908	0.2492	0.2476
rs10999338	T	0.9436	0.7750	0.7877	0.7336
rs74139636	C	0.5413	1.00	0.4978	0.3833

SNP, single nucleotide polymorphism.

[1] χ^2 test *P* value for genotype as a 3-level categorical variable. Fisher exact test performed for any group $n < 5$.

[2] χ^2 test *P* value for genotype as a 2-level categorical variable, heterozygous grouped with homozygous. Fisher exact test performed for any group $n < 5$.

[3] χ^2 test *P* value for genotype as a 2-level categorical variable, heterozygous grouped with wildtype. Fisher exact test performed for any group $n < 5$.

[4] Two-sided *P* value for Cochran-Armitage test for trend toward preterm birth with increasing variant alleles.

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Chapter 5: **General Discussion and Conclusions**

5.1 General Discussion and Conclusion

Overview

Preterm birth is a global health problem. It is the second leading cause of child death (Blencowe et al 2012) and affects approximately 12% of pregnancies worldwide (Blencowe et al 2012). The incidence of preterm birth in European countries is approximately 5% while in some African countries it can be as high as 18% (Blencowe et al 2012). Although the rate is very high in some countries and the burden is also increasing in those countries, the causes and molecular mechanisms that underlie the majority of preterm births remain largely unknown. This poor understanding is due in part to a lack of proper animal models for preterm birth research.

Previous studies in our laboratory have shown that the lack of NODAL causes a significant reduction in fertility in conditional uterine specific Nodal knockout mice. In addition, 80% of those mice that do get pregnant, deliver on day 17.5 instead of at day 19.5 which is considered as preterm birth (Park et al 2012). Nodal was the first gene deletion in mice to result in preterm birth as mice either deliver to phenotypically abnormal pups at term or the embryos undergo resorption. In the present study, I investigated the hypothesis that NODAL plays a crucial role in the timing of parturition and the decreased expression of this gene increases susceptibility to preterm birth in both mice and humans. In this thesis, I demonstrate that: (1) NODAL is required to maintain the uterine immunological environment in an anti-inflammatory state during late pregnancy; (2) NODAL regulates the LPS-induced inflammatory response in macrophages through the modulation of the NFkB and MAPK pathways; and, (3) there is gene-environment interaction of NODAL variants and inflammation in preterm birth in humans suggesting that Nodal is involved in human pregnancies.

In the first manuscript, **chapter 2 in this thesis,** we highlighted that Nodal is necessary to balance the immunological environment during pregnancy and that conditional uterine-specific Nodal heterozygous mice have a 50% reduction in fertility, but pregnant females delivered at term. Although these mice did not have preterm birth, they were more sensitive inflammation (LPS), where 50% of Nodal heterozygous mice deliver prematurely 12-14h on LPS injection while the same dose did not affect the control mice. We also demonstrated that these Nodal heterozygous mice have a high basal level of pro-inflammatory cytokines and chemokines, including IL- 1β, TNF-α, IL-6, IL-12p70, and IFN-γ, in maternal placental decidual tissue. It is known that cytokine production is part of the labor process and is responsible for activating important factors, such as Cox2, that participate in the parturition cascade (Bryant et al 2017, Salminen et al 2008). Other studies have reported that the main cellular sources of cytokines, including IL-1ß, IL-6, IL-12p70, and TNF-α, are macrophages and monocytes (Arango Duque & Descoteaux 2014, Bryant et al 2017). Macrophages also release other agents that lead to activation of the labor cascade, such as specific chemokines, including CXCL10, CCL5, and CCL12, prostaglandins and complement proteins (Arango Duque & Descoteaux 2014).

Supporting a role Nodal plays, a role in modulating the immunological environment during pregnancy. Other studies have shown that NODAL and its signaling components are involved in the regulation of the immune system. NODAL and its co-receptor CRIPTO polarize cultured macrophages towards an anti-inflammatory phenotype(Wang et al 2014, Zhang et al 2016). Interestingly, when using BMDM as a model to study the role of Nodal in immunity, we demonstrated that the presence of rNodal is able to inhibit cytokine production after challenging cells with LPS.

In a wildtype uterus on day 15 of pregnancy, uterus is in an anti-inflammatory state. At this time, low concentrations of pro-inflammatory cytokines and chemokines are present. At parturition, the uterine environment switches to a pro-inflammatory state with an increase in the level of pro-inflammatory cytokines and chemokines as well as leucocytes infiltration. In contrast, Nodal heterozygous mice on day 15 of pregnancy already have a higher level of pro-inflammatory cytokines and chemokines. At this stage, no leukocyte infiltration was detected. However, injection of the inflammatory agent LPS resulted in a significant increase in infiltration of leukocytes and a further increase in pro-inflammatory cytokines and chemokines. This increase in leukocyte infiltration and pro-inflammatory cytokines were most likely responsible for 50% of the mice delivering preterm (see the model in Figure 5.1)

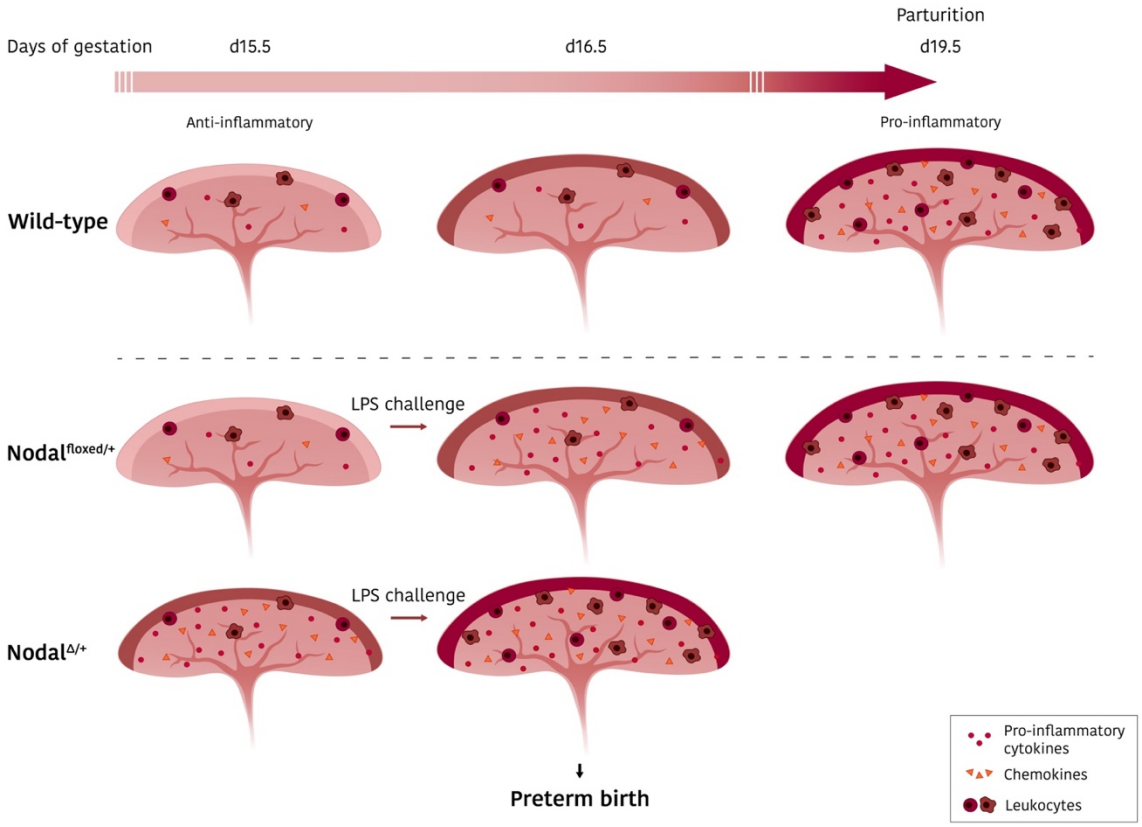

Figure 5.1 A Model showing the immunological state during late pregnancy in wildtype, control Nodal mice and Nodal heterozygous mice.

Collectively, our study demonstrates that the decrease in of Nodal in Nodal heterozygous mice results in a disturbance in the immunological environment during late pregnancy and labor. Nodal heterozygous mice have very high basal level for several cytokines (IL-1ß, IL-1α, IL-6, IL12, TNF-α), and chemokines (CXCL10, CCL5, and CCL12). This increase in the pro-inflammatory agents was only in our model Nodal heterozygous mice not in the control mice.

Alongside the specific gene approach that we undertook, a more general approach to identify all factors that are differentially expressed in Nodal heterozygous mice compared to controls mice needs to be performed. As an example, RNAseq on the decidua maternal tissues on day 16.5 of pregnancy should be performed to identify all genes that show differential regulation in the Nodal heterozygous versus controls. This global experiment will help us to identify other potential pathways, aside from the immune pathways, that are dysregulated in Nodal heterozygous mice, which may be responsible for inducing preterm parturition. The RNAseq results will give us a general idea of the dysfunction when Nodal, is decreased.

In conclusion, our study demonstrates that a decrease of Nodal disturbs the immunological environment during late pregnancy and labor. The Nodal heterozygous mouse is a suitable model to study gene-environment interactions that are important in human preterm births.

In the second manuscript, **Chapter 3 of this thesis**, we examine the mechanisms through which Nodal downregulates cytokine production in both BMDM as well as the RAW264.7 macrophages cell line. Our study highlights a novel role for Nodal, namely its ability to decrease the global phosphorylation of proteins after treating macrophages with rNodal for two minutes. Compared to the untreated macrophages by proteomics analysis, this analysis has shown that

Nodal modulates NFκB and MAPK, both well-known mediator of the inflammation cascade (Ruland 2011, Tammi et al 2011, Zhang & Dong 2005).

A growing number studies have shown that TGF-ß modulates the immune response by targeting several immune cells resulting in several human immune orders (Sheng et al 2015). *In vitro* studies have shown the TGF-ß effects on NK cells, dendritic cells, and macrophages (Sheng et al 2015). Also, a deficiency in the TGF-ß signaling pathway in NK cells results in proliferation of the NK cells and their secretion of IFN-γ, which is fundamentally essential for the differentiation of the Th1 cells, indicating the involvement of TGF-ß in cytokine expression (Laouar et al 2005, Sheng et al 2015). Also, *in vivo* studies showed that TGF-ß signaling could be involved in inhibiting autoimmunity in dendritic cells (Sheng et al 2015) and is preventing macrophage activation by blocking the expression of macrophage activation markers, including nitric-oxide (iNO), and matrix metalloproteinase (MMP)-12 (Sheng et al 2015). Despite the clear inhibitory role of TGF-ß in modulating the immune response, the molecular and cellular mechanisms need more studies to be better understood.

It has been shown that Nodal and its co-receptor CRIPTO are involved in modulating the macrophage polarization (Wang et al. 2014, Zhang et al. 2016). Not much is known about the role of Nodal in the immune response. However, in our study, using the macrophage the RAW264.7 cells line, we showed that Nodal can modulate the production of several cytokines after inductionol inflammation with LPS.

Nodal, like other members in the TGF-ß superfamily, signals through two pathways, either the SMAD dependent one (canonical) or the SMAD independent pathway (non-canonical). (Feng & Derynck 2005, Shi & Massague 2003). The non-canonical pathway includes ERK, JNK, and

p38, PI3K, and AKT (Derynck & Zhang 2003, Massague 2012). Individually, ERK, JNK, p38 are activated by the kinase kinases (MKKs), MKK4, and MKK3/6, respectively(Weston & Davis 2002, Zhang 2009). That might help us in our study to investigate the inhibitory effect of Nodal through non-Smad signaling, and we can perform western blot analysis for these downstream components. Moreover, in our model, we were challenging the cells with the inflammatory mediator LPS; it is well known that LPS signals through only the TLR4 receptor. However, we do not know if Nodal affects signaling through other TLRs. To investigate if Nodal is playing an inhibitory role through other TLRs, western blot analysis could be performed to examine the protein phosphorylation levels of the downstream effectors of different TLRs in the presence and absence of Nodal.

In the third manuscript, **Chapter 4 in this thesis**, we demonstrated the association between NODAL genetic variants and the frequency of preterm births in humans via a secondary analysis of a nested case-control study from a prospective birth cohort. No association between NODAL SNPs and preterm birth was found in this population. However, when we subdivided the NODAL SNPs according to incidence of Bacterial Vaginosis or placental inflammation, there were three SNPs associated with increased risk of preterm birth. Notably, women who have SNP rs2231947 and a BV diagnosis are at an increased risk of preterm birth. In contrast, women who have SNP rs1904589, but no placental inflammation were at an increased risk of preterm birth, whereas women who have rs10999338 SNP and did have placental inflammation had a decreased risk of preterm birth.

Furthermore, several SNPs in the NODAL gene have been identified in the population, and some of the SNPs have been found to decrease Nodal activity (Roessler et al 2009). Population analysis

has shown that these SNPs are implicated in predisposing patients to heart defects and holoprosencephaly (Roessler et al 2009). Consequently, this raises the possibility that NODAL SNPs could also predispose patients to pregnancy-associated disorders.

Previously, our lab collaborated with Thulluru et al. (2013), at VU University Medical Centre, who have been investigating a familial form of early-onset, IUGR-complicated pre-eclampsia. We sequenced the NODAL gene in the mothers who had IUGR-complicated pre-eclampsia and determined that all the mothers had the NODAL H165R variant that had been shown to reduce Nodal activity by 50% (Thulluru et al 2013). This was the first evidence that NODAL SNPs predisposed mothers to pregnancy-associated disorders. It has also been shown, in humans, that the downregulation of Nodal in decidua increases NODAL expression in the -fetal placental tissues (Thulluru et al 2013).

Growing evidence has shown that genetics is a critical factor that influences PTB (Muglia 2000, Muglia & Katz 2010, Rappoport et al 2018). Zhang et al. (2018) revealed the genetic influence of PTB in European ancestry. This study identified six genes that are significantly associated with PTB, including EBF1, EEFSEC, AGTR2, ADCY5, RAP2C, and WNT4 (Zhang et al 2018). Our gene of interest, NODAL, was not included in this list of genes in this study. Our results show that NODAL is not directly associated with PTB. However, an interaction between NODAL SNPs and PTB in the presence of infection/inflammation increases the risk of PTB. Although our study consisted of a limited population from Montreal, we were able to identify possible gene-environment interactions. Indeed, we found three NODAL SNPs involved in the risk of preterm birth depending on BV and placental inflammation status. Thus, applying a similar analysis to a larger sample size may well provide additional evidence of the relationship between genetics and environment in very early, early, and late preterm births. We also suggest determining

inflammation/infection in other tissues in the female tract, which will hopefully provide us with a global idea of the different types of inflammation/infection that might be involved in preterm birth. Additional studies are needed to identify the mechanisms by which interactions between NODAL and infection/inflammation influence preterm birth.

A growing body of studies demonstrates that other SNPs exert effects similar to NODAL SNPs. In particular, the Toll-like receptor pathway adapter variant (TIRAP rs8177314) (Karody et al 2013) and the IL-6-174 C/C variant (Simhan et al 2003) have been implicated in decreasing inflammation-induced preterm births. These variants play an anti-inflammatory role and reduce inflammation-induced preterm birth. On the other hand, studies have suggested that preterm birth might be a positive condition for the mother in some cases, as the presence of infection when carrying an infected fetus might put her in danger (Muglia & Katz 2010).

5.2 Conclusion

The results described in this thesis have essential implications in expanding our knowledge of the role of Nodal in immune system regulation during late pregnancy. We demonstrated how the loss of one allele of Nodal increases the cytokine production, which consequently results in activation of the parturition cascade. Thus, our research uncovered a key role for Nodal in modulating the immunological state during late pregnancy and preventing the occurrence of preterm births.

We elucidated the mechanisms through which Nodal is activating and playing this inhibitory role. Specifically, our data showed that NODAL attenuates the LPS-induced inflammation via downregulation of the NFkB and MAPK pathways. Besides, our data revealed an interaction between NODAL SNPs and infection/inflammation. Which increases the incidence of preterm birth in human.

Ultimately, our research underscores the centrality of Nodal in modulating the immunological environment during late pregnancy in both mice and humans. Our findings open a new avenue for the use of Nodal as a preventative therapy for several common, inflammatory disorders.

Chapter 6: References

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